Metabolic engineering of the yeast Yarrowia lipolytica for the production of even- and odd-chain fatty acids

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Metabolic engineering of the yeast *Yarrowia lipolytica* for the production of even- and odd-chain fatty acids

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ABBREVIATION

E. coli *Escherichia coli*
B. subtilis *Bacillus subtilis*
C. curvatus *Cryptococcus curvatus*
C. necator *Cupriavidus necator*
C. violaceum *Chromobacterium violaceum*
L. starkeyi *Lipomyces starkeyi*
R. eutropha *Ralstonia eutropha*
R. glutinis *Rhodotorula glutinis*
R. toruloides *Rhodosporidium toruloides*
S. cerevisiae *Saccharomyces cerevisiae*
T. cutaneum *Trichosporon cutaneum*
To. delbrueckii *Torulaspora delbrueckii*
Y. lipolytica *Yarrowia lipolytica*

3-HB 3-hydroxybutyrate
3-HV 3-hydroxyvalerate

AA arachidonic acid
AAA aromatic amino acid
ACC acetyl-CoA carboxylase
ACL ATP-citrate lyase
ACS acetyl-CoA synthetase
AcTAGs acetylated TAGs
AEP alkaline extracellular protease
ARS autonomously replicating sequence
ATP adenosine triphosphate

CEN centromere
CFA cyclopropanated fatty acid
CHD coronary heart disease
CLA conjugated linoleic acid
C/N ratio carbon to nitrogen ratio
CRISPR clustered regularly interspaced short palindromic repeats
CRM *cis*-regulatory module

DAG diacylglycerol
DCW dry cell weight
DHA docosahexaenoic acid

ECFA even-chain fatty acid
EPA eicosapentaenoic acid

FA fatty acid
FAEE fatty acid ethyl ester
FAME fatty acid methyl ester
FAS fatty acid synthase
FFA free fatty acid

G3P glycerol-3-phosphate
GC Gas chromatography
GG Golden Gate Assembly
GRYC Genome Resources for Yeast Chromosomes

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h (hr) hour
HPLC high-performance liquid chromatography
HR homologous recombination

KAS beta-ketoacyl-ACP synthase
KB (Kb) kilo base pair

LB lipid body
LB Luria-Bertani medium
LCFA long-chain fatty acid
LiAc lithium acetate
LPA lysophosphatidic acid

MB (Mb) mega base pair
MCFA medium-chain fatty acid
ME malic enzyme
MFE multifunctional enzyme
MFS major facilitator superfamily

NAD+ nicotinamide adenosine triphosphate (oxidized form)
NADH nicotinamide adenosine triphosphate (reduced form)
NADP+ nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH nicotinamide adenine dinucleotide phosphate (reduced form)
NHEJ non-homologous end joining

OCFA odd-chain fatty acids
OD_{600nm} optical density at 600 nm
ORF open reading frame

PA phosphatidic acid
PCR polymerase chain reaction
PCT propionyl-CoA transferase
PE 2-phenyl ethanol
PEG polyethylene glycol
PDH pyruvate dehydrogenase
PDC pyruvate decarboxylase
PHA polyhydroxyalkanoate
PHB polyhydroxybutyrate
PHBV Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
POM pyruvate-oxaloacetate-malate

PP2A protein phosphatase 2A
PPP pentose phosphate pathway
PUFA polyunsaturated fatty acid
PYC pyruvate carboxylase

RA Ricinoleic acid

SCFA short-chain fatty acid
SE steryl esters
SEP sticky-end PCR
SFU specific fluorescence unit
ssDNA single stranded DNA

T2D type II diabetes
TAE tris/acetate/EDTA
TAG triacylglycerol
TAL triacetic acid lactone
TALEN transcription activator-like effector nuclease
TCA tricarboxylic acid
TU transcription unit

UAS upstream activation sequence
UniProt universal protein resource database

VLCFA very long-chain fatty acid
VFA volatile fatty acid

WT wild-type

YNB yeast nitrogen base medium
YP yeast extract and peptone medium

CHAPTER 1. INTRODUCTION

1.1. Context of the study

Biotechnology has a very long history since the use of yeast as the biocatalyst in bread making, which is thought to have begun around 10,000 BC [McGovern *et al*. 2004]. After several important discoveries such as antibiotics by Fleming in 1928, biotechnology has steadily developed and now plays a key role in several industrial sectors, providing products with high value. In 2003, a color scheme has been adopted which provides a specific definition of biotechnology according to its application area by Dr. Rita Colwell and the National Science Foundation [Heux *et al*. 2015]. White biotechnology (or industrial biotechnology) describes the application of enzymes, cell extracts, or whole microorganisms in industrial processes that lead to the production of a wide variety of products, such as fuel, food ingredients, and chemical compounds [Frazzetto, 2003; Heux *et al*. 2015]. There are several advantages of white biotechnology over chemical processes. It uses renewable resources such as sugars, vegetable oils, and agricultural crops instead of dwindling fossil resources, providing sustainable and environmentally friendly processes and supporting the agricultural sector. Moreover, it frequently shows superior performance compared to conventional chemical technology in terms of conversion efficiency, product purity, energy consumption, waste generation, and so on [Soetaert and Vandamme, 2006]. In this regard, white biotechnology has been bringing many innovations to the chemical, textile, food, packaging, and health care industries.

The remarkable advances in white biotechnology have come along with the progress of metabolic engineering since the 1990s following a period of intensive researches of genetic engineering and applied molecular biology [Stephanopoulos, 2012]. Metabolic engineering is a multidisciplinary scientific field as well as technology being defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of the new one(s) with the use of recombinant DNA technology [Stephanopoulos 2012; Koffas *et al*. 1999].

Metabolic engineering involves the manipulation of enzymatic, transport, and regulatory functions of the cell by using recombinant DNA technology. First, various analytical techniques are used to identify and subsequently determine fluxes through critical metabolic pathways in the cell. This knowledge provides the rational basis for applying, in the second step, molecular biological techniques to enhance metabolic flux through a pathway of interest and minimize metabolic flow to undesired by-products. Although a certain sense of direction is inherent in all strain improvement programs, the directionality of effort is a primary point of metabolic engineering which is distinguishable to random mutagenesis. Up to now, the modulation of pathways for the production of high-volume products including biopolymers, fuels (ethanol, isobutanol, n-butanol, lipids), chemicals (succinic acid, acrylic acid, lactic acid), and numerous specialty chemicals was broadly explored.

One of the important points in metabolic engineering is the selection of the chassis (working horse microorganism). The availability of basic information, techniques for engineering, and the particular properties (such as ethanol fermentation in *Saccharomyces cerevisiae*, lipid accumulation in oleaginous yeasts, or resistance to certain conditions) are the important criteria for choosing chassis and facilitating the development of a project [Brophy and Vioght, 2014; Khoury *et al*. 2014]. The toxicity of intermediates and/or final products to host microorganisms also should be considered in chassis selection, which alternatively can be resolved through optimization of fermentation condition or evolutionary adaptation [Sopko *et al*. 2006; Forster and Gescher, 2014; Keasling, 2012].

Another crucial point in metabolic engineering is the sustainability and feasibility of the whole process. For this, many interests and efforts have directed to the engineering of chassis to utilize inexpensive renewable resources such as cellulosic biomass and industrial wastes. Responding to the market forces, progresses in metabolic engineering for expanding substrate ranges have been providing the sustainable and economically viable platform [Stephanopoulos, 2012; Ledesma-Amaro and Nicaud, 2016a].

There is no doubt that the advances in synthetic biology have helped to move metabolic engineering forward. Synthetic biology provides genetic components (promoters, coding sequences, terminators, transcriptional factors, and their binding sequences, and more), the assembly of devices, genetic circuits, and even entirely tailor-made microorganisms [Garcia-Granados *et al*. 2019; Heux *et al*. 2015]. Because metabolic engineering and synthetic biology, both, seek to achieve basic requirements such as the generation of specific mutations, the assembly of parts or components in biosynthetic pathways, and the integration of DNA in the genome of chassis, both areas of research rely on the advances in the actual methods, techniques, and tools for DNA modification [Boyle and Silver, 2012; Garcia-Granados *et al*. 2019]. In addition, it is undeniable that there are many synergies between metabolic engineering and synthetic biology [Stephanopoulos, 2012; Garcia-Granados *et al*. 2019]. Metabolic engineering will benefit from the tools of synthetic biology in the synthesis and control of non-natural pathways. Synthetic biology also will profit the approaches of metabolic engineering in the areas of pathway design, analysis, and optimization. Therefore, the development of both research areas altogether will be crucial to develop the chassis microorganisms as cell factories that produce value-added compounds with higher yield in white biotechnology.

1.2. Background

1.2.1. *Yarrowia lipolytica*

1.2.1.1. Taxonomy

Yarrowia lipolytica is a yeast belonging to the Ascomycota phylum, Dipodascaceae family, Fungi kingdom [Kurtzman, 2011]. The generic name '*Yarrowia'* was proposed by van der Walt and von Arx in acknowledgment of a new genus identified by David Yarrow from the Delft Microbiology Laboratory [Van der Walt, 1980; Yarrow, 1972]. The species name '*lipolytica*' originated from the ability of this yeast to hydrolyze lipids. It was first identified as *Candida lipolytica* in the late 1960s and was then reclassified as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica*, and finally *Yarrowia lipolytica* [Barth and Gaillardin, 1996]. It is denoted as a "non-conventional yeast" with respect to its distinctive genome structure and its relatively large phylogenetic distance to other yeasts while sharing common properties with higher eukaryotes [Barth and Gaillardin, 1996].

1.2.1.2. Natural habitats

This yeast was originally isolated from lipid-rich or protein-rich environments such as (fermented) dairy products, cheese (e.g. Camembert, Livarot, Rokpol-Polish Roquefort), yogurt, meat, poultry, and olive oil. Strains have also been isolated from various environments such as lipid-rich sewage, oil-polluted media, and hypersaline environments [Beopoulos *et al*. 2009]. Most *Yarrowia* strains are unable to grow above 32 °C and the species is strictly aerobic.

Y. lipolytica is considered non-pathogenic and has been classified as Generally Regarded as Safe (GRAS) by the American Food and Drug Administration (FDA) for the production of citric acid, erythritol, and eicosapentaenoic acid [Groenewald *et al*. 2014]. In line with this, it is regarded as a 'safe-to-use' organism, a class 1 established by the project between the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA) [Bourdichon *et al*. 2012; Groenewald *et al*. 2014; EFSA NDA Panel *et al*. 2019].

1.2.1.3. Morphology

Y. lipolytica exhibits various colony shapes ranging from smooth and glistening to heavily convoluted and mat. Dimorphism is one of the characteristic features of *Y. lipolytica* (Figure 1.1). This yeast is able to undergo a true yeast-hyphae transition, which partly contributes to the diverse colony morphologies of *Y. lipolytica.* It is suggested that the regulation of yeast-to-hyphae transition is a multifactorial and complex mechanism in response to stressful environments and nutritional conditions [Perez-Campo and Dominguez, 2001; Torres-Guzman and Dominguez, 1997]. It is reported that there are several complexed factors on a yeast-to-hyphae transition like different genotypes, carbon sources, pH, nitrogen sources,

aeration, *etc*. In addition, it was recently determined that three Ras proteins (Ras1p, Ras2p, and Ras3p) are critical for dimorphic transition [Li *et al*. 2014]. The yeast form is preferred in submerged fermentation since it is associated with better rheological properties, while the hyphae forms may be desired in solid-state fermentation [Timoumi *et al*. 2017; Bellou *et al*. 2014]. Lipid bodies (LBs), often called lipid droplets or oleosomes, are another particular feature of *Y. lipolytica*. LBs are comprised mainly of triacylglycerides (TAG) as the inner core, steryl esters (SE) as the outer core, and phospholipid monolayer encapsulating the entire structure [Athenstaedt, 2010; Beopoulos *et al*. 2009]. Previously, it was known that LBs function as the storage of energy as a form of TAGs. More recent studies unveiled that LBs serve as dynamic organelles more than storage one involving the remobilization of fatty acids out of LBs by TAG lipases and SE hydrolases for providing biomass precursors and energy in response to the circumstance of the cells [Athenstaedt, 2010; Abghari and Chen, 2014].

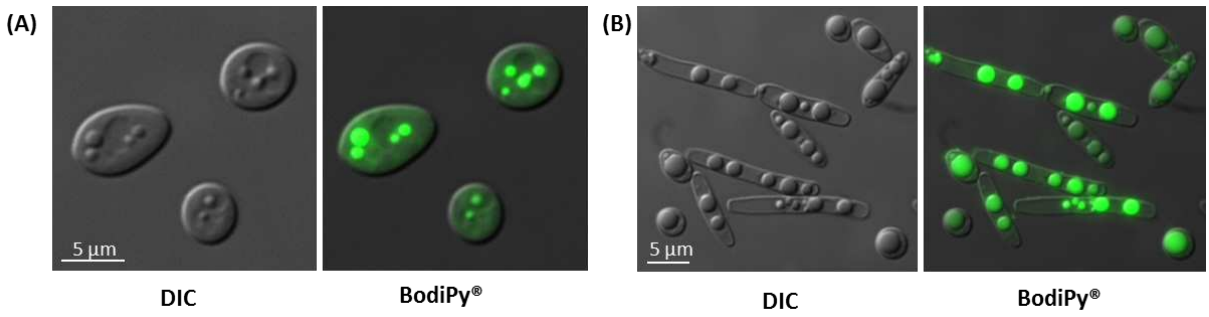


Figure 1.1. The morphology of *Y. lipolytica*.

(A) The wild-type (JMY2900) strain with yeast form. (B) The engineered strain (JMY3501) for higher lipid accumulation with hyphae form. Lipid bodies are stained in green with Bodipy® reagent.

1.2.1.4. Genome

Y. lipolytica has six chromosomes with high G+C contents of 49.6% - 51.7% and a genome size of approximately 20.1 to 22.1 MB [Barth and Gaillardin, 1996]. In addition, the sequence of the mitochondrial genome with a size of 47.9 KB from *Y. lipolytica* was reported [Kerscher *et al*. 2001]. Several inbred lines of *Y. lipolytica* have been obtained by different groups from the German (H222), French (W29), and American (CBS6214-2) [Nicaud, 2012]. The genome sequences of three strains CLIB122, WSH-Z06, and Po1f are available on the NCBI website (http://www.ncbi.nlm.nih.gov/genome) and the genomes of A101, E150, and H222 are available on GRYC (http://gryc.inra.fr), up to date. Due to their diversity and different properties, several strains are being used for research.

Genome annotations of CLIB122 and W29 show a total of 7144 and 8746 genes, respectively. Among them, 6472 and 7949 are protein-coding genes in CLIB122 and W29, respectively. Such numbers of ORFs are unexpectedly low given the large genome size of *Y. lipolytica* (20.5 MB) compared to that of *S. cerevisiae* (12 MB) [Dujon, 2015]. This reflects on a lower gene density

(46.3% in *Y. lipolytica vs.* 70.3% in *S. cerevisiae*), but also the large intergenic regions allowing to identify chromosomal sites can be used for gene integration [Morin *et al*. 2011].

Y. lipolytica is a typical heterothallic yeast that has two mating types, *Mat* A and *Mat* B. Most strains isolated in nature are haploid, a diploid strain will be produced only if two types mate. It was shown that both haploid and diploid states are stable under laboratory conditions [Thevenieau *et al*. 2009].

1.2.1.5. Carbon sources

Y. lipolytica has a characteristic ability to assimilate a wide range of hydrophilic and unusual hydrophobic carbon sources. Hydrophilic carbon sources include glucose, fructose, acetyl-D-glucosamine, ethanol, polyols (e.g. glycerol, erythritol, mannitol, and glucitol), and organic acids (acetate, lactate, succinate, and citrate) [Michely *et al*. 2013; Barth and Gaillardin, 1997]. The hydrophobic substrates are alkanes (C12 to C16), alkenes, fatty acids, fatty acid methyl esters, and triglycerides [Barth and Gaillardin, 1996; Kurtzman, 2011; Michely *et al*. 2013]. There have been studies on the identification of the substrate transporting- and consuming-pathways, and the engineering of these native pathways in order to maximize the consumption of carbon sources. For example, glycerol is a very attractive substrate for biolipid production because it serves as a scaffold in the generation of triacylglycerol (TAG), and it is low-cost substrate which could be obtained from industrial processes as a byproduct. Therefore, the utilization of glycerol *via* the glycerol-3-phosphate (G3P) pathway has been extensively studied in *Y. lipolytica*. The conversion of glycerol to biomass and lipid synthesis is improved in *Y. lipolytica* through metabolic engineering, such as overexpression of *GUT1* (glycerol kinase), deletion of *GUT2* (G3P dehydrogenase), and overexpression of heterologous genes (*dhaB1* (glycerol dehydratase) and *dhaB2* (reactivator of DhaB1) from *Clostridium butyricum, dhaT* (alcohol oxidoreductase) from *Shimwellia blattae*) [Workman *et al*. 2013; Beopoulos *et al*. 2008; Celińska and Grajek, 2013].

The efforts to broaden the substrate range of *Y. lipolytica* have been made in order to enable more sustainable and feasible production (Figure 1.2). Sucrose which can be obtained from industrially relevant materials like molasses cannot be utilized by *Y. lipolytica* wild-type strain. The introduction of *SUC2* gene encoding invertase from *S. cerevisiae* made *Y. lipolytica* consume sucrose and produce lipids and citric acid [Nicaud *et al*. 1989; Lazar *et al*. 2011; Lazar *et al*. 2013; Moeller *et al*. 2013; Hapeta *et al*. 2017]. Various engineering strategies such as introducing heterologous genes or awakening native genes resulted in the utilization of diverse sugars from monosaccharides to polysaccharides (i.e. galactose, xylose, cellobiose, starch, inulin, xylan, cellulose) by *Y. lipolytica* as depicted in Figure 1.2 [Ledesma-Amaro and Nicaud, 2016a; Abdel-Mawgoud *et al*. 2018]. Recently, the use of volatile fatty acids such as acetic, propionic, and butyric acids can be obtained from industrial waste treatment also has been gaining interests as low-cost substrates and building blocks for lipid-based bioproducts [Llamas *et al*. 2019].

1.2.2. Metabolic engineering tools for *Y. lipolytica*

Metabolic engineering of *Y. lipolytica* for biotechnological applications has required the development of efficient genetic tools. Previously, the efficiency and abundancy of genetic engineering tools in *Y. lipolytica* were relatively lower than the conventional yeast *S. cerevisiae*, which made a barrier of engineering to the researchers. However, the efficient engineering tools for *Y. lipolytica* have been greatly developed with the advances of synthetic biology, and now it is regarded as a model yeast among the non-conventional yeasts.

1.2.2.1. Plasmid

As a first step in developing *Y. lipolytica* genetically tractable, the episomal plasmids were constructed. As there is no natural episome in *Y. lipolytica*, replicative plasmids have been designed using chromosomal autonomously replicating sequence/centromere (*ARS*/*CEN*) replication origins [Fournier *et al*. 1993; Matsuoka *et al*. 1993]. Replicative plasmids are useful for transient protein expressions, such as Cre recombinase expression for marker excision [Fickers *et al*. 2003] and Cas9 expression for genome editing [Schwartz *et al*. 2016; Larroude *et al*. 2020]. The developed replicative plasmid by fusing a promoter upstream from the CEN element was shown to increase the plasmid copy number by 80% [Liu *et al*. 2014].

For the purpose of metabolic engineering, the integrative plasmid is preferable because of its stability and higher copy number more than 30 copies than those of the episomal plasmid [Juretzek et al. 2001; Nicaud et al. 2002]. Gene integration by multi-copy was achieved in *Y. lipolytica* by homologous recombination at specific target sites like targeting repeated sequences (e.g. zeta, rDNA) or a defective marker sequence [Juretzek *et al*. 2001; Nicaud *et al*. 2002; Le Dall *et al*. 1994; Bordes *et al*. 2007]. Zeta sequence (*YLT1*, the long terminal repeated sequence of *Y. lipolytica*'s retrotransposon) has been used for either zeta-targeting insertion in zeta-containing strains or random integration in non-zeta-containing strains [Pignède *et al*. 2000]. In the strains do not have *YLT1*, a specific locus integration platform has been developed that using *LEU2* locus *via* a single cross-over and homologous recombination [Bordes *et al*. 2007; Juretzek *et al*. 2001].

1.2.2.2. Transformation

It was reported that *Y. lipolytica* uptakes exogenous DNA with moderate efficiency compared to *S. cerevisiae*, with a transformation efficiency exceeding 10^7 transformants per µg DNA [Kawai *et al*. 2010]. Most transformation methods in *Y. lipolytica* use the chemical transformation starting with a lithium acetate (LiAc) treatment, followed by incubation in polyethylene glycol (PEG). An early protocol using the LiAc method required an excessive amount of carrier DNA to achieve 10^4 transformants per µg DNA per 10^8 viable cells [Davidow *et al*. 1985]. Later studies have optimized this method by developing a one-step protocol where cells can be directly prepared from a YPD plate [Chen *et al*. 1997], increasing site-

specific integration by using a strain with a zeta docking platform [Bordes *et al*. 2007] and more developed protocol for high-throughput transformation in a 96-well plate has also been reported [Leplat *et al*. 2015]. Each of these methods reports maximum transformation efficiencies ranging from 10^3 - 10^4 transformants per µg DNA, but limited efficiency for site-directed integration. As well as chemical transformation methods, an electroporation method has been developed for *Y. lipolytica* with efficiency up to 10^4 transformants per µg DNA [Wang *et al*. 2011]. Recently, developed electroporation method without carrier DNA was reported with a transformation efficiency of 1.6 x 10^4, 2.8 x 10^4 transformants per µg linear DNA, episomal plasmid DNA, respectively [Markham *et al*. 2018].

1.2.2.3. Promoter

In metabolic engineering, a fine-tuned gene expression is required to ensure optimal flows in related pathways or to avoid metabolic burdens. Among several possible ways to modulate the expression of target gene, selecting appropriate promoters depending on their strength has been mostly used in metabolic engineering. Therefore, there have been various studies on the identification of promoter functional sequence and its engineering for different levels of expression.

In *Y. lipolytica*, the promoter from the *XPR2* gene coding an alkaline extracellular protease was first isolated and characterized [Ogrydziak and Scharf, 1982]. The constitutive promoter pTEF from *TEF1* encoding translation elongation factor-1 was identified and has been used the most largely until now [Müller *et al*. 1998; Larroude *et al*. 2018]. The characterization of other native promoters, mostly associated with lipogenic pathway, was followed and used for gene expression in many studies.

As well as native promoters, hybrid promoters have been gaining interests through synthetic biology. Madzak and colleagues identified the upstream activation sequences (UAS) of pXPR2, and firstly developed hybrid promoters containing up to four copies of UAS fused with a core *LEU2* promoter [Madzak *et al*. 2000]. These hybrid promoters showed a linear increase in promoter strength as a function of the number of tandem UAS elements. After this study, the construction of hybrid promoters by increasing UAS tandem repeats or fusing different core promoters to improve the expression level has been one of the main strategies to develop the promoter system [Shabbir-Hussain *et al*. 2016; Trassaert *et al*. 2017; Larroude *et al*. 2018]. From the synthetic biological view, the library of promoters with different combinations of UAS and core promoter is a great strategy to screen the optimal expression.

The development of an inducible promoter system is also necessary for the fermentation process which required the separation of growth and production phase. The pXPR2 promoter is induced by hydrophobic substrates such as alkanes and lipids. Other inducible promoters (pPOX2, pPOT1, pLIP2, and pALK1) are also identified as lipid inducible [Juretzek *et al*. 2000; Sassi *et al*. 2016]. Recently, the genes (*EYK1* and *EYD1*) involved in the erythritol catabolic pathway were identified, and the promoters of each gene were characterized and developed

as inducible promoters [Carly *et al*. 2017a, b; Trassaert *et al*. 2017]. The same group further constructed the hybrid promoter of pEYK1 with additional tandem copies of $UAS1_{EYK1}$, showing an improved expression level [Trassaert *et al*. 2017].

1.2.2.4. Terminator

Terminators being essential for completing the transcription process are also known as the important element of gene expression. Similar to the promoter, the terminator of *XPR2* and *LIP2* were identified and used for gene expression, and the heterologous terminator Tcyc1 from *S. cerevisiae* was also shown to be functional in *Y. lipolytica* [Franke *et al*. 1988; Pignède *et al*. 2000; Mumberg *et al*. 1995]. Though there are much fewer studies on terminators than promoters, the importance of terminators on gene expression has been emerging recently. A subset of short synthetic *S. cerevisiae* terminators was assessed in *Y. lipolytica* [Curran *et al*. 2015].

1.2.2.5. Selection marker

In *Y. lipolytica*, both auxotrophy and dominant markers are available. Auxotrophy markers, widely used in yeast, remain as the best performing selective markers in *Y. lipolytica* [Barth and Gaillardin, 1996]. Up to now, *URA3* encoding orotidine 5-phosphate decarboxylase, *LEU2* encoding β-isopropyl malate dehydrogenase, and *LYS5* encoding phosphopantetheinyl transferase are available, which can be complemented by uracil, leucine, and lysine, respectively. Dominant markers such as *hph* from *E. coli (*conferring hygromycin resistance), *nat1* from *Streptomyces noursei* (conferring nourseothricin resistance), AHAS W572L mutant of *Y. lipolytica* (conferring chlorimuron ethyl herbicide resistance), *guaB* from *E. coli* (conferring mycophenolic acid resistance), and *ble* from *Streptoalloteichus hindustanus* (conferring zeocin resistance) are available in *Y. lipolytica* [Otero *et al*. 1996; Kretzschmar *et al*. 2013; Wagner *et al*. 2018; Tsakraklides *et al*. 2018]. The *SUC2* gene encoding invertase and *EYK1* gene encoding erythrulose kinase can also be used as a dominant marker as well as the utilization of a specific carbon source [Nicaud *et al*. 1989; Vandermies *et al*. 2017]. Recently, *ptxD* encoding phosphite dehydrogenase from *Pseudomonas stutzeri* and *AMD1* (*YALI0E34771g,* encoding acetamidase) was also shown as a functional marker in *Y. lipolytica* [Shaw *et al*. 2016; Hamilton *et al*. 2020].

In order to use the selective markers several times for multi-gene editing in metabolic engineering, the marker rescue and reuse system has been developed [Fickers *et al*. 2003]. By combining the Cre-lox recombination system and the sticky-end polymerase chain reaction (SEP) method, the marker was excised at a frequency of 98% allowing multiple gene insertions and deletions in *Y. lipolytica*. Very recently, the genome-editing without marker became possible with CRISPR/Cas9 system but the efficiency still should be improved (More details on CRISPR/Cas9 system is described in the part 1.2.6).

1.2.2.6. Gene assembly tools

Traditionally, the genes were cloned to the plasmid that already harboring all the essential elements (origin of replication, selective marker, promoter, terminator, and optionally homologous sequence for the integration) by restriction enzyme digestion and ligation. As metabolic engineering developed, the multiple gene integration at the same time is highly required which is not efficient through the traditional cloning method regarding time and cost. Consequently, significant efforts are being made to develop DNA assembly techniques that would allow a more efficient multi-gene cloning.

The Gateway cloning method employs site-specific recombination [Hartley, 2000]. The gene of interest, which has been cloned into the entry vector, is transferred into the destination vector at *att* recombination sites. The obtained expression vector is then digested to release the expression cassette and used to transform *Y. lipolytica* [Leplat *et al*. 2018].

The Gibson assembly method requires the overlap of around 30 bp between each part. Their ends are fused by the sequential processing of an exonuclease, a polymerase, and DNA ligase [Gibson *et al*. 2009]. This method allows multiple DNA fragments to be assembled regardless of their length or end compatibility. In *Y. lipolytica*, the Gibson method was used to construct the plasmid with the genes of the xylose pathway [Rodriguez *et al*. 2016]. It was also used for the deletion of *GSY1* encoding glycogen synthase in *Y. lipolytica* [Bhutada *et al*. 2017].

The BioBricks method uses four compatible restriction enzyme sites (EcoRI, XbaI, SpeI, and PstI) that enable the modular genetic engineering and the reuse of parts [Knight, 2003]. In 2017, BioBrick-based vectors for *Y. lipolytica*, named YaliBricks, were developed [Wong *et al*. 2017]. From this system, the authors showed the construction of a five-gene biosynthetic pathway of violacein within one week.

The Golden Gate (GG) modular cloning system utilizes type IIS restriction enzyme cutting outside of their recognition sites [Engler *et al*. 2008]. Each part has 4-nt overhangs being compatible to other parts, therefore, the parts can be assembled all together in a defined order. Celińska and colleagues developed GG system for *Y. lipolytica* and validated the system through the assembly of the genes in the carotenoid pathway [Celińska *et al*. 2017].

1.2.2.7. Gene deletion tools

The traditional gene deletion of *Y. lipolytica* was done by homologous recombination with homologous flanking fragments (up to 1 kb). Fickers and colleagues developed a knock-out system for multiple gene deletions in *Y. lipolytica* [Fickers *et al*. 2003]. The system includes the homologous sequence of the promoter and terminator of the target gene, and an excisable marker flanked by a *loxP* and a *loxR* element permitting the excision *via* the action of the Cre recombinase.

More recently, engineered nucleases that cleave specific DNA sequences *in vivo* have been developed for targeted mutagenesis. The programmable nucleases that recognize and bind to specific DNA sequences, cause double-strand breaks (DSBs), and then induce non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ introduces random insertions and deletions into the genome.

Transcription activator-like effector nucleases (TALENs) were created by fusing transcription activator-like effectors (TALE) to the catalytic domain of the *FokI* endonuclease. By customizing the TALE DNA binding domain, the DNA DSBs can be directed to occur at a specific target site [Christian *et al*. 2010]. This technique was recently applied in *Y. lipolytica* to generate fatty acid synthase (FAS) mutants and proved to be efficient in inducing targeted genome modifications [Rigouin *et al*. 2017]. The mutants from NHEJ repair at the targeted locus shown to be very efficient, 97% of transformants, while HR-mediated repair occurred in 40% of transformants.

A clustered regularly interspaced short palindromic repeats CRISPR/Cas9 system is another example using a nuclease, Cas9-targeted nuclease [Jinek *et al*. 2012]. The Cas9 can be programmed with guide RNA to generate DSBs at a specific DNA site. Schwartz and colleagues reported the marker-free gene disruption and integration by the CRISPR/Cas9 system adapted from *Streptococcus pyogenes* in *Y. lipolytica* [Schwartz *et al*. 2016]. The codon-optimized human Cas9 was also shown to be functional in the genome editing of *Y. lipolytica* [Gao *et al*. 2016]. Holkenbrink and colleagues created a toolbox, EasyCloneYALI, for easily performing genome editing in *Y. lipolytica* through CRISPR/Cas9 technology [Holkenbrink *et al*. 2018]. The EasyCloneYALI includes standardized promoters, genes, and plasmids that can be reused and exchanged. Dual CRISPR/Cas9 strategy using paired guide RNA to create complete gene knockout was also described in *Y. lipolytica* [Gao *et al*. 2018]. There are also variants of the CRISPR/Cas9 system, CRISPRi for repressing the target gene and CRISPRa for activating transcription of the target gene [Schwartz *et al*. 2017; Schwartz *et al*. 2018].

With the development of synthetic biology, a series of emerging tools have changed the scope of engineering possibilities in *Y. lipolytica* which was a major drawback compared to the conventional hosts. Among several, the GG assembly, TALEN, and CRISPR/Cas9 systems helped to advance the engineering of *Y. lipolytica* with simplicity, efficiency, and affordability. It is expected that further progress of engineering tools customized to *Y. lipolytica* will accelerate the development of *Y. lipolytica* as one of the most promising chassis for biotechnological applications.

1.2.3. The biotechnological applications of *Y. lipolytica*

Y. lipolytica has been gaining a lot of interest as a chassis for the production of valuable compounds. Thorough studies of this host on engineering tools, optimization of fermentation, and system biology resulted in broad range of compounds can be produced in *Y. lipolytica* from lab-scale to commercial scale. Representative products are described in Figure 1.2. and this part.

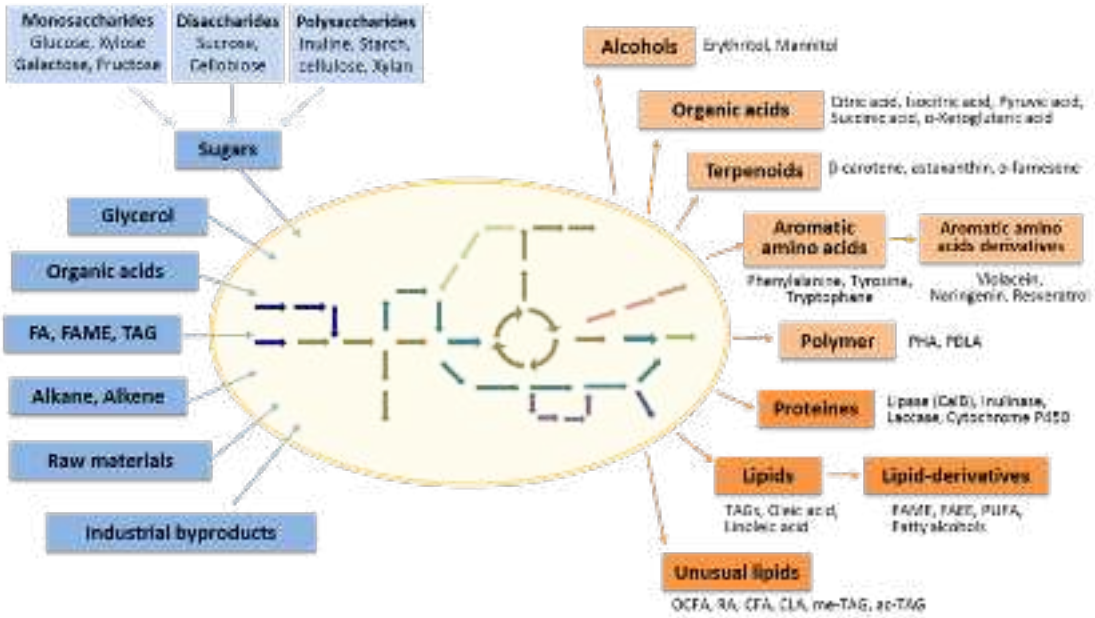


Figure 1.2. A brief description of *Y. lipolytica* as a chassis in biotechnological application.

The substrates that can be utilized by native or engineered pathways in *Y. lipolytica* are indicated in the blue boxes. The type of products obtainable from *Y. lipolytica* is indicated in the orange boxes, the detailed products are written near the boxes. Abbreviations: FA, fatty acids; FAME, fatty acids methyl ester; TAG, triacylglycerol; FAEE, fatty acid ethyl ester; PUFA, polyunsaturated fatty acid; PHA, polyhydroxyalkanoate; PDLA, poly-D-lactic acid; OCFA, odd-chain fatty acid; RA, ricinoleic acid; CFA, cyclopropanated acid; CLA, conjugated linoleic acid; me-TAG, methylated TAG; ac-TAG, acetylated TAG.

1.2.3.1. Proteins

Y. lipolytica can produce and secrete many endogenous enzymes such as alkaline protease, extracellular protease, RNase, phosphatase, lipase, and esterase. Under convenient inducing conditions, *Y. lipolytica* secrets very large amounts (1 - 2 g/L) of alkaline extracellular protease (AEP) encoded by the *XPR2* gene [Tobe *et al*. 1976], which is a model protein in protein secretion research. Lipase (EC 3.1.1.3) hydrolyzing TAGs into free fatty acids and glycerol is also one of representative protein which can be produced in *Y. lipolytica*. Among several lipases, Lip2p, Lip7p, and Lip8p are well studied in *Y. lipolytica*. Lip2p can be used as a therapeutic tool for exocrine pancreatic insufficiency treatment. Nicaud and colleagues showed the high production level of lipase (11,500 U/ml in batch and 90,500 U/ml in fed-

batch, respectively) with glucose as a carbon source [Nicaud *et al*. 2002]. Turki *et al*. reported over 10,000 U/ml of lipase activity by *Y. lipolytica* grown on glucose *via* a stepwise fed-batch strategy [Turki *et al*. 2010]. Lipase production with raw material as the carbon source has received increasing interest to lower the cost of production. Several oils such as rapeseed oil, olive oil, methyl oleate, and crude glycerol were assessed, the highest production level of lipase activity (2,760 U/ml) was obtained from rapeseed oil [Kamzolova *et al*. 2005].

Apart from endogenous enzymes, *Y. lipolytica* is regarded as an ideal expression host for heterologous protein production such as inulinase, laccase, epoxide hydrolase, many other functional proteins. Inulinase (EC 3.2.1.7) hydrolyzing inulin is used as a renewable raw material for industrial applications. The introduction of *INU1* gene coding exo-inulinase from *Kluyveromyces marxianus* in *Y. lipolytica* resulted in the production of inulinase up to 47.5% of crude protein content and 20.1 g/L of DCW under optimized conditions [Cui *et al*. 2011]. The production of endo-inulinase from *Arthrobacter* sp. S37 in *Y. lipolytica* was also reported with the 16.7 U/ml of endo-inulinase activity [Li *et al*. 2012]. Another example of heterologous protein production in *Y. lipolytica* is cytochrome P450, playing an important role in oxidative and reductive metabolism. Nthangeni and colleagues successfully obtained the human cytochrome P450 CYP1A1 by expressing its functional coding gene in *Y. lipolytica* [Nthangeni *et al*. 2004].

1.2.3.2. Organic acids

The overall oleaginous nature of *Y. lipolytica* and its metabolism also mean that this host has a high potential to produce TCA cycle intermediates. The intermediates of TCA cycles, such as citric acid, isocitric acid, α-ketoglutaric acid, and succinic acid are important building-block chemicals with potential applications. This ability is most pronounced in the industrial production of citric acid using *Y. lipolytica*. Since the first report of citric acid production in *Y. lipolytica* ATCC 20114 wild-type strain [Akiyama *et al*. 1973], the *Y. lipolytica*-based fermentation process for citric acid production has been extensively investigated. It is known that limited nitrogen condition induces a rapid drop in the AMP concentration, which ultimately leads to isocitrate dehydrogenase inactivation, then the high amount of citric acid is synthesized and transported into the cytosol. Arzumanov and colleagues presented that 105.4 g/L of citric acid production by mutant *Y. lipolytica* grown on ethanol in repeat-batch cultivation [Arzumanov *et al*. 2000]. Using glucose as the sole carbon source, 42.9 g/L of citric acid was produced by *Y. lipolytica* ACA-DC 50109 in nitrogen-limited cultures [Papanikolaou *et al*. 2006]. Many different carbon substrates, such as ethanol, n-paraffin, glucose, glycerol, rapeseed oil, sunflower oil, and olive mill wastewater have been used for citric acid accumulation. Kamzolova *et al*. reported the high citric acid concentration of 150 g/L from mutant strain *Y. lipolytica* N15 grown on sunflower oil as a carbon source [Kamzolova *et al*. 2008]. The highest production of citric acid (197 g/L) was obtained from long-term repeated-batch cultures from crude glycerol [Rywinska and Rymowicz, 2010].

Isocitric acid, a useful chiral building block, has also been produced in *Y. lipolytica*. It can be produced during the citric acid accumulation process. The ratio of isocitric acid to citric acid always reflects the potential yield and can be shifted using different strategies such as changing culture conditions and generating the mutant for a high yield of isocitric acid production. The overexpression of *ICL1* (isocitrate lyase) or *ACO1* (aconitase) has shown to affect the ratio of citric acid/isocitric acid [Förster *et al*. 2007, Holz *et al*. 2009]. Kamzolova and colleagues screened 60 yeast strains that could produce isocitric acid using rapeseed oil, and the high production of isocitric acid (70.6 g/L) was obtained from *Y. lipolytica* VKM Y-2373 strain [Kamzolova *et al*. 2013]. The same group demonstrated the improved production of isocitric acid up to 86 g/L and 20 g/L of citric acid by mutagenesis.

α-Ketoglutaric acid, can be synthesized by isocitrate dehydrogenase in the TCA cycle, is an important metabolic intermediate in industrial applications. Generally, α-ketoglutaric acid can be produced in large amounts under carbon excess and thiamine limitation. The 49 g/L of α-ketoglutaric acid was produced by a thiamine-auxotrophic mutant *Y. lipolytica* N1 under optimized conditions [Chernyavskaya *et al*. 2000]. In order to improve the α-ketoglutaric acid yield, Zhou and colleagues regulated the carbon flux through expressing *ACS1* (acetyl-CoA synthetase) from *S. cerevisiae* and *ACL* (ATP-citrate lyase) from *Mus musculus* in *Y. lipolytica*, resulting in 56.5 g/L of α-ketoglutaric acid [Zhou *et al*. 2012]. The authors further engineered this strain by introducing *PYC* (pyruvate carboxylase) from *Rhizopus oryzae* to decrease pyruvic acid, the competitive intermediates. This resulted in an increase of α-ketoglutaric acid production up to 62.5 g/L [Yin *et al*. 2012]. Another group developed a biotechnological process with overexpression of *IDP1* (isocitrate dehydrogenase) and *PYC1*, which produced the highest level of α-ketoglutaric acid, 186 g/L from raw glycerol under optimized condition [Yovkova *et al*. 2014].

Beyond the intermediates in TCA cycle, non-native organic acid, itaconic acid, was produced in *Y. lipolytica*. As a platform chemical being converted to a variety of petroleum-derived products, itaconic acid is gaining a lot of interest. The heterologous expression of *cis*-aconitic acid decarboxylase from *Aspergillus terreus* and cytosolic expression of native aconitase allowed the production of itaconic acid, 4.6 g/L from bioreactor fermentation [Blazeck *et al*. 2015].

1.2.3.3. Sugars

Erythritol that can be natively produced in *Y. lipolytica*, has been an attractive product for the food industry because of its use as a low-calorie sweetener. By screening strain which does not produce citric acid, a substantial erythritol titer (170 g/L) was achieved on crude glycerol [Rymoxicz *et al*. 2009]. Recently, the genes involved in the erythritol catabolism were identified [Carly and Fickers, 2018], and engineering these genes in *Y. lipolytica* CGMCC7326 strain allowed the production of an erythritol titer of 178 g/L in the medium from 300 g/L of initial glucose within 84 h with a productivity of 2.1 g/(L·h) and a yield of 0.59 g/g [Cheng *et*

al. 2018]. Very recently, 180 g/L of erythritol was produced at a 500-L scale in raw glycerol fed-batch mode after 144 h of cultivation and a yield was 0.53 g/g [Chi *et al*. 2019]. Using the same strain, 165 g/L of erythritol was obtained within 146 h in a 20 L bioreactor, showing the possibility of the erythritol production process at pilot-scale [Fickers *et al*. 2020].

1.2.3.4. Aromatic amino acid (AAA) derivatives

The aromatic amino acids, L-phenylalanine, L-tryptophan, and L-tyrosine constitute a considerable market volume in food, chemical, and pharmaceutical industries as a functional compound by itself or as a precursor of derivatives [Bongaerts *et al*. 2001]. The biosynthetic pathways of AAAs and their regulation have been extensively studied, and several metabolic engineering approaches have been applied in order to improve the production overcoming the limitation of traditional production from plant or chemical synthesis.

2-Phenylethanol (2PE) is an aromatic compound having rose-like odor, applicable to cosmetics and food production. It can be synthesized *via de novo* or bioconversion of L-phenylalanine (Ehrlich pathway) [Celińska *et al*. 2013]. Celińska and colleagues screened the best 2PE producing-strain among six *Y. lipolytica* wild-type strain, 1.98 g/L of 2PE was produced from L-phenylalanine bioconversion *via* the Ehrlich pathway. The same group further engineered the Ehrlich pathway to expand product range (2PE, 3-methylbutanol, 3-methylbutanoic acid, *etc*.) depending on the amino acid precursors [Celińska *et al*. 2019].

Violacein is a purple pigment that can be used in the cosmetics and fabric industry as well as the pharmaceutical industry due to its interesting bioactivities such as antibacterial, anticancer, antiviral, and antioxidant properties [Wong *et al*. 2017]. It is revealed that the gene cluster *vioABCDE* or shortened operon *vioABCE* is responsible for the biosynthesis of violacein from L-tryptophan. The recombinant expression of *vioABCE* cluster from *Chromobacterium violaceum* in *E. coli* resulted in the accumulation of deoxyviolacein [Rodrigues *et al*. 2013]. In *Y. lipolytica*, the gene cluster *vioABCDE* from *C. violaceum* took advantage of violet color from violacein for quick visual screening tools during the evaluation of DNA assembly strategies [Wong *et al*. 2017]. The extraction and separation of violacein from engineered *Y. lipolytica* strains were optimized, showing the possibility of the downstream process for microbial violacein production [Kholany *et al*. 2019].

Naringenin is the key intermediate flavonoid that exhibits a wide variety of antioxidant, antimicrobial, anti-inflammatory, and human health-related properties [Koopman *et al*. 2012]. Naringenin is synthesized from L-phenylalanine or tyrosine with multiple steps. In *S. cerevisiae*, the production of naringenin was reported by introducing naringenin biosynthesis genes from *Arabidopsis thaliana* [Koopman *et al*. 2012]. This study further engineered *S. cerevisiae* by increasing the copy number of naringenin biosynthesis genes, improving precursor supply, and reducing byproduct formation, resulted in extracellular production of naringenin over 400 µM [Koopman *et al*. 2012]. In *Y. lipolytica*, *de novo* production of naringenin was reported very recently [Lv *et al*. 2019; Palmer *et al*. 2020]. Lv and colleagues optimized copy number of

naringenin synthesis genes (chalcone synthase, *CHS*; cytochrome P450 reductase, and *CPR*) and engineered precursor pathway, resulting in 252.4 mg/L of naringenin in flask level. The strategy rewiring β-oxidation for improved triacetic acid lactone and the introduction of naringenin biosynthesis genes were demonstrated in *Y. lipolytica* [Palmer *et al*. 2020]. From fed-batch fermentation, 898 mg/L of naringenin was produced in the engineered *Y. lipolytica* strain. This study also exhibited the production of other AAA derivatives like resveratrol and bisdemethoxycurcumin which also have various applications.

1.2.3.5. Non-native products

Polyhydroxyalkanoates (PHA) are important biodegradable chemical biopolymers. PHA can be synthesized from 3-hydroxyacyl-CoA, which is an intermediate in the β-oxidation pathway. Therefore, the modification of the β-oxidation cycle to produce PHA was reported in *Y. lipolytica* [Haddouche *et al*. 2010]. The same group also described that the expression of β-oxidation multifunctional enzyme (MFE2) subunits to increase precursor of PHA, 3-hydroxyacyl-CoA, allowed the synthesis of PHA up to 7% of dry cell weight in *Y. lipolytica* [Haddouche *et al*. 2011]. More recently, the mutation of PhaC synthase from *Pseudomonas aeruginosa* showed the improved synthesis of PHA composed of 3-hydroxyoctanoic, decanoic, dodecanoic, and tetradecanoic acids in *Y. lipolytica*. The accumulation of PHA reached more than 25% of DCW in this study [Rigouin *et al*. 2019].

The oleaginous nature of *Y. lipolytica* effectively stems from a high flux through acetyl-CoA and malonyl-CoA that can be diverted into diverse valuable compounds. Particularly, the production of terpenoids was achieved through overexpression of the key native mevalonate pathway genes, *HMG1* and/or *GGS1*. When these strains were combined with heterologous pathways, modest titers of terpenoids have been produced. The expression of *CarB* and *CarRP* from *Mucor circinelloides* led to 2.22 mg/g dry cell weight of β-carotene [Gao *et al*. 2014]. A multi-functional gene, *carS* from *Schizochytrium* sp. led to produce 0.41 mg/g DCW of β-carotene [Gao *et al*. 2017]. The same group further engineered the strain by overexpressing *ERG10* and deleting the genes in the competitive pathway, resulting in 4 g/L of β-carotene production. The screening of high β-carotene producing strain through promoter shuffling and integrating the biosynthetic genes (*carB*, *carp*, and *GGS1*) in multi-copy were shown to produce 6.5 g/L of β-carotene production from *Y. lipolytica* in a bioreactor [Larroude *et al*. 2018]. Other terpenoids of interest as well as carotenoids also have been explored in *Y. lipolytica*. The introduction of an additional gene from *Pantoea ananatis* to carotenoid-producing strain resulted in 54.6 mg/L of astaxanthin production which can be used as a food and feed additive [Kildegaard *et al*. 2017]. The production of α-farnesene in *Y. lipolytica* reached a titer of 260 mg/L by fusing α-farnesene synthase to *ERG20* [Yang *et al*. 2016].

1.2.3.6. Lipids

Microbial lipids have emerged in recent years as alternative sources of fuels and chemicals. They present several advantages over plant oils, such as faster growth, less dependent on environmental conditions, better adaptability to market needs, and the use of renewable substrates. The detailed lipid synthesis and the status of metabolic engineering for lipid production in *Y. lipolytica* is described in the next part 1.2.4.

1.2.4. Lipid production in *Y. lipolytica*

Y. lipolytica has been gaining a lot of interest as a chassis for the production of value-added compounds as described in the part 1.2.3. and Figure 1.2. Particularly, its oleaginous phenotype makes *Y. lipolytica* as the most attractive host for the production of lipids and lipid-derivatives. Therefore, there has been a thorough understanding of lipid metabolism as described in Figure 1.3 and extensive engineering strategies in order to produce these oleochemicals at high titer, rate, and yield for commercial exploitation.

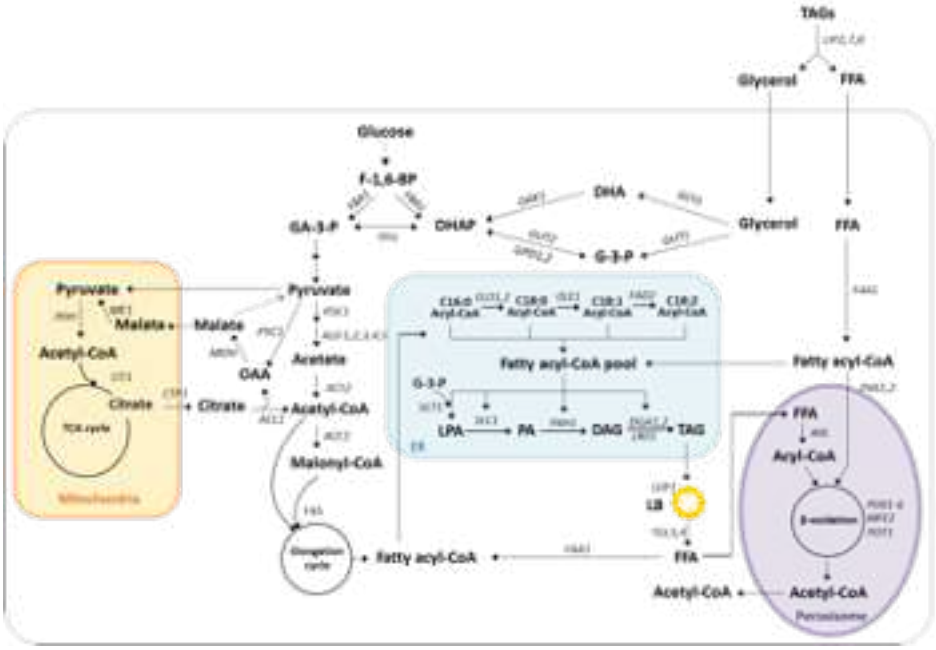


Figure 1.3. Overview of metabolic pathways involved in the synthesis of fatty acids in *Y. lipolytica*.

Abbreviations: F-1,6-BP, fructose-1,6-biphosphate; GA-3-P, Glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; G-3-P, glycerol-3-phosphate; OAA, oxaloacetate; FFA, free fatty acid; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; ER, endoplasmic reticulum; LB, lipid body; *FBA1*, fructose biphosphate aldolase; *TPI1*, triosephosphate isomerase; *ME1*, malic enzyme; *PYC1*, pyruvate carboxylase; *MDH*, malate dehydrogenase; *PDH*, pyruvate dehydrogenase; *CIT1*, citrate synthase; *CTP1*, citrate/malate transporter; *PDC1*, pyruvate decarboxylase; *ALD1-5*, aldehyde dehydrogenase; *ACS2*, acetyl-CoA synthetase; *ACC1*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *FAA1*, fatty acyl-CoA synthetase; *GPD1* and *GPD2*, glycerol-3-phosphate dehydrogenase; *GUT1* and *GUT2*, glycerol kinase; *DAK1*, Dihydroxyacetone kinase; *GCY1*, glycerol dehydrogenase; *ELO1, 2*, elongase; *OLE1*, *Δ*-9 desaturase; *FAD2*, *Δ*-12 desaturase; *SCT1*, Glycerol-3-phosphate O-acyltransferase; *SLC1*, fatty acyl transferase; *PAH1*, Phosphatidate phosphatase; *DGA1, 2*, DAG acyltransferase; *LRO1*, Phospholipid:diacylglycerol acyltransferase; *LDP1*, lipid droplet protein; *TGL3, 4,* TAG lipase 3 and 4; *AAL*, Acyl/aryl-CoA ligase; *POX1-6*, acyl-CoA oxidases 1-6; *MFE2*, peroxisomal multifunctional enzyme 2; *POT1*, 3-ketoacyl-CoA thiolase; *LIP2,7*,and *8*, lipase; *PXA1, 2*, peroxisomal ATP-binding cassette transporter complex; Dash lines, multistep metabolic route.

1.2.4.1. Lipid metabolism

1.2.4.1.1. Lipid accumulation

There are two different pathways for the accumulation of lipids in *Y. lipolytica* (i) the *de novo* synthesis, starting from the acetyl-CoA and malonyl-CoA building blocks and (ii) the *ex novo* lipid accumulation pathway involving the uptake of fatty acids, oils and TAGs from the culture medium and their accumulation in an unchanged or modified form within the cell (Figure 1.3).

The *de novo* FAs synthesis occurs in the cytosol from the building block acetyl-CoA, which can come from the amino acid degradation pathway and the reaction of ACS (acetyl-CoA synthetase encoded by *YALI0F05962g*), PDH (pyruvate dehydrogenase complex), or ACL (ATP citrate lyase encoded by *YALI0E34793g* and *YALI0D24431g*). Interestingly, *ACL* genes are present only in the genome of oleaginous yeast, being a hallmark of oleaginous microorganisms. The synthesized acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase encoded by *YALI0C11407g* (*ACC1*).

The fatty acid synthase (FAS) enzymatic complex (encoded by *YALI0B15059g* and *YALI0B19382g*) produces acyl-CoA using acetyl-CoA as an initiation molecule and malonyl-CoA as an elongation unit by adding two carbons to the fatty acid backbone. Most of the released acyl-CoA corresponds to the chain lengths with 16 or 18 carbons. Thereafter, these 16:0 and 18:0 activated molecules can be the substrates of elongases and desaturases. Elongases are responsible for chain elongation and produces long-chain fatty acids (ELO1 encoded by *YALI0B20196g*) or very long-chain fatty acids (ELO2 encoded by *YALI0F06754g*). Desaturases, located in the ER, catalyze either the first desaturation (OLE1; Δ9 desaturase encoded by *YALI0C05951g*) to produce palmitoleic (16:1) or oleic acid (18:1), or the second double bond formation (Δ12 desaturase; FAD2 encoded by *YALI0B10153g*) to produce primarily linoleic acid (18:2).

The resulting acyl-CoA products are condensed with one glycerol-3-phosphate (G3P) to generate lysophosphatidic acid (LPA) by SCT1 (G3P acyltransferase encoded by *YALI0C00209g*), then phosphatidic acid (PA) by SLC1 (LPA acyltransferase encoded by *YALI0E18964g*), and finally diacylglycerol (DAG) by PAH1 (phosphatidate phosphatase encoded by *YALI0D27016g*) before forming triacylglycerol (TAG). TAGs are formed through the Kennedy pathway, where DAG is converted into TAG either from phospholipid by *LRO1* (phospholipid: diacylglycerol acyltransferase encoded by *YALI0E16797g*) or from acyl-CoA by *DGA1* and *DGA2* (DAG acyltranferase encoded by *YALI0E32769g* and *YALI0D07986g*, respectively). As well as TAG (85% in LB), little amounts of SEs (8% in LB) are also formed from acyl-CoA by ARE1 (encoded by *YALI0F06578g*) being present in LB.

The *ex novo* lipid accumulation requires hydrolysis of the hydrophobic substrate and incorporation of the released fatty acids. *Y. lipolytica* secretes an emulsifying agent to the medium (liposan) to reduce the size of hydrophobic droplets. Then, TAGs are hydrolyzed into glycerol and FFAs by lipases, the main ones being Lip2p, Lip7p, and Lip8p encoded by

YALI0A20350g, *YALI0D19184g*, and *YALI0B09361g*, respectively. FFAs enter cells *via* single or multiple transporters that remain to be identified. Internal FFAs can either (1) be activated by Faa1p encoded by *YALI0D17864g*, and stored in lipid bodies as TAGs, or (2) be transported into peroxisome *via* the heterodimer Pxa1p/Pxa2p (encoded by *YALI0A06655g* and *YALI0D04246g*, respectively), and go through β-oxidation.

1.2.4.1.2. Lipid remobilization

Apart from lipid synthesis pathway, oleaginous yeasts store their lipids mostly in the form of TAG (80-90% of the neutral lipid fraction) and the rest in the form of steryl esters (SE). These storage molecules are not suitable for integration into phospholipid bilayers; therefore, they are accumulated in a specialized compartment for neutral lipid storage, the lipid bodies (LBs). This organelle consists of the lipid core encased in a phospholipid monolayer in which many proteins with diverse functions are embedded. Recent proteomic studies revealed the important role of these proteins in lipid metabolism (synthesis, storage, trafficking, and degradation of lipids) as well as lipid storage [Athenstaedt, 2010; Brown, 2001; Fujimoto *et al*. 2008; Zweytick *et al*. 2000].

Lipid bodies are dynamic structures that represent an additional carbon source once the nutrients of the media have been depleted. Therefore, TAGs are substrates of intracellular lipases, which will release free fatty acids at the surface of the lipid bodies. In contrast to *S. cerevisiae*, there are two genes in *Y. lipolytica*: *TGL4* (*YALI0F10010g*), an active lipase localized in the interface of the lipid bodies, and *TGL3* (*YALI0D17534g*), a positive regulator of Tgl4p. Notably, the spatial organization of these two enzymes varies depending on the media composition and the physiological state of the cell, suggesting a complex regulation [Dulermo *et al*. 2013].

1.2.4.1.3. FA transport and activation

FFAs, from either the extracellular media or from TGL lipase activity, must subsequently be activated and transported into the peroxisome for β-oxidation. FFAs in the peroxisome must be activated by the *AAL* genes to be degraded by the β-oxidation enzymes. However, AAL activation requires ATP, which enters the peroxisome through the Ant1p transporter (encoded by *YALI0E03058g*). An alternative remobilization pathway mediated by Fat1p (encoded by *YALI0E16016g*) directly transporting FA from the lipid bodies to the peroxisome is also suggested. Notably, alternative pathway shows the different chain-length preferences [Dulermo *et al*. 2014].

1.2.4.1.4. FA degradation

Fatty acid degradation takes place in the peroxisome following the β-oxidation pathway, which is a four-reaction cycle resulting in the shortenings of the FA backbone by two carbons, accompanied by the release of acetyl-CoA. In *Y. lipolytica*, the first step is carried out by six acyl-CoA oxidases encoded by *POX* genes (*YALI0E32835g*, *YALI0F10857g*, *YALI0D24750g*, *YALI0E27654g*, *YALI0C23859g*, and *YALI0E06567g*). The *POX* genes show different chain-length preferences, and the sextuple knockout strain is unable to degrade fatty acids, which leads to a lipid over-accumulation phenotype [Wang et al. 1999]. The second and third steps in β-oxidation are catalyzed by the multi-functional enzyme Mfe2p (encoded by *YALI0E15378g*), which, in contrast to *POX*, is encoded by a single gene. The deletion of *MFE2* has been extensively studied in engineered strains for lipid production due to its technical simplicity compared to *POX1-6* deletion [Dulermo and Nicaud, 2011]. The fourth and last step in β-oxidation are carried out by the thiolase Pot1p (encoded by *YALI0E18568g*). Because β-oxidation takes place in the peroxisome, several strategies to block this pathway address the abolition of peroxisome biogenesis through the deletion of *PEX3*, *PEX10*, and *PEX11 (YALI0F22539g, YALI0C01023g, and YALI0C04092,* respectively) [Xue et al. 2013; Dulermo et al. 2015].

1.2.4.2. Metabolic engineering to improve lipid production

As mentioned earlier, the oleaginous phenotype and the comprehensive studies on the lipid metabolism of *Y. lipolytica* make this yeast as a favorable host strain for producing lipids. The emergence and advancement of metabolic engineering provided a huge opportunity to improve the lipid production at high titer, rate, and yield in *Y. lipolytica* (Table 1.1).

1.2.4.2.1. Increasing the availability of precursors

A common starting point for metabolic engineering is increasing the supply of metabolic precursors: acetyl-CoA and malonyl-CoA in case of fatty acids. *Y. lipolytica* has a natural capacity of high-level production of cytosolic acetyl-CoA provided by the ATP citrate lyase (*ACL1,2*), particularly under nitrogen limitation. The association between lipogenesis and nitrogen starvation resulted in prolonged cultivation. Xu and colleagues tried to decouple acetyl-CoA flux from nitrogen starvation by engineering several alternative cytosolic acetyl-CoA pathways including the pyruvate-acetate route (*pdc, aldH, acs*), pyruvate-aldehyde route (*pdc* and *aad*), pyruvate formate lyase (*pflA* and *pflB*), acetyl-CoA shuttling pathway (*Cat2*), and nonoxidative pentose-phosphate pathway (phosphoketolase and *pta*) [Xu *et al*. 2016]. The overexpression of native *ACL* resulted in only a minor increase in lipid titer as already seen in other study [Dulermo *et al*. 2015], but most of the other strategies led to a significant improvement. Particularly, the overexpression of peroxisomal carnitine acetyltransferase

(*Cat2*) from *S. cerevisiae* for enhanced export of mitochondrial acetyl-CoA into the cytosol led to a 75% improvement of the lipid titer. Moreover, the engineered strain began accumulating lipids in the exponential growth phase before nitrogen became limiting. High level of lipids (66.4 g/L) and oil content (81.4%) with an overall yield of 0.229 g/g glucose was achieved in fed-batch cultivation.

Malonyl-CoA is converted from acetyl-CoA by acetyl-CoA carboxylase (ACC). The overexpression of *ACC1* showed the increase of lipid content by 2-fold, with more linoleic acid in total lipids [Tai and Stephanopoulos, 2013]. In the same study, co-expression of *ACC1* and *DGA1* (DAG acyltransferase) showed a significant increase of lipid content by 4.7- and 2.3-fold compared to control and *ACC1*-overexpressing strain. In a bioreactor, the lipid production of *ACC1*- and *DGA1*-overexpressing strain reached 61.7% lipid content, with the overall yield and productivity from glucose of 0.195 g/g and 0.143 g/L/h, respectively. This simple but efficient method showed the successful push-and-pull strategy for lipid production.

The same group tried to overcome the potential allosteric inhibition of ACC1 by saturated FAs, the overexpression of *SCD* (delta-9 stearoyl-CoA desaturase, *OLE1*, *YALI0C05951g*) converting from saturated to monounsaturated FAs in *ACC1* and *DGA1* co-expressing strain led to a greatly increased lipid titer, 55 g/L from glucose with the oil content of 67%, the overall yield of 0.234 g/g, and productivity of 0.707 g/L/h [Qiao *et al*. 2015]. The authors hypothesized that the action of SCD released the inhibitory effect on ACC1 caused by palmitoyl-CoA and stearoyl-CoA through converting them to monounsaturated versions. The monounsaturated acyl-CoAs were then rapidly incorporated into TAG through the action of DGA1 without further inhibition of the desaturase itself.

1.2.4.2.2. Increasing lipogenic pathway flux

Several studies showed the strategy diverting flux and driving force to TAG in order to improve lipid production. The push and pull strategy described above is a good example of this approach. It is proved that the roles of DGA1 and DGA2 catalyzing the ultimate step in TAG synthesis are very important to increase lipogenesis in many studies [Beopoulos *et al*. 2012; Tai and Stephanopoulous, 2013; Blazeck *et al*. 2014; Gajdos *et al*. 2015]. The overexpression of these enzymes has successfully improved lipid production with different phenotypes of LB, generating smaller but more numerous LBs from *DGA1* overexpression or forming large LBs from *DGA2* overexpression. Therefore, the strategy enhancing the last step of TAG synthesis has been broadly combined with other engineering strategies as described in this chapter.

Heterologous expression of *DGA1* and *DGA2* in *Y. lipolytica* has also been largely successful in engineering the lipid-overproducing phenotype. For instance, the *DGA1* from *Rhodosporidium toruloides* and *DGA2* from *Claviceps purpurea* were found to outperform the native ones of *Y. lipolytica* [Friedlander *et al*. 2016]. The overexpression of these heterologous genes combined with the deletion of *TGL3*, which encodes the regulatory protein for TAG remobilization, resulted in lipid content of 77% and a yield of 0.21 g/g during batch culture.

Applying fed-batch mode with glucose allowed the strain to produce 85 g/L of lipids with a productivity of 0.73 g/L/h.

Another successful strategy for improving lipid accumulation by redirecting carbon flux was shown by Dulermo and Nicaud [Dulermo and Nicaud, 2011]. The G3P (glycerol-3-phosphate) shuttle pathway is closely related to TAG synthesis since G3P provides a backbone of TAG. The increase of G3P concentration by the overexpression of *GPD1* and/or the deletion of *GUT2* resulted in the increase of TAG synthesis. The further engineering by inactivating β-oxidation with the overexpression of *GPD1* for coupling the increase of G3P level and FA availability showed the great increase of lipid accumulation up to 65% (g/g DCW) of lipids.

1.2.4.2.3. Inhibiting lipid remobilization and degradation

TAGs stored in LBs are transported to the peroxisome and degraded by β-oxidation. These processes are regarded as an antagonistic pathway to lipid synthesis and accumulation. Therefore, the genes involved in the transport and degradation of lipids were the targets for deletion in order to improve lipid production.

It is known that intracellular lipases encoded by *TGL3* and *TGL4* are involved in TAG remobilization. The inactivation of Tgl3p and/or Tgl4p led to higher lipid accumulation, with increasing the amounts of both TAG and FFA, but without affecting the FA profile [Dulermo *et al*. 2013]. The detailed function of each enzyme was also shown in this study showing that Tgl4p is the major intracellular lipase and Tgl3p is an activator of Tgl4p in *Y. lipolytica*. The increase of lipid accumulation was obtained by combining the deletion of *TGL3* and the overexpression of heterologous *DGA1* and *DGA2* in *Y. lipolytica* [Friedlander *et al*. 2016].

When the β-oxidation pathway was inhibited *via* the inactivation of *POX* genes, the corresponding strains displayed greater lipid accumulation. The deletion of the *POX1- 6* genes combined with *GUT2* deletion resulted in a 3.2-fold increase of lipid content (from 12.76% of DCW to 41.92% of DCW), and the engineered strain exhibited a hyper lipid-accumulating phenotype with extremely large LBs [Beopoulos *et al*. 2008]. The knockout of β-oxidation combined with the overexpression of *DGA2* and *GPD1* yielded lipid accumulation of 55% of DCW under nitrogen-limited conditions [Lazar *et al*. 2014; Sagnak *et al*. 2018].

It is shown that the inactivation of MFE catalyzing the second and the third step in the β-oxidation also improved the lipid accumulation. Moreover, deletion of *GUT2*, *POX1-6*, and *MFE1*, combined with overexpression of *GPD1*, triggered the accumulation of higher lipid levels, which reached 65-75% of DCW [Dulermo *et al*. 2011].

As well as inhibiting β-oxidation, abolishing peroxisome biogenesis where β-oxidation occurs was also studied. The *PEX3*, *PEX10*, and *PEX11* responsible for peroxisome biogenesis have been deleted to increase the lipid accumulation [Xue *et al*. 2013; Dulermo *et al*. 2015]. Blazeck and colleagues obtained the 60-fold increase in lipid titer (25 g/L) with lipid content approaching 90% by combining the deletion of *PEX10* and *MFE2* and the overexpression of

DGA1 [Blazeck *et al*. 2014]. The same group improved the lipid production titers up to 39.1 g/L with 77.6% of lipid content by an evolutionary approach linked with a floating cell enrichment process [Liu *et al*. 2015].

1.2.4.2.4. Engineering the redox metabolism

Availability of the FAS cofactor, NADPH, converting acetyl groups to fully reduced acyl-chain is crucial for lipid synthesis. Cytosolic, $NADP^+$-dependent malic enzyme has traditionally been put forward as the major producer of lipogenic NADPH in a *trans* hydrogenation mechanism termed the pyruvate-oxaloacetate-malate (POM) cycle [Wynn *et al*. 2001; Zhang *et al*. 2016]. However, a malic enzyme in *Y. lipolytica* is predicted to be localized into the mitochondria inconsistent with other oleaginous yeasts. Also, it is reported that the overexpression of the native malic enzyme in *Y. lipolytica* did not increase the lipid production and the enzyme prefers NAD^+ over $NADP^+$ [Zhang *et al*. 2013].

Many studies suggest that *Y. lipolytica* regenerates NADPH almost exclusively through the oxidative pentose phosphate pathway (oxPPP) [Wasylenko *et al*. 2015]. The metabolic flux within oxPPP was significantly enhanced, as determined by ^{13}C metabolic flux analysis, in the engineered strain overexpressing *DGA1* and *ACC1*. Importantly, the estimated rate of NADPH synthesis from the oxPPP was consistent with the estimated rate of NADPH consumption in the TAG synthesis pathway in engineered strains. Furthermore, studies that focused on enhancing the oxidative PPP flux through overexpression of related enzymes have shown an increase in lipid synthesis, further strengthening this argument [Silverman *et al*. 2016; Yuzbasheva *et al*. 2017]. Qiao and colleagues identified an imbalance in electron cofactors with NADH in excess and NADPH being limiting from the metabolic network in lipid production. To convert the excess NADH to NADPH for lipid production, the authors constructed several pathway modules [Qiao *et al*. 2017]. Two $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenases, *GapC* from *Clostridium acetobutylicum* and *GPD1* from *Kluyveromyces lactis*, were overexpressed in an *ACC1*- and *DGA1*-overexpressing background strain, and the lipid accumulation was improved by 20.0 % and 17.8 %, respectively. A cytosolic $NADP^+$-dependent malic enzyme, MCE2 from *Mucor circinelloides*, was also tested to activate the pyruvate–oxaloacetate–malate cycle for further conversion from NADH to NADPH. The introduction of *MCE2* also improved the yield by 23%. The *GapC* introduction was further coupled to *YEF* (endogenous NAD^+/NADH kinase) overexpression, which resulted in multiple functional synthetic pathways working simultaneously converting NADH to NADPH. From the final engineered strain, the lipid titer, productivity, and yield reached 99 g/L, 1.2 g/L/h, and 0.27 g/g respectively.

1.2.4.2.5. Removing the competing by-products

Besides TAG, glycogen serves as a second storage form for excess carbon. The accumulation of glycogen is only observed after the complete depletion of the nitrogen source, which is a

similar condition of TAG accumulation. Therefore, redirecting carbon flux from glycogen to TAG synthesis was studied to improve the lipid accumulation [Bhutada *et al*. 2017]. In this study, the authors verified that glycogen contributes up to 16% to the biomass in *Y. lipolytica* W29 strain background. The strain deleted for *GSY1* (glycogen synthase, *YALI0F18502g*) accumulated 60% higher amounts of TAGs compared to the wild-type strain. When this strategy was combined with the deletion of *TGL4* and the overexpression of *DGA2* and *GPD1*, the accumulation of TAGs was increased from 44.9% to 52.4% of DCW.

1.2.4.2.6. Engineering lipid profiles

As well as the production of lipids in general, tailoring lipid profiles to produce specific fatty acids having industrial application were studied. Tsakraklides and colleagues engineered the chain lengths and degree of desaturation of fatty acids in *Y. lipolytica* to produce high-oleate TAG having applications in the food and chemical industries [Tsakraklides *et al*. 2018]. The lipids produced from *Y. lipolytica* WT are composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). In order to increase oleic acid and remove other compounds, native Δ9 fatty acid desaturase (*OLE1*, *YALI0C05951g*) and glycerol-3-phosphate acyltransferase (*SCT1*, *YALI0C00209g*) were exchanged with heterologous genes (*OLE1* from *Rhodotorula graminis* and *SCT1* from *Arxula adeninivorans*, respectively). Also, the Δ12 fatty acid desaturase (*FAD2*, *YALI0B10153g*) was deleted to eliminate polyunsaturated fatty acids. By combining these engineering steps, high-oleate producing *Y. lipolytica* strain (more than 90% in total TAGs) was constructed. Further boosting overall TAG synthesis by overexpressing *DGA1* from *R. toruloides* and *DGA2* from *C. purpurea* resulted in 45% of lipid content and 77 g/L of DCW from glucose fed-batch fermentation.

Other lipid derivatives which are barely produced in native microorganisms, unusual lipids, are also gaining a lot of interest as target products of metabolic engineering because of their useful applications in industry. The engineering approaches to produce unusual lipids are described in 1.2.4.3.

Table 1.1. The studies on the lipid production in *Y. lipolytica* by metabolic engineering approaches described in this chapter.

Parental strain	Genotype	Subsstrate	Process	Lipid titer (g/L)	Lipid content % (g/g DCW)	Yield (g/g)	Productivity (g/L/h)	Reference
Po1f	*ACC1, DGA1*	Glucose	Batch	17.6	61.7	0.195	0.14	Tai and Stephanopoulos. 2013
Po1f	*pex10Δ, mfe1Δ, DGA1*	Glucose	Batch	25.3	75	0.16	0.11	Blazeck *et al*. 2014
Po1f	*(pex10Δ, mfe1Δ, DGA1) evolved*	Glucose	Batch	39.1	77.6	0.244	0.42	Liu *et al*. 2015
Po1f	*ACC1, DGA1, SCD*	Glucose	Fed-batch	55	67	0.23	0.71	Qiao *et al*. 2015
Po1d	*tgl4Δ, pox1-6Δ, DGA2, GPD1*	Glucose	Batch	4.88	40	0.25		Lazar *et al*. 2014
Po1g	*ACC1, DGA1, ScperCat2*	Glucose	Batch	66.4		0.23	0.56	Xu *et al*. 2016
Po1d	*pex10Δ, ZWF1, ACBP*		Flask		30			Yuzbasheva *et al*. 2017
Po1g	*ACC1, DGA1, MCE1, GapC*	Glucose	Fed-batch	98.9			1.30	Qiao *et al*. 2017
NS18	*rtDGA1, cpDGA2, tgl3Δ*	Glucose	Fed-batch	84.5	66.8	0.20	0.73	Friedlander *et al*. 2016
Po1d	*tgl4Δ, gsy1Δ, DGA2, GPD1*	Glycerol	Flask	2.62	52.4			Bhutada *et al*. 2017

1.2.4.3. Metabolic engineering to produce unusual lipids

As well as FAs themselves, the derivatives of FAs are also products of interest in the biotechnology field. As metabolic engineering developed, the production of a wide range of oleochemicals has been possible in both bacteria and yeast. Many of them can be produced by engineering the termination enzymes in lipid synthesis, such as thioesterase, ester synthase, methyl transferase, PHA synthase, acyl-thioester reductase, and aldehyde deformylating oxidase which convert acyl-CoA to FFA, FAEE, FAME, PHA, fatty aldehydes, and fatty alcohols, respectively [Yan and Pfleger, 2020].

Among the FA-derivatives, there is a class called 'unusual lipids' which is atypical, deviated from the normal lipids due to the differences in chain length (short, very long, and odd-numbered), position or number of double bonds (conjugated and polyunsaturated), or the presence of other functional groups (hydroxy, epoxy, keto, branched-chain, cyclic, *etc*.) [van de Loo *et al*. 1993; Napier, 2007]. Some unusual FAs are regarded as high-value products as described in Figure 1.4 because of their useful applications in pharmaceutical, food, and chemical industries but low abundancy in nature, in which the microbial production can provide more sustainable and economically viable options [Ledesma-Amaro and Nicaud, 2016b; Xie, 2017; Aznar-Moreno and Durrett, 2017].

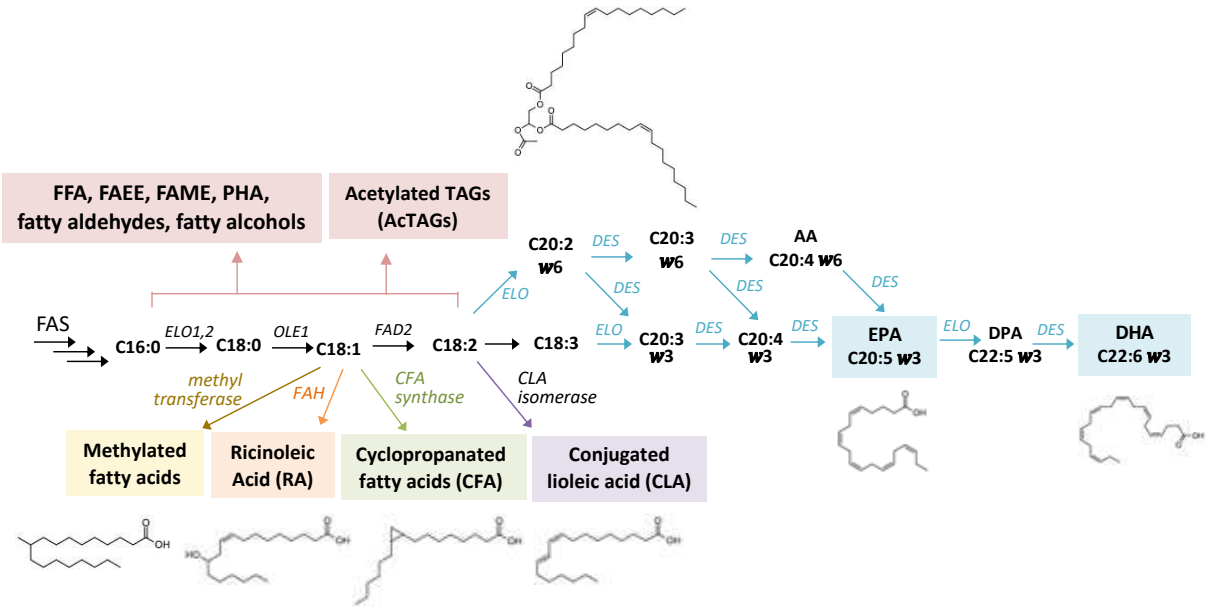


Figure 1.4. Synthesis of the lipid derivatives in *Y. lipolytica*.

Lipid derivatives as the target of interest are in colored boxes, and the heterologous reactions are indicated in colored arrows. FFA, free fatty acids; FAEE, fatty acid ethyl esters; FAME, fatty acid methyl esters; PHA, polyhydroxyalkanoates; AcTAGs, acetylated TAGs; RA, ricinoleic acid; CFA, cyclopropanated fatty acid; CLA, conjugated linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; *ELO1,2*, elongase1,2; *OLE1*, Δ9 desaturase; *FAD2*, Δ12 desaturase; *FAH*, Δ12-oleate hydroxylase; *ELO*, elongase; *DES*, desaturase.

1.2.4.3.1. Conjugated linoleic acid (CLA)

CLA is a group of octadecadienoic acids which are the isomers of linoleic acid (C18:2, n=9, 12). It is known that CLA can be used for the prevention of metabolic diseases and cancer, anti-atherogenic and anti-obesity effects, and immune-system modulation. Therefore, the production of CLA is of interest in the food and pharmaceutical industries. The production of recombinant CLA has been tried in *Lactobacillus planetarium*, *Delacroixia coronate*, *S. cerevisiae*, and plants [Kishino *et al*. 2004; Ando *et al*. 2004; Ando *et al*. 2009; Hornung *et al*. 2005]. In *Y. lipolytica*, the CLA production was obtained by expressing CLA-producing isomerase from *Propionibacterium acnes* in high copy number [Zhang *et al*. 2012]. The production of *trans*-10-*cis*-12-CLA was 0.23% of DCW, 5.6% of total FAs. The best strain showed an 80% conversion rate of linoleic acid (LA) to CLA in a biotransformation condition. The same group developed the strain by co-expressing CLA-producing isomerase and *FAD2* from *Mortierella alpine* producing CLA up to 0.4% of DCW, 10% of total FAs [Zhang *et al*. 2013]. From soybean oil-based media, the production of CLA reached 4 g/L representing 30% of DCW and 44% of total FAs. Another metabolic engineering approach to produce CLA was recently reported [Imatoukene *et al*. 2017]. In this study, β-oxidation and TAG storage were blocked and CLA-producing isomerase from *P. acnes* and native *FAD2* were overexpressed in *Y. lipolytica*. The production of CLA reached 6.5% of total FAs in the flask, and 302 mg/L in a bioreactor.

1.2.4.3.2. Cyclopropanated fatty acids (CFA)

CFAs which can be simply converted to methylated fatty acids are useful in the lubrication and oleochemical industries because of their long-term resistance to oxidization and low-temperature fluidity. Natively, CFAs are produced in a variety of different organisms from bacteria to plants and comprise varying percentages of the total FA pool, up to 46% [Markham and Alper, 2018]. Since the native producer of CFAs accumulates CFA typically in membrane-bound phospholipids, the strategies aimed to produce CFAs in the form of TAGs which is a more desired pool for production by using oleaginous yeast *Y. lipolytica* were reported [Markham and Alper, 2018; Czerwiec *et al*. 2019]. The CFA synthase from *E. coli* was overexpressed in the engineered strain accumulating a high amount of lipids by disrupting β-oxidation and overexpressing *DGA1*, and 200 mg/L of C19CP (C19:0 cyclopropanated) was produced at flask level. Through fed-batch fermentation, the titer of C19CP was reached to 3.13 g/L representing 32.7% of total FAs. In another study, 10 different CFA synthase coding genes from various organisms were assessed for the CFA production in *Y. lipolytica*. Instead of multi-copy expression of CFA synthase shown in the previous study [Markham and Alper, 2018], different hybrid promoters were tested to find the optimal promoter for the production of CFA. The final strain blocked in the TAG degradation and remobilization and

overexpressed CFA synthase from *E. coli* under Hp8d promoter produced 2.32 g/L of CFA representing 45.8% of total FAs [Czerwiec *et al*. 2019].

1.2.4.3.3. Ricinoleic acid (RA)

Ricinoleic acid, a hydroxylated FA (12-hydroxy-octadeca-*cis*-9-enoic acid, C18:1-OH), can be used as a substrate for double-bond reactions and for hydroxyl-group reactions, an intermediate of plasticizers, lubricants, dyes, inks, soaps, pharmaceuticals, food additives, cosmetics, biofuels, *etc*. [Mutlu and Meier, 2010; de Silva *et al*. 2006; Ogunniyi, 2006]. The recombinant production of RA was tried in plants remaining at lower level of RA than the one from native producer, castor seed [Broun and Somerville, 1997; Smith *et al*. 2003; Lu *et al*. 2006; Kumar *et al*. 2006; Burgal *et al*. 2008; Lee *et al*. 2015]. The microbial production of RA was previously studied in *S. cerevisiae* and *Schizosaccharomyces pombe* [Smith *et al*. 2003; Holic *et al*. 2012; Yazawa *et al*. 2014]. Holic and colleagues produced 52.6% of RA, a total of 137.4 mg/L by overexpressing *FAH12* (Δ12-oleate hydroxylase) from *C. purpurea*. In order to suppress the toxicity of RA, the intracellular phospholipase (*PTL2*) was overexpressed, thus the increased secretion and intracellular RA levels were obtained by 1.2- and 1.3-fold, respectively [Yazawa *et al*. 2014]. Beopoulos and colleagues constructed a multiple engineered *Y. lipolytica* strain that could accumulate RA up to 43% of its total lipids and more than 60 mg/g of DCW [Beopoulos *et al*. 2014]. In order to provide the substrate of RA, β-oxidation was blocked and *FAD2* gene was deleted. By deleting three DAG acyltransferases and overexpressing *LRO1*, the TAG was synthesized only through phospholipid pathway. Together with the overexpression of *FAH12* from *C. purpurea*, the production of RA reached 12 g/L representing 60% of total lipids in a bioreactor. The crucial domain of CpFAH12 for the hydroxylation activity was recently studied by secondary structure prediction and mutagenesis in *Y. lipolytica* [Robin *et al*. 2019].

1.2.4.3.4. Acetylated lipids

The 3-acetyl-1,2-diacylglycerols (acTAGs) is structurally similar to common TAGs but different at the sn-3 position being esterified by acetate not FA [Gajdos *et al*. 2019]. The acTAG naturally found from plants of the family *Celastraceae,* and animals like *Cervus nippon* and *Eurosta solidaginis*. Due to an acetyl moiety, acTAGs have lower viscosity and calorific value than TAGs which provides advantages in industrial applications like biodiesels, emulsifiers, lubricants, plasticizers, and so on. Gajdos and colleagues introduced heterologous diacylglycerol acetylransferase (DAcT) from *Eunonymus europaeus* and obtained about 20% of acTAGs in total lipids in *Y. lipolytica* WT strain. When *DAcT* was overexpressed in non-TAG-accumulating strain, 10% of acTAGs in total lipids were accumulated as the only stored lipid.

1.2.4.3.5. Methylated lipids (branched lipids)

Saturated branched (methyl) lipids like 10-methylstearic acid have desirable properties such as low-temperature fluidity and favorable oxidative stability, which are useful properties for lubricants and specialty fluids. The oleic acid and methionine substrates can be converted to 10-methylenestearic acid by methyltransferase (tmsB), then further reduced to 10-methylstearic acid by reductase (tmsA). Shaw and colleagues expressed *tmsB* and *tmsA* from different origins in several microorganisms including *Y. lipolytica* [Shaw *et al*. 2017]. It was found that co-expression of *tmsA* and *tmsB* from *Thermomonospora curvata* can produce branched 10-methyl and 10-methylene fatty acids in *Y. lipolytica*. The fusion enzymes having the activity of two enzymes (tmsA-B) resulted in the production of 10-methylstearic acid, representing more than 20% of total fatty acids.

1.2.4.3.6. Polyunsaturated fatty acids (PUFA)

PUFAs, particularly eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) also known as long-chain omega-3 FAs, have attracted scientific attention because of their several health benefits. They reduce the incidence of cardiovascular diseases and prevent myocardial infarction, bronchial asthma, inflammatory bowel disease, major depression, and several types of cancer. Though the natural source of omega-3 FAs is fish oils, numerous studies have focused on the production of EPA and DHA in microorganisms to prevent the competition with fish supply and the environmental pollution of marine ecosystems [Domingo *et al*. 2007; Martins *et al*. 2013]. A few products from microalgae (*Crypthecodinium cohnii* and *Schizochytrium* sp.) are commercially available [Martins *et al*. 2013]. In *Y. lipolytica*, a metabolic engineering strategy by introducing Δ6 desaturase, C18/20 elongase, Δ5 desaturase, and Δ17 desaturase was successful for the production of EPA. Further engineering including the overexpression of an elongase from *M. alpina* and a Δ12 desaturase from *Fusarium moniliforme* made *Y. lipolytica* to produce up to 40% of EPA [Macool *et al*. 2008; Yadav *et al*. 2009; Zhu *et al*. 2010]. The same group gave a lot of effort to improve the production of EPA by eliminating the competitive pathway producing by-products and introducing the crucial enzymes described above in multi-copy, finally constructed the strain producing EPA approximately 25% of DCW and 50% of FAME [Hong *et al*. 2011]. This strategy allowed the commercialization of EPA produced from *Y. lipolytica*, New Harvest™ EPA oil and Verlasso® salmon, showing a successful example of *Y. lipolytica* as a chassis for biotechnological application. Recently, Gemperlein and colleagues constructed transgenic *Y. lipolytica* strains that produce specific PUFAs, such as AA (arachidonic acid, 20:4, n-6), EPA, DPA (docosapentaenoic acid, 22:5, n-3), and DHA [Gemperlein *et al*. 2019]. The multifunctional polyketide synthase (PKS)-like PUFA synthases from *Aetherobacter fasciculatus* and *Minicystis rosea* were introduced with a diverse hybrid form in *Y. lipolytica*, mainly producing DHA and AA, respectively. Notably, one of these strains is able to produce DHA at a concentration of 350 mg/L, 16.8% of total FAs under improved fermentation conditions.

1.2.5. Odd-chain fatty acids (OCFAs)

OCFAs, a type of unusual lipids, are synthesized naturally less than 3% of total fatty acids in microorganisms, plants, and animals. Recently, it has been gaining a lot of interest as the target compound of microbial production because of its broad application in the industry (Figure 1.5). Up to now, microbial oil production has mostly focused on the production of even-chain fatty acids (ECFA), therefore, the studies of OCFA production from oleaginous yeast, even from microorganisms are very limited, so far. Therefore, the microbial production of OCFAs through metabolic engineering is highly demanding regarding its various application and scarcity.

1.2.5.1. Properties and applications of OCFAs

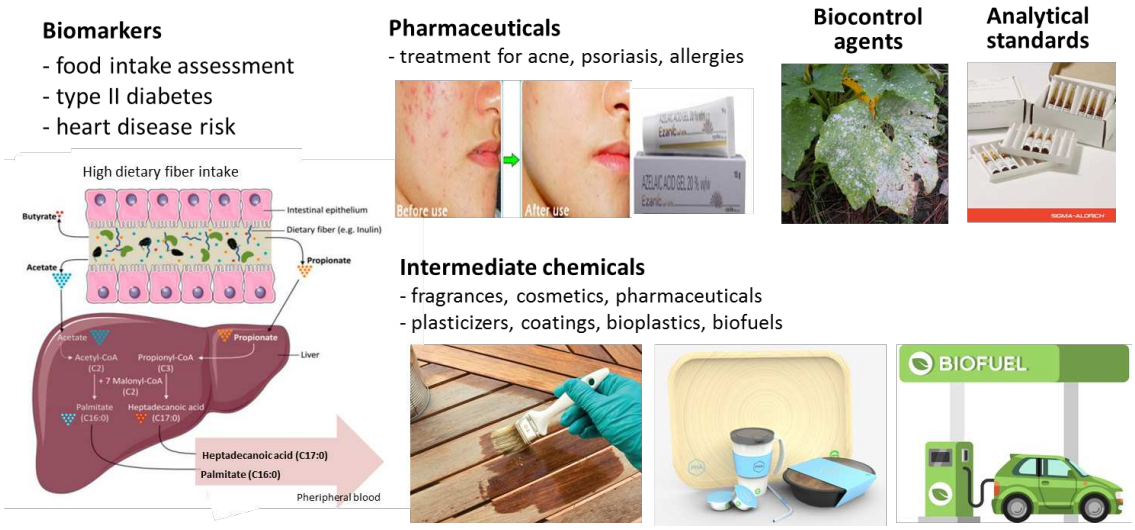


Figure 1.5. Applications of OCFAs in pharmaceutical, food, cosmetics, chemical and fuel industries.

OCFAs have shown the various applications in food, medical, and chemical industries while the very limited amount in microorganisms, plants, and animals. Notably, research has revealed that OCFAs with chain lengths of 15 and 17 carbons may have functional importance for the nutrition and medical field.

It is reported that *cis*-9-heptadecenoic acid (C17:1) has anti-inflammatory effects and can help treat psoriasis, allergies, and autoimmune diseases [Degwert *et al*. 1994]. *cis*-9-Heptadecenoic acid showed the inhibition of the activation of lymphocytes and thus influences processes in which lymphocytes participate, for example, allergies, psoriasis, and autoimmune diseases. Also, *cis*-9-heptadecenoic acid showed a certain inflammatory macrophage-stimulating potency in the macrophage differentiation test. Thus, a certain absence of acutely inflammatory macrophages (e.g. psoriasis), *cis*-9-heptadecenoic acid has the ability to normalize the inflammatory process. It is also verified to reduce the production

of TNFα by macrophages to a stimulus. This is of importance in the prophylaxis of cytokine-induced processes, such aspsoriasis, atopy, allergies or autoimmune diseases. All the effects showed the high potential of this compound in pharmaceutical or cosmetic composition.

The level of pentadecanoic acid and heptadecanoic acid in serum is known to be associated with all adiposity indicators, which make it use as a biomarker of the obesity [Forouhi *et al*. 2014; Aglago *et al*. 2017]. The relationship between OCFA levels and the risk of type II diabetes (T2D) also has been observed, which seems to be related to dietary fiber intake [Pfeuffer and Jaudszus, 2016; Weitkunat *et al*. 2017]. OCFAs also can be used as biomarkers for the risk of coronary heart disease (CHD). There are case studies showing the inverse association of the OCFAs with cardiovascular disease [Khaw *et al*. 2012; Jenkins *et al*. 2015].

Pentadecanoic acid and heptadecanoic acid (C15:0 and C17:0) can be used as indicators of dairy product intake by humans and rumen function (e.g. rumen fermentation pattern, bacterial number) [Pfeuffer and Jaudszus, 2016; Alves *et al*. 2006]. Levels of these OCFAs in adipose tissue and serum have been used as markers of intake of ruminant fat by humans. This has been useful in identifying relationships between intake of ruminant products and disease outcomes. In addition, it is shown their potential as markers to quantify bacterial matter in the rumen, to provide the description of the proportions of microbes in the rumen, and to predict the ratios of volatile fatty acids. Also, OCFAs has been used as softer fat in lambs because of the low melting points [Vlaeminck, 2006].

As well as straight OCFAs, odd- and branched-chain fatty acids (OBCFAs) have anti-cancer activity. *iso*-Pentadecanoic acid (C15:0) from a fermented soybean resulted in the inhibited growth of various cancer cell lines and *anteiso*-pentadecanoic acid showed the activity against human breast cancer cells [Yang *et al*. 2000; Wongtangtintharn *et al*. 2004]. It is revealed that branched-chain fatty acids hindered fatty acid synthesis in tumor cells, thus provide a meaningful clue for developing cancer treatments [Vlaeminck, 2006]. The chemical properties and potential biological activities of OCFAs are now being more extensively studied [Rezanka and Sigler, 2009], so it is expected that novel nutritional and pharmaceutical applications could be more discovered.

As well as nutritional, pharmaceutical, and medical applications, it can be used in the chemical industry. It is known that *cis*-9-heptadecenoic acid has antagonistic activity against powdery mildew, a fungal disease affecting a wide range of plants [Avis, 2000; Avis. 2001]. *cis*-9-Heptadecenoic acid showed the partitioning into fungal membranes and causing an elevation in membrane fluidity in its free form. Thus, it resulted in the inhibited growth of fungi by ensuing loss of membrane integrity. When there is a high amount of *cis*-9-heptadecenoic acid, the elevation in membrane fluidity leads to the changes in membrane permeability. This would cause the release of intracellular electrolytes and proteins, and cytoplasmic disintegration of mycelia and spores. This antifungal effect of *cis*-9-heptadecenoic acid makes to be used as biocontrol agents.

In addition, OCFAs and their derivatives are precursors for manufacturing substances such as flavor and fragrance compounds, hydraulic fluids, plasticizers, coatings, and other industrial chemicals [Avis *et al*. 2000; Clausen *et al*. 2010; Kockritz *et al*. 2010; Fitton and Goa, 1991]. Furthermore, adding OCFAs or their derivatives into biodiesels can be helpful for their quality by enhancing transesterification reactions or storage conditions [Knothe, 2008; Knothe, 2009].

1.2.5.2. Occurrence of OCFAs in nature

It seems that OCFAs occur ubiquitously in small amounts in microorganisms, plants, and animals including humans. Higher values are found in animals and men with B12 deficiency (pernicious anaemia). In microorganisms, OCFAs are widespread but in little amounts (nearly up to 3%) with the different position of the double bond of the unsaturated FAs (C11 to C25). Even very-long-chain OCFAs (VLOCFA, C27 to C33) are found in bacteria, grass, or sponges (*Desmospongiae*) [Diedrich and Henschel, 1990; Rezanka and Sigler, 2009].

In bacteria and yeast, long-chain OCFAs (LCOCFAs) such as pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and nonadecanoic acid (C19:0), are identified. VLOCFAs e.g. C23:0 or C25:0 are also detected in specific bacteria like *Streptomyces cinnamonensis* or *Franchisella tularensis* [Rezanka *et al*. 1984; Nichols *et al*. 1985]. In fungi and plant seed oil, monounsaturated OCFAs with 17- 21 carbon chains have been identified. The waxes were also found to contain OCFAs, with much lower content than that of ECFAs. OCFAs such as 21-29 carbon atoms and even longer carbon chains are reported from fish oils which seem to come from algal FAs. In Thraustochytrid, various polyunsaturated OCFAs (17:2, 17:3, 19:2, 19:3, 19:4, 21:4, 21:5, 21:6) were identified, the further functional study of these PUFAs regarding human health might be interesting. Up to now, most attention has been paid to the effects of long-chain OCFAs, such as C15:0, C17:0, and C17:1 because of their abundance and the effect on human health.

1.2.5.3. Synthesis of OCFAs

Generally, *de novo* fatty acid synthesis in microorganisms begins with the condensation of acetyl-CoA and malonyl-CoA (Figure 1.6 (A)). Then, the elongation step occurs in which long-chain FAs are synthesized in a reaction catalyzed by fatty acid synthase (FAS). For OCFAs, propionyl-CoA is a primer for fatty acid synthesis. The condensation of both propionyl-CoA and malonyl-CoA results in the formation of 3-oxovaleryl-ACP, which is the launching point for OCFA synthesis. This five-carbon compound goes through elongation where two carbons are added in each cycle, then OCFAs can be synthesized as described in Figure 1.6 (B).

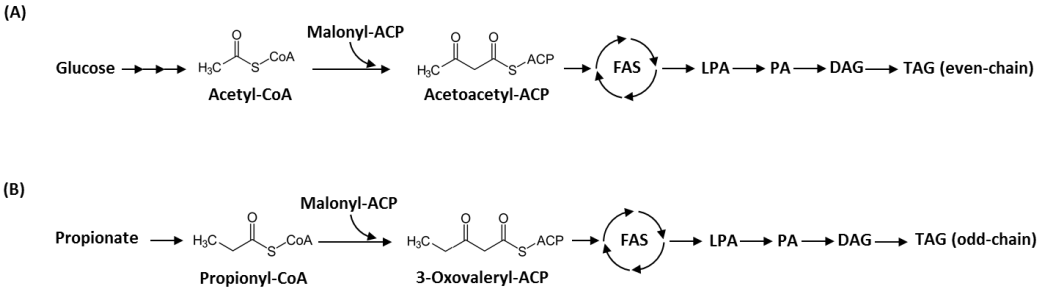


Figure 1.6. Lipid synthesis in *Y. lipolytica*. (A) Synthesis of even-chain fatty acids (ECFAs) from glucose. (B) Synthesis of OCFAs from propionate.

Depending on the primer of FA, ECFA from acetoacetyl-ACP or OCFA from 3-oxovaleryl-ACP is synthesized. After the elongation steps by fatty acid synthase (FAS), FAs are converted to triacylglycerol (TAG) for the storage in the LBs. LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol.

It is known that propionyl-CoA, a key primer of OCFAs, is synthesized from several pathways. It can be directly converted from propionate provided to the media (Figure 1.6 (B)) or synthesized through other metabolisms such as β-oxidation from propionate or long-chain FAs. It also can be created *via* the citramalate/2-ketobutyrate pathway, the aspartate/2-ketobutyrate pathway, the methylmalonyl-CoA pathway, the 3-hydroxypropionate pathway, and the isoleucine or valine degradation pathway as described in Figure 1.7 [Lee *et al*. 2013; Han *et al*. 2013]. The pathways relating amino acid synthesis are conserved and most genes are identified in *Y. lipolytica*. However, other pathways of propionyl-CoA and the genes involved in *Y. lipolytica* remained to be studied, so far.

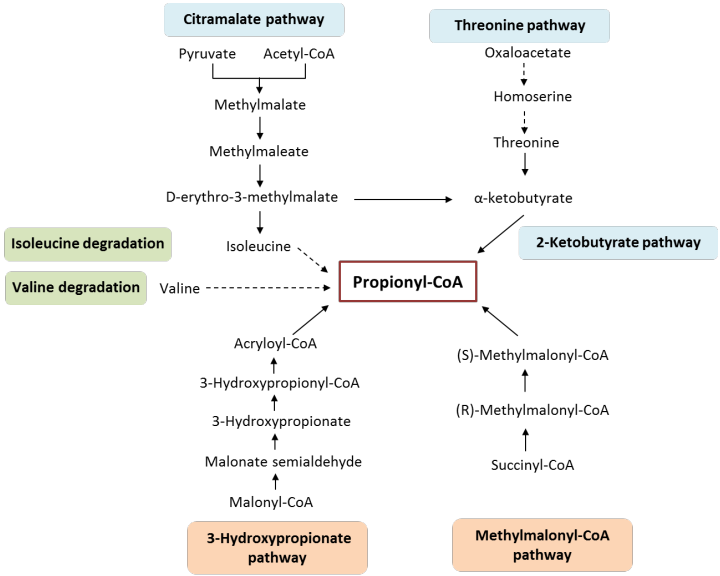


Figure 1.7. Metabolic pathways leading to propionyl-CoA synthesis.
(The dashed arrows indicate multiple reactions.)

1.2.5.4. Production of OCFA by fermentation

A key step in the synthesis of fatty acids is the production of a biosynthetic precursor, i.e., acyl-CoA. OCFAs with straight chains are synthesized mostly from odd-numbered chain precursors, i.e., propionyl-CoA. In order to provide propionyl-CoA, many studies used the strategy to utilize three-carbon chain compounds such as propionate, propionic acid, or 1-propanol as the substrate. Due to their toxicity, many efforts have directed to find the strains which are more tolerant, thus can grow well and produce OCFAs more, or to optimize the combination of substrates together with C3 substrates.

In 2015, the lipid production of several yeast species both oleaginous (*Candida* sp., *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Y. lipolytica*) and non-oleaginous (*Kluyveromyces polysporus*, *S. cerevisiae*, *Torulaspora delbrueckii*) was investigated with various VFAs [Kolouchova *et al*. 2015; Rezanka *et al*. 2015]. Nearly all species, when cultured on propionic acid, showed a high content of the desirable OCFAs, especially C17:1. The higher level of C17:1 in *Candida* sp., *K. polysporus*, *To. delbrueckii* and *T. cutaneum* up to 37% of total FAs was observed in comparison with *S. cerevisiae* (5.1%) or *R. glutinis* (18.8%). From the cultivation of *Candida* sp. on propionic acid, the titer of C17:1 reached 0.11 g/L. In the presence of other substrates together with propionic acid, the content of C17:1 was decreased in all strains ranged from 0 to 3.8% of total FAs.

In *Y. lipolytica*, several studies on the growth and lipid accumulation on propionate are reported [Fontanille *et al*. 2012; Kolouchova *et al*. 2015; Chakraborty, 2015]. The synthesis of OCFAs in *Y. lipolytica* was shown during growth on propionate and valerate. The ratio of OCFAs in total lipids on propionate was higher than one on valerate at the same concentration (43% *vs.* 17%) which might be due to lower propionate fraction after degradation of valerate to acetate and propionate.

In the study of Bhatia and colleagues, three different *Rhodococcus* species (*Rhodococcus* sp. YHY01, *Rhodococcus* sp. 1918, and *Rhodococcus* sp. 19938) were investigated with diverse carbon sources for OCFA accumulation [Bhatia *et al*. 2019a]. The production of OCFA was 0.69 g/L which representing 69% in total lipids in *Rhodococcus* sp. YHY01 on propionate, whereas very minor levels of OCFAs were obtained in other strains. Notably, *Rhodococcus* sp. YHY01 could produce OCFAs up to 1 – 10% of total FAs even on the conventional carbon source (glucose). The same group optimized the carbon and nitrogen sources to maximize the OCFA production through response surface design. From 20 different combinations, maximum biomass (1.98 g/L), FAs accumulation (70.78% g/g DCW), and optimum concentration of OCFAs (1.19 g/L, 85.1% in total FAs) were obtained with glycerol, propionate, and NH4Cl at a ratio of 0.5%:0.5%:0.05%. In terms of biomass yield ($Y_{X/S}$) and OCFA yield ($Y_{P/S}$), the combination of glycerol and propionate resulted in higher $Y_{X/S}$ (0.20 g/g) and $Y_{P/S}$ (0.12 g/g) than the single carbon source glycerol ($Y_{X/S}$=0.09 g/g, $Y_{P/S}$=0.01) or propionate ($Y_{X/S}$=0.16 g/g, $Y_{P/S}$=0.08).

Using 1-propanol for the production of OCFAs by *Rhodococcus opacus* PD630 was described [Zhang *et al*. 2019]. After adding 0.5-1.5% (v/v) of 1-propanol, the production of lipids was increased from 1.27 g/L to 1.31 - 1.61 g/L, and the OCFAs content in total lipids is also increased by 46.7-55.1%. The maximum ratio of OCFAs in total lipids, 84.51%, was obtained on 1.0% of 1-propanol. Interestingly, the increase of OCFAs on 1.0% of 1-propanol mostly came from the increase of pentadecanoic acid (29.44% in total lipids), while the level of other OCFAs in total lipids remained similar.

Recently, several studies using volatile fatty acids (VFAs) as substrates for the production of OCFA have been reported. VFAs obtainable from wastes of biotechnological and industrial processes have been interested as substrates regarding the economic balance of biolipid production. It was shown that VFAs from wastes can be used as substrates for biomass and lipid production in yeast including non-oleaginous and oleaginous. VFAs mostly consist of acetic acid, propionic acid, and butyric acid as main components. Their profiles are various depending on the substrate and fermentation conditions [Zhang *et al*. 2015]. More notably, it also can be used as substrates for OCFA production because of the higher portion of propionic acid which is a provider of propionyl-CoA, a crucial primer of OCFAs.

The utilization of VFAs for the microbial lipid production was reported in *Cryptococcus curvatus* [Liu *et al*. 2017]. In this study, two different conditions of substrates, propionic acid as a single carbon source or mixture of VFAs were tested. *C. curvatus* could use 5 – 40 g/L of pure propionic acid as the sole carbon source to accumulate lipid. The highest production of OCFAs was 0.65 g/L from 15 g/L of propionic acid. When the mixture of VFAs was utilized, the highest OCFAs reached 1.37 g/L, when the ratio of acetic acid: propionic acid: butyric acid was 5:15:10. The highest content of OCFAs, 45.1% in total lipids, was obtained when the ratio of VFAs was 0:15:15, which showed the contribution of butyric acid on the biosynthesis of OCFAs as well as propionic acid.

Another possibility of synthesizing OCFA is using long-chain precursors. The study of using n-alkanes with a chain length of 15 and 17 carbons for the production of OCFAs was reported [Matatkova *et al*. 2017]. Three yeast strains (*Candida krusei* DBM 2136, *T. cutaneum* CCY 30-5-10, and *Y. lipolytica* CCY 30-26-36) were tested to see if the yeasts can transform the odd-chain alkanes to OCFAs without their degradation to short carbon chain compounds. *Y. lipolytica* was found as the most suitable strain for the growth on n-alkanes (n-pentadecane and n-heptadecane) as a sole carbon source. It seems that the addition of biosurfactant, rhamnolipids, is crucial for increasing OCFAs contents, the highest content of OCFAs in *Y. lipolytica* (44.5% in total lipids) was obtained from 3 g/L of heptadecane with rhamnolipid.

As the interest of microbial production of OCFAs has been increasing recently, many studies aimed to screen the strains, to test different substrates, and to optimize the conditions of fermentation for OCFA production have been published [Zhang *et al*. 2020]. However, it is known that the production of OCFAs from fermentation with native microorganisms is highly dependent on the strains, substrates, and culture conditions. In order to realize the scale-up of OCFA production, there are several issues to be solved such as 1) the toxicity of propionate,

propionic acid, propanol, and VFAs, 2) the lack of understanding about propionyl-CoA metabolism in each platform strain, and 3) the low productivity.

1.2.5.5. Production of OCFA by metabolic engineering

It was firstly reported that propionate can induce synthesis of a very small amount of OCFAs by wild-type *E. coli* K-12 [Ingram *et al*. 1977]. After this, some studies about propionate metabolism, OCFA production from propionate, and OCFA production from other conventional substrates through synthetic pathways have been described in *E. coli*.

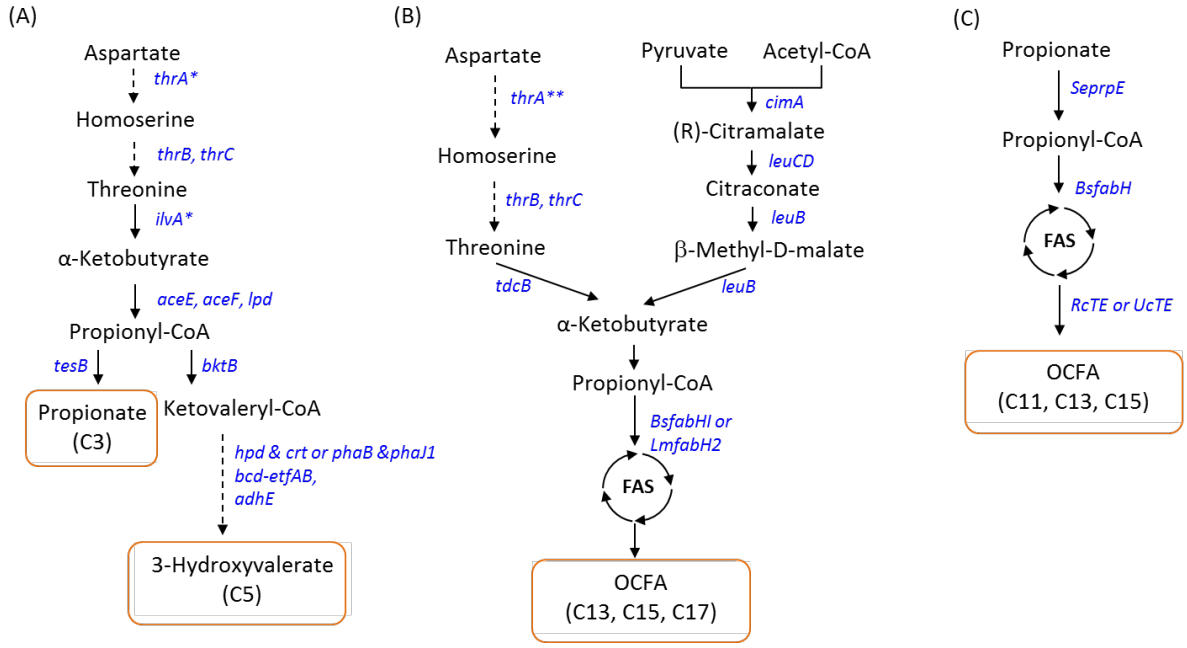


Figure 1.8. Metabolic engineering approaches for OCFA production in *E. coli*.
(A) Up-regulating threonine pathway for the production of very short-chain OCFAs [Tseng and Prather, 2012]. (B) Increasing propionyl-CoA synthesis for OCFA production [Lee *et al*. 2013]. (C) Increasing propionyl-CoA and exchanging β-ketoacyl-ACP synthase III for OCFA production [Wu and san, 2014a; Wu and San, 2014b]. The engineered steps are indicated with the corresponding genes in blue (native and heterologous, with or without mutation), the final products are indicated in the orange boxes. Dashed lines indicate the multiple steps.

Tseng and Prather demonstrated the controlled production of odd-numbered carbon chains like pentanol and very short-chain OCFAs such as propionate, *trans*-2-pentenoate, and valerate by constructing a modular pathway in *E. coli* as described in Figure 1.8 (A) [Tseng and Prather, 2012]. They tried to prevent for feeding propionate, a pathway for propionyl-CoA synthesis from glucose or glycerol through up-regulation of threonine biosynthesis was constructed by overexpressing seven *E. coli* genes (module (a), *thrA**, *thrB*, *thrC*, *ilvA**, *aceE, aceF, and lpd*). From module (a), 0.804 g/L of propionate was produced from glycerol with the overexpression of *tesB* thioesterase. The next modules were also constructed and validated; (b) overexpressing *bktB* gene of *Cupriavidus necator*, *hbd*, *crt*, and *bcd-etfAB* genes from *C. acetobutylicum*, (c) overexpressing *phaB* from *C. necator* H16, *phaJ1* from *Pseudomonas*

aeruginosa, and *adhE* from *C. acetobutylicum*. When three modules were combined, 0.963 g/L of 3-hydroxyvalerate was produced from glycerol. Through several combinations with these modules, the authors showed the synthesis of various odd-numbered compounds, up to C5.

Lee *et al*. showed the genetic engineering of *E. coli* to increase propionyl-CoA by overexpressing endogenous pathways, threonine intermediate pathway and citramalate pathway as described in Figure 1.8 (B) [Lee *et al*. 2013]. Threonine intermediate pathway includes the enzymes (aspartokinase, homoserine dehydrogenase, ThrA** with the mutation for conferring reduced feedback inhibition; homoserine kinase, ThrB; threonine synthase, ThrC; threonine deaminase, TdcB) involved in the biosynthesis and degradation of threonine, which increased metabolic flux to α-ketobutyrate. The overexpression of four genes resulted in the increased OCFA content in total FAs from 1% to 18%. The further increase of OCFAs was obtained by the exchange of β-ketoacyl ACP synthase (FabH) with *FabHI* from *Bacilllus subtilis* having a higher specificity towards propionyl-CoA. The OCFA production reached to 0.832 g/L, representing 72% in total FAs. The OCFAs produced in this study generally included C13:0, C15:0, C17:0, and C17:1, with C15:0 being the predominant OCFAs. The same group also explored the citramalate pathway for increasing propionyl-CoA, thus improving the production of OCFAs. The overexpression of three genes (citramalate synthase, *cimA*; isopropyl malate isomerase, *leuCD*; β-isopropyl malate dehydrogenase, *leuB*) was less efficient on OCFA production than the threonine intermediate pathway. The same group also investigated the β-ketoacyl synthase (FabH) from other origins (*B. subtilis*, *Listeria monocytogenes*, *Propionibacterium freudenreichii*, *Stenotrophomonas maltophila, Alicyclobacillus acidocaldarius*, and *Desulfobulbus propionicus*) which are known to utilize the propionyl-CoA in the priming reaction for fatty acid biosynthesis. Each *FabH* gene was overexpressed in the strain overexpressing threonine intermediate pathway, the highest OCFAs (0.622 g/L, 32% in total FAs) was obtained in the strain overexpressing *fabH2* from *L. monocytogenes* which was increased by 1.6 times (in g/L) compared to the control strain.

In 2014, two different strategies for improving OCFA production were reported through propionate supplementation in *E. coli* as described in Figure 1.8 (C) [Wu and san, 2014a; Wu and San 2014b]. One study evaluated the heterologous thioesterase terminating fatty acyl group extension by hydrolyzing the acyl moiety from the acyl-ACP at the appropriate chain length, to see if the higher specificity to odd-chain compounds improves the synthesis of OCFAs [Wu and San, 2014a]. The strain carrying the acyl-ACP TE genes from *Umbellularia californica* (*UcTE*) produced undecanoic acid (C11) and tridecanoic acid (C13) as the major OCFAs, while the strains carrying the acyl-ACP TE genes from *Ricinus communis* (*RcTE*) preferred pentadecanoic acid (C15). Introducing the propionyl-CoA synthetase gene (*prpE*) from *S. enterica* into these strains has been shown to further improve the OCFA production by increasing the intracellular propionyl-CoA availability. The final strain overexpressing *prpE* and *UcTE* produced 0.276 g/L of OCFAs with a ratio of 23.4% in total FAs. In the case of the strain overexpressing *prpE* and *RcTE*, the OCFAs titer (0.297 g/L) was a little higher but the

ratio of OCFAs (17.6%) in total FAs was lower than those of the strain overexpressing *prpE* and *UcTE*. The possibility of engineering the profiles of OCFAs was firstly described here, which can be applied to other systems.

The same group further engineered *E. coli* strain to improve the OCFA production by evaluating the heterologous β-ketoacyl-ACP synthase III (KAS III encoded by *fabH*) [Wu and San, 2014b]. KAS III is responsible for initiating both straight- and branched-chain fatty acid biosynthesis, some fabH shows the specificity to propionyl-CoA as described in other studies [Tseng and Prather, 2012; Lee *et al*. 2013]. In this study, four KASIII from *B. subtilis* (BsFabH1, BsFabH2), *Staphylococcus aureus* (SaFabH), and *Streptomyces peucetius* (SpDpsC) were investigated to enhance the propionyl-CoA specificity, respectively. The strain overexpressing *BsfabH2* and *SaFabH* produced significantly more OCFAs, by 7.43 and 6.57 times compared to the control strain at 48 h. When the *fabH* and *prpE* are overexpressed simultaneously, the strain carrying *SeprpE-BsfabH2* and *SeprpE-BsfabH1* accumulated large quantities of OCFAs, 0.567 and 0.387 g/L, respectively. The authors disrupted the native *fabH* in *E. coli* and confirmed the highest amount of OCFAs in *SeprpE-SafabH* expressing strain, 1.205 g/L (60.25% in total lipids) with the major component C15, 84.65% of total OCFAs. The highest percentage of OCFAs in total lipids was obtained by the strain overexpressing *SeprpE* and *BsfabH1*, about 85.1% in total FAs at 24 h. This study showed the importance of the specificity of β-ketoacyl-ACP synthase for improving the production of OCFAs.

Compared to the study in *E. coli*, the production of OCFAs in yeast has more focused on the screening of the strains or the optimization of the fermentation as described in the part 1.2.5.4. Very recently, the synthesis of OCFAs together with DHA in unmodified oleaginous heterotrophic microalgae (*Schizochytrium*) was reported [Wang *et al*. 2019]. The elevation of NADPH supply by overexpressing a malic enzyme (ME) from *Crypthecodinium cohnii* and the relieved feedback inhibition on acetyl-CoA carboxylase (ACC) by overexpressing *ELO3* from *Mortierella alpina* resulted in the increase of OCFA and DHA by 2.57- and 1.08-fold, reaching a level of 3.32 g/L (25.08% in total FAs) and 3.54 g/L (26.70% in total FAs), respectively. The titer of OCFAs composed of C15:0 and C17:0 is the highest among the studies on the production of OCFAs, so far. Interestingly, the significant increase of propionyl-CoA concentration was observed in the engineered strain without engineering the pathway of propionyl-CoA or supplementing propionate as the substrate. Further studies on the synthesis and regulation of propionyl-CoA in *Schizochytrium* sp. S31 will be helpful to increase the production of OCFAs. This study shows the advantage of selecting oleaginous microorganisms for OCFA production compared to bacteria, regarding the level of OCFAs (in g/L).

The previous studies on fermentation and metabolic engineering for OCFA production as summarized in Table 1.2. showed the limitation of low titer, therefore, the selection of suitable chassis and the efficient metabolic engineering strategy are necessary to increase the production of OCFAs. Regarding the availability of engineering tools, the oleaginous properties, the easily controllable culture conditions, and the range of substrates, *Y. lipolytica* is a promising platform organism for producing OCFAs.

Table 1.2. Production of OCFAs in microorganisms.

Strategy	Strains	Substrate (g/L)	Biomass (g/L)	Lipid (g/L)	Lipid content % (g/g DCW)	OCFAs /Total lipids (%)	OCFAs (g/L)	Reference
Fermentation	*Candida sp.*	Propionic acid 4 g/L					0.111	Rezanka *et al*. 2015
Fermentation	*Trichosporon cutaneum*	Propionic acid 4 g/L	1.60	0.38	23.90	~35	0.13	Kolouchova *et al*. 2015
Fermentation	*Trichosporon cutaneum*	Glucose 20 g/L Propionate 4 g/L	1.76	0.64	36.10	< 20	< 0.13	Kolouchova *et al*. 2015
Fermentation	*Cryptococcus curvatus*	Propionate 16.5 g/L	6.7	2.28	34.1	38.7	0.88	Zheng *et al*. 2012
Substrate optimization	*Cryptococcus curvatus*	VFA 30 g/L	8.24	3.83	46.47	42.3	1.37	Liu *et al*. 2017
Substrate optimization	*Rhodococcus sp.YHY01*	Glycerol 5 g/L Propionate 5 g/L	1.98	1.40	70.78	85.10	1.19	Bhatia *et al*. 2019a
Fermentation	*Rhodococcus opacus*	Glucose 12 g/L 1-Propanol 15 g/L		1.58		80.46	1.27	Zhang *et al*. 2019
Fermentation	*Yarrowia lipolytica*	Propionate 4 g/L	3.53	0.31	8.90	~30	<0.093	Koluchova *et al*. 2015
Fermentation	*Yarrowia lipolytica*	Glucose 20 g/L Propionate 4 g/L	3.83	0.39	10.20	<15	<0.040	Koluchova *et al*. 2015
Fermentaion	*Yarrowia lipolytica*	Pentadecane 3 g/L Rhamnolipids	2.75	0.47	17.20	44.5	0.209	Matatkova *et al*. 2017
Engineering precursor pools and thioesterase	*Escherichia coli*	Propionate 8 g/L	1.178			23.43	0.276	Wu and San, 2014a
Engineering FAS	*Escherichia coli*	Glucose 14.4 g/L Propionate 8 g/L	2.0			60.25	1.205	Wu and San, 2014b
Engineering cofactor and precursor pools	*Schizochytrium* sp. S31	Glucose 40 g/L	13.24	9.48	71.68	25.08	3.32	Wang *et al*. 2019

1.3. Objectives

The first objective of this thesis is the development of the engineering tools, specifically erythritol-inducible promoters that can be utilized for the fine-tuning of gene expression at a defined time in *Y. lipolytica*. Novel erythritol-inducible promoters with native, mutated, and biosynthetically hybridized forms are constructed and their functions are characterized in *Y. lipolytica*.

The second objective is the production of OCFAs from propionate in *Y. lipolytica*. Since propionate, the main substrate for OCFA synthesis, shows toxicity to the cells, the identification of native propionate-tolerant gene is one of the strategies to improve OCFA production. In addition, metabolic engineering of *Y. lipolytica* such as inhibiting competitive pathway and enhancing carbon flux to lipid synthesis is another goal for the increase of OCFA production.

The third objective is the enhancement of precursor pool availability of ECFA and OCFAs in *Y. lipolytica*. During the assessment of the candidate pathways, the rate-limiting step of OCFAs synthesis is identified and targeted for rational engineering to improve OCFA production. Also, the substrates are optimized for more balanced supply of precursor pools.

The fourth objective is the production of OCFAs without propionate supplementation. The candidate pathway synthesizing the main precursor of OCFAs, propionyl-CoA, is explored and verified by overexpression in *Y. lipolytica.*

Finally, the ultimate objective of this thesis is to develop *Y. lipolytica* as a promising platform strain for the production of lipids, especially odd-chain fatty acids (OCFAs), by metabolic engineering.

CHAPTER 2. MATERIALS AND METHODS

2.1. Strains, media, and growth conditions

Media and growth conditions for *E. coli* were as described by Sambrook and Green [Sambrook and Green, 2012]. *E. coli* strain DH5α was used for cloning and plasmid propagation. Cells were grown at 37 °C with constant shaking on 5 mL Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), and ampicillin (100 µg/mL) or kanamycin (50 µg/mL) were added for plasmid selection. Solid media were prepared by adding 1.5% (w/v) agar. All strains used in this thesis are described in Appendix A by chapter.

Media and growth conditions for *Y. lipolytica* were as described by Barth and Gaillardin [Barth and Gaillardin, 1996]. *Y. lipolytica* strains were grown at 28 °C with constant shaking (180 rpm) in different media depending on the objective of experiments as below.

- Rich medium (YPD) - 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose
- Minimal medium (YNB) - 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww), 0.5% (w/v) NH_4Cl, 50 mM KH_2PO_4-Na_2HPO_4 (pH 6.8)
  Depending on the carbon source and the concentration added to YNB, we named the media "YNB and abbreviation of substrates with the concentration in percentage".
  e.g. YNBD1 (1% (w/v) glucose), YNBP1 (1% (w/v) propionate), and YNBD0.5P1A0.5 (0.5% (w/v) glucose, 1% (w/v) propionate, 0.5% (w/v) acetate).
  To complement auxotrophic processes, 0.1 g/L of uracil or leucine (Difco) was added as necessary. To screen for hygromycin resistance, 250 µg/mL of hygromycin was added to the appropriate media. Solid media were prepared by adding 1.5% (w/v) agar.

For lipid biosynthesis in minimal media, cultures were prepared as follows: an initial pre-culture was established by inoculating cells to 10 mL of YPD medium in 50 mL Erlenmeyer flasks. This was followed by overnight cultivation at 28 °C and 180 rpm. The resulting cell suspension was washed with sterile distilled water and used to inoculate to 50 mL of YNB medium with various concentrations of carbon sources and/or nitrogen source depends on the genotype of strains and the purpose of the experiments in 250 mL Erlenmeyer flasks, grown at 28 °C and 180 rpm. Detailed conditions for each experiment are described in the result chapter. For each strain and set of conditions, we used two or three biological replicates and calculated average and standard deviation values.

2.2. Cloning

2.2.1. General molecular biology

Standard molecular genetic techniques were used in this study [Sambrook and Green, 2012]. Restriction enzymes were obtained from New England Biolabs (MA, USA). The PCRs were performed using an Applied Biosystems 2720 Thermal Cycler and employing GoTaq DNA

Polymerase (Promega, WI, USA) or Q5 High-Fidelity DNA Polymerase (New England Biolabs). PCR fragments were purified with a PCR Purification Kit (Macherey-Nagel, Duren, Germany), and plasmids were purified with a Plasmid Miniprep Kit (Macherey-Nagel).

2.2.2. Gene synthesis

Native genes were amplified from genomic DNA of *Y. lipolytica* W29 strain through PCR for cloning. Overlap PCR was performed to remove the undesired recognition site of restriction enzyme. Heterologous genes were synthesized by GeneScript Biotech (NJ, USA), TWIST Bioscience (CA, USA), or GeneCust (Boynes, France). Codon optimizations were done through the customized software by each provider. Sequences of primers and synthesized genes used in this thesis are described in Appendix B and C, respectively.

2.2.3. Plasmid construction

2.2.3.1. Gene expression plasmid by regular cloning

Classical restriction enzyme digestion and ligation method were used for one gene expression. JMP62-based plasmids containing zeta sequences, selective marker (URA3, LEU2, Hygro), *TEF* promoter, and *LIP2* terminator were used [Nicaud *et al*. 2002]. The gene of interest was cloned into JMP62 plasmid by using BamHI and AvrII recognition sites. Selective marker or promoter can be replaced by using I-SceI or ClaI and BamHI restriction enzymes, respectively. The gene expression cassette should be linearized by NotI digestion for the integration into *Y. lipolytica* genome.

2.2.3.2. Gene expression plasmid by Golden Gate assembly

Golden Gate assembly (GG) using type II restriction enzyme (BsaI) was developed for *Y. lipolytica* in previous studies [Celińska *et al*. 2017; Larroude *et al*. 2019]. Each part necessary for assembling to gene expression plasmid has specific overhang as described in Table 2.1 which makes the assembly in order and interchangeable. Then GG assembly was done with the following protocols.

Table 2.1. Description of specific overhangs designed for GG assembly in *Y. lipolytica*.

Position	5' overhang	3' overhang
InsertUp (NotI)	GCCT	AGGT
Marker	AGGT	ACGG
Promoter1	ACGG	AATG
Gene1	AATG	TCTA
Terminator1	TCTA	GCTT
Promoter2	GCTT	ACAA
Gene2	ACAA	GGAT
Terminator2	GGAT	GTCA

Promoter3	GTCA	CCAC
Gene3	CCAC	GTAT
Terminator3	GTAT	GAGT
InsertDown (NotI)	GAGT	TGCG
Destination vector	TGCG	GCCT

a. Cloning the gene of interest into TOPO plasmid (Invitrogen, Waltham, US) with specific overhang depends on the position (G1: AATG-TCTA, G2: ACAA-GGAT, G3: CCAC-GTAT).
b. Prepare the GG reaction mixture with each GG component adjusted to 50 pmoles, the destination plasmid, BsaI, T4 ligase buffer, and T4 ligase.
c. Run the GG reaction with the following thermal profile.
* When assembling three transcription units (promoter-gene-terminator), the GG reaction was done through two separate reactions in order to increase the efficiency. Three or Four parts in order were grouped and assembled respectively in the first GG reactions. Then all parts were mixed into one tube for the second GG reactions.

Table 2.2. Thermal profiles of GG assembly.

Cycle	50		1	1	1
Temperature	37 °C	16 °C	60 °C	80 °C	15 °C
Time	5 min	5 min	10 min	10 min	hold

d. Transform the GG reaction mixture to *E. coli* and screen the white colonies for further verification.

2.2.3.3. Gene disruption plasmid for PUT method

The general principle of gene disruption in *Y. lipolytica* was the replacement of the target locus, by double cross-over homologous recombination, with a NotI-digested cassette that consisted of a selection marker flanked by 1 kb of promoter and terminator region of the target gene [Fickers *et al*. 2003]. Each fragment of promoter and terminator was cloned with an appropriate order of NotI and I-SceI recognition sites to TOPO plasmid, respectively. Then the selective marker was cloned between the two fragments with I-SceI recognition site. The disruption cassette was prepared by NotI digestion before the transformation.

2.2.3.4. Gene disruption plasmid for CRISPR/Cas9

The Cas9 plasmid compatible with any designed guide RNA was set up in the previous study [Larroude *et al*. 2020]. Selecting target sequence, designing the guide RNA, and constructing Cas9 plasmid were done using the following protocol to construct Cas9-guide RNA plasmid based on GG assembly.

a. Select the targeting guide RNA with the CRISPOR program (http://crispor.tefor.net/).

b. Design the primer for cloning the guide RNA into Cas9-harboring plasmid.

Table 2.3. Primer sequence for Cas9 plasmid construction.

Name	Sequence (5' – 3')	Use
GGP_gRNA_BsmbI_Gene_Fw	TTCGATTCCGGGTCGGCGCAGGTTG(g)xxxxxxGTTTTA	Cloning gRNA
GGP_gRNA_BsmbI_Gene_Rv	GCTCTAAAACxxxxxx (c)CAACCTGCGCCGACCCGGAAT	
VerifsgRNA_Fw	CTTTGAAAAATACCTCTAATGCGCC	Verification
VerifsgRNA_Rv	AAGCACCGACTCGGTGCCA	

* xxxx is the targeting guide RNA sequence from the prediction except PAM.

c. Construct the double stranded gRNA insert by phosphorylation of the two designed primers. The phosphorylation mixture (10 µL) includes T4 kinase (1 µL), 100 µM Forward primer (1°µL), 100 µM Reverse primer (1 µL), T4 ligase buffer (1 µL). Incubate the mixture at 37 °C for 30 minutes and then heat the mixture to 95 °C for 5 min. Cooling the mixture very slowly.

d. Run the GG reaction with the mixture (20 µL) including gRNA insert (diluted 200 times, 2°µL), pGGA_CRISPRl (100 ng), T4 ligase buffer (2 µL), BsmBI (1 µL), T7 ligase (1 µL). Verify the assembly by colony PCR with primer set VerifsgRNA_Fw/Rv.

2.2.3.5. Verification of construction

2.2.3.5.1. Colony PCR

Around 8 - 16 transformants on selective media were selected for verification by colony PCR with the following methods.

a. Prepare PCR mixture with appropriate primer set and 2X GoTaq Master Mix.
b. Transfer the colony from selective media to PCR mixture and resuspend.
c. Run the PCR reaction with the following thermal profiles.

Table 2.4. Thermal profiles of *E. coli* colony PCR.

Cycle	1	25			1	1
Temperature	95 °C	95 °C	X °C	72 °C	72 °C	10 °C
Time	10 min	30 sec	30 sec	1 min/kb	5 min	hold

*Annealing temperature (X) is adjusted by the primer set.

2.2.3.5.2. Digestion by restriction enzyme

Plasmids, extracted from *E. coli* after overnight culture on selective media, are cut by restriction enzymes. Time, temperature, and buffer for the reaction are specific for each enzyme and should be verified on the specification sheet. After the incubation, the size of fragments is verified by electrophoresis in an agarose gel at 0.8% with ethidium bromide.

2.3. Construction of *Y. lipolytica* strain

2.3.1. Transformation

2.3.1.1. LiAc method

Transformation of *Y. lipolytica* was performed using the lithium-acetate method adapted from Barth and Gaillardin [Barth and Gaillardin, 1996]. *Y. lipolytica* strains are streaked on YPD plates and grown for 16 h at 28 °C. Fresh cells around a scoop by 5 µl loop are washed with 1 mL sterile water. Cells are then resuspended in 600 µl LiAc 0.1 M pH 6.0 and incubated 1 h at 28 °C. Cells are centrifuged for 2 min at 3000 rpm and resuspended in 60 µl of LiAc 0.1 M pH 6. Afterward, 40 µl of competent cells are mixed with 3 µl of carrier DNA (5 mg/mL, Dualsystems AG Biotech) and 10 µl of DNA to be transformed (200 - 1000 ng), and incubated 15 min at 28 °C. 350 µl of PEG solution (Polyethylene glycol in 0.1 M LiAc pH 6) is added to the transformation mix. After 1 h of incubation at 28 °C, a 10 min heat shock at 39 °C is performed. After 600 µl of LiAc solution is added, the transformation mixture is plated on selective media. Transformants are selected on YNB, YNBLeu, YNBUra, or YNBHygro, depending on their genotype.

2.3.1.2. Transformation kit

Frozen-EZ Yeast Transformation Kit (Zymo Research, CA, USA) was also used for the transformation of *Y. lipolytica*, according to the manufacturer protocol.

2.3.1.3. Transformation for CRISPR/Cas9

The basic method of transformation is same as described in 2.3.1.1. Around 500 – 1000 ng of Cas9-guide RNA plasmid is used for transformation. After heat shock and addition of 600 µl of LiAc solution, the transformation mixture can be spread either on YNB selective media directly or transferred to liquid selective media for 1 – 2 days outgrowth and then spread to YPD media with appropriate dilution.

2.3.2. Verification of construction in *Y. lipolytica*

Around 8 - 16 of *Y. lipolytica* transformants on selective media were selected for verification by colony PCR with the following methods.

a. Resuspend each colony with 2 µl of sterile water.

b. Run the cell lysis of the yeast suspensions on a PCR machine with the following thermal profile described in Table 2.5.

Table 2.5. Thermal profiles for *Y. lipolytica* cell lysis.

Temperature	65 °C	8 °C	65 °C	97 °C	8 °C	65 °C	97 °C	65 °C	80 °C
Time	30 sec	30 sec	1 min 30s	3 min	1 min	3 min	1 min	1 min	15 min

c. Mix the cell suspension with PCR mixture (primers and 2X GoTaq Master Mix) and run the PCR reaction with the following thermal profile described in Table 2.6.

Table 2.6. Thermal profiles of *Y. lipolytica* colony PCR.

Cycle	1	8			32			1	1
Temperature	94 °C	94 °C	60 °C	68 °C	94 °C	X °C	68 °C	68 °C	10 °C
Time	6 min	35 sec	45 sec	2 min 30 sec	35 sec	45 sec	1 min/kb	10 min	Hold

*Annealing temperature (X) is adjusted by the primer set.

2.3.3. Re-use of marker

The removal of the selective marker was carried out *via* the Cre-LoxP system as described in Fickers *et al*. [Fickers *et al*. 2003]. After transformation with the Cre-expressing plasmid (replicative plasmid, JME547), the loss of the marker gene was verified on the selective medium. The loss of the Cre-expressing plasmid was checked using replica plating on YPD with and without hygromycin after culturing on YPD for 24 hours.

2.4. Analysis

2.4.1. Growth

2.4.1.1. 96-well plate assay (liquid)

Pre-cultures were inoculated into 96-well plates containing 200 µl of YPD medium, and cultured overnight (28 °C, 180 rpm). Cell suspensions were transferred to the minimal YNB medium with an initial OD_{600nm} of 0.1 and cultivated at 28 °C with constant shaking. Different substrates were used depending on the experiments. OD_{600nm} was measured every 30 minutes for 120 hours by a microtiter plate reader (Biotek Synergy MX, Biotek Instruments, Colmar, France). For each strain and set of conditions, we used two or three biological replicates. The growth rate was calculated in the exponential phase for each strain and condition.

2.4.1.1. Spot assay (solid)

The cells were inoculated in 3 ml of YNBD1 and grown overnight. After their optical density (OD_{600nm}) values were adjusted to 1.0, these cell suspensions and three sequential dilutions (1:10, $1:10^2$, and $1:10^3$) were applied (3 µl) to the surface of YNB solid medium supplemented with adequate substrates (with or without organic acids) and were incubated at 28 °C for 3–5 days.

2.4.1.2. Dry cell weight (DCW)

To determine DCW in flask experiments, 2 mL of the culture were washed and lysophilized in a pre-weighed tube. The differences in weight corresponded to the mg of cells found from 2 mL of culture. For each data point, we used at least two biological replicates and calculated average and standard deviation values.

2.4.2. Fluorescence

The preparation of culture and cells are same as growth assay as described in 2.4.1.1. Red fluorescence was analyzed at the following wavelength settings: excitation at 558 nm and emission at 586 nm. Fluorescence was expressed as specific fluorescence value (SFU, Fluorescence/OD_{600nm}) or mean specific fluorescence rate (SFU/h, the mean value of SFU per hour). For the RedStarII measurements, no intrinsic fluorescence was detected. For each strain and set of conditions, we used at least two biological replicates and calculated average and standard deviation values.

2.4.3. Metabolites

All metabolites (glucose, propionate, and acetate) were identified and quantified by HPLC. Filtered aliquots of the culture medium were diluted by 10 times and analyzed by the UltiMate 3000 system (Thermo Fisher Scientific, UK) using an Aminex HPX-87H column (300 mm x 7.8 mm, Bio-RAD, USA) coupled to UV and RI detectors. Glucose is analyzed through RI detector, and other acids were analyzed through UV detector (210 nm). The mobile phase used was 0.01 N H_2SO_4 with a flow rate of 0.6 mL/min and the column temperature was 35 °C. Identification and quantification were achieved *via* comparisons to standards. For each data point, we used at least two biological replicates and calculated average and standard deviation values.

2.4.4. Lipids

Lipids were extracted from 10 - 20 mg of freeze-dried cells and converted into their fatty acid methyl esters (FAMEs) according to Browse *et al*. [Browse *et al*. 1986], and FAMEs were analyzed by gas chromatography (GC) analysis. GC analysis of FAMEs was carried out by a Varian 3900 instrument equipped with a flame ionization detector and a Varian FactorFour vf-23ms column, where the bleed specification at 260 °C is 3 pA (30 m, 0.25 mm, 0.25 µm). Fatty acids were identified by comparison to commercial FAME standards (FAME32, Supelco) and quantified by the internal standard method, involving the addition of 100 µg of commercial dodecanoic acid (Sigma-Aldrich). Commercial OCFAs (9 Odd carbon fatty acids, OC9, Supelco) were converted to their FAMEs with the same method for yeast samples and analyzed by GC to identify and compare OCFAs from yeast samples.

2.4.5. Morphology

Images were obtained by using a Zeiss Axio Imager M2 microscope (Zeiss, Le Pecq, France) with a 100x objective lens and Zeiss filter sets 45 and 46 for fluorescence microscopy. Axiovision 4.8 software (Zeiss, Le Pecq, France) was used for image acquisition. To make the lipid bodies (LBs) visible, BodiPy® Lipid Probe (2.5 mg/mL in ethanol, Invitrogen) was added to the cell suspension (OD_{600nm} = 5) and the samples were incubated for 10 min at room temperature.

2.4.6. Gene and protein sequence

Gene and protein sequences were obtained from NCBI (www.ncbi.nlm.nih.gov), UniprotKB (http://www.uniprot.org/help/uniprotkb), and the yeast genomic database Génolevures (http://gryc.inra.fr/). The alignment of peptide sequences was performed using MultAlin (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html).

Blast searches were carried out on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Benchling software was employed for the gene sequence analysis and *in silico* plasmid construction (https://benchling.com/). Transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)

CHAPTER 3. DEVELOPMENT OF SYNTHETIC BIOLOGICAL TOOLS - SYNTHETIC INDUCIBLE PROMOTER

3.1. Introduction

In synthetic biology, gene expression must be fine-tuned to ensure optimal flows in related pathways or to avoid metabolic burdens. *cis*-Regulatory modules (CRMs) are non-coding DNA elements that help regulate gene expression *via* the binding of transcription factors to motifs in CRM sequences, thus facilitating cell adaptation to internal conditions and the exterior environment. Predicting CRMs is thus a key part of understanding the complex processes underlying cell regulation; it is also necessary for designing efficient cellular factories, notably by engineering promoters with context-specific expression. As indicated in a review by Aerts [Aerts, 2012], many computational strategies have been developed throughout the years to identify CRMs. Among them, the phylogenetic footprinting exploits the fact that regulatory modules have been evolutionarily conserved among related species. Motifs identified in the promoters of orthologous genes can be tested for functionality, and the corresponding upstream activating sequences (UASs) can then be used to construct hybrid promoters.

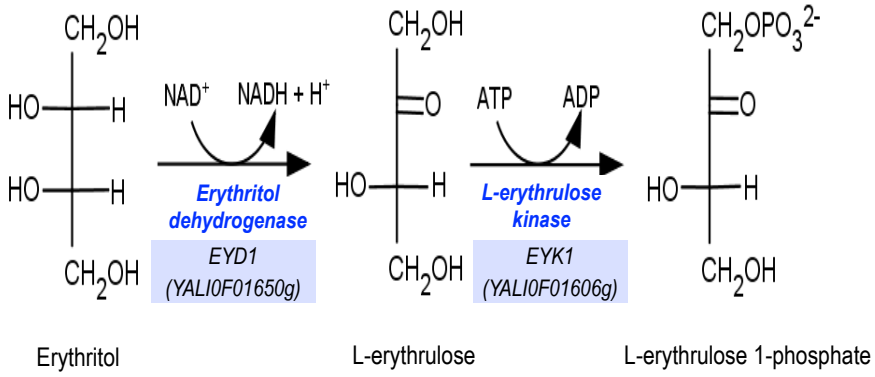


Figure 3.1. Pathways of erythritol catabolism in *Y. lipolytica*.

Erythritol is converted into erythrulose by the erythritol dehydrogenase encoded by *EYD1* (*YALI0F01650g*). The erythrulose then becomes erythrulose-phosphate *via* a phosphorylation reaction catalyzed by the erythrulose kinase encoded by *EYK1* (*YALI0F01606g*) [Carly *et al*. 2017a; Carly *et al*.2017 b; Carly *et al*. 2018].

Recently, the catabolic pathway of erythritol was identified (Figure 3.1). Erythritol is catalyzed to erythrulose by the erythritol dehydrogenase encoded by *EYD1* (*YALI0F01650g*) [Carly *et al*. 2018]. Then, erythrulose is converted into erythrulose-1-phosphate by the erythrulose kinase encoded by *EYK1* (*YALI0F01606g*) [Carly *et al*. 2017a; Carly *et al*. 2017b]. Expression of both genes is induced by erythritol; the *EYD1* gene displayed 46-fold higher expression on erythritol medium than on glucose medium, a similar induction by erythritol (41-fold increase) was observed for *EYK1* gene [Carly *et al*. 2017a; Carly *et al*. 2017b; Carly *et al*. 2018; Carly and Fickers 2018]. Consequently, both genes might contain CRMs that respond to erythritol or

erythrulose. From the previous study, two CRMs were identified within the *EYK1* promoter region using sequence conservation among members of the *Yarrowia* clade, and the first erythritol-induced hybrid promoters were developed in *Y. lipolytica* [Trassaert *et al*. 2017].

However, to engineer complex pathways, a large set of promoters with different strengths and expression profiles is necessary. The different expressions depending on the growth phase (eg. pTEF in the exponential phase, hp4d in the late exponential phase) or inducible expression will be favorable in that the expression can be controlled at a defined time.

In this chapter, the CRMs for *EYK1* and *EYD1* were identified and a set of inducible promoters was constructed as a form of biobricks that could be used in Golden Gate assembly (GGAS) in *Y. lipolytica*. The expression with newly constructed promoters was characterized by the reporter protein (RedStarII) and the heterologous protein (CalB lipase) applicable in the enzyme industry.

3.2. Engineering of erythritol-inducible promoters

3.2.1. Identification of CRMs within *EYK1* and *EYD1* promoters

Multiple alignments of the nucleotide sequences of the *EYK1* and *EYD1* gene promoters among the *Yarrowia* clade (*Y. lipolytica* [YALI], *Yarrowia phangngensis* [YAPH], *Yarrowia yakushimensis* [YAYA], *Yarrowia alimentaria* [YAAL], and *Yarrowia galli* [YAGA]) was performed using the program Clustal Omega [http://www.ebi.ac.uk/Tools/msa/clustalo/, Larkin *et al*. 2007]. The alignment results highlighted the CRM motifs that have been conserved through evolution and that are likely to have a regulatory function. The conserved motifs were named Box A and Box B, and the region containing these motifs plus 5 to 17 bases on either side of the motifs were selected for constructing new promoters.

Trassaert and colleagues identified two CRMs in the promoter region of *EYK1*, named UAS1-eyk1 (Box A) having the consensus sequence [CGGNANCNNNANNNGGAAAGCCG], and UAS2-eyk1 (Box B) having the minimal consensus sequence [CNTGCATNATCCGANGAC] (Figure 3.2 (A)) [Trasseart *et al*. 2017]. It was also identified a UAS1-eyk1 motif that responded to erythritol, thus allowing the development of the erythritol-inducible hybrid promoters [Trassaert *et al*. 2017].

To identify the regulatory element (i.e., UAS) within the *EYD1* promoter region, we analyzed the intergenic region between *YALI0F01650g* (*EYD1*) and the upstream gene *YALI0F01672g*, using a similar CRM search. Since this intergenic region was longer than 5500 bp (i.e., 5591 bp; Figure 3.3), we analyzed the upstream region using the 800 bp nucleic acid sequence found upstream from *EYD1*. We examined how the promoter region of the *EYD1* gene in *Y. lipolytica* is conserved with that of other species in the *Yarrowia* clade (Figure 3.3). This alignment process highlighted the existence of three putative conserved elements within the region 300 bp upstream; these elements were a putative TATA box (Box TATA; GATATAWA)

and two CRMs. The first box, which had the main signature [ANTTNNNTTTCCNNATNNGG], was named CRM1-eyd1 (Box A). The second box, which had the main signature [CGGNNCTNNATTGAGAANNC], was named CRM2-eyd1 (Box B) and a variable number of CA repeats were found. Like the *EYK1* promoter, the *EYD1* promoter also had two CRMs, which may also represent motifs required for erythritol and/or erythrulose regulation.

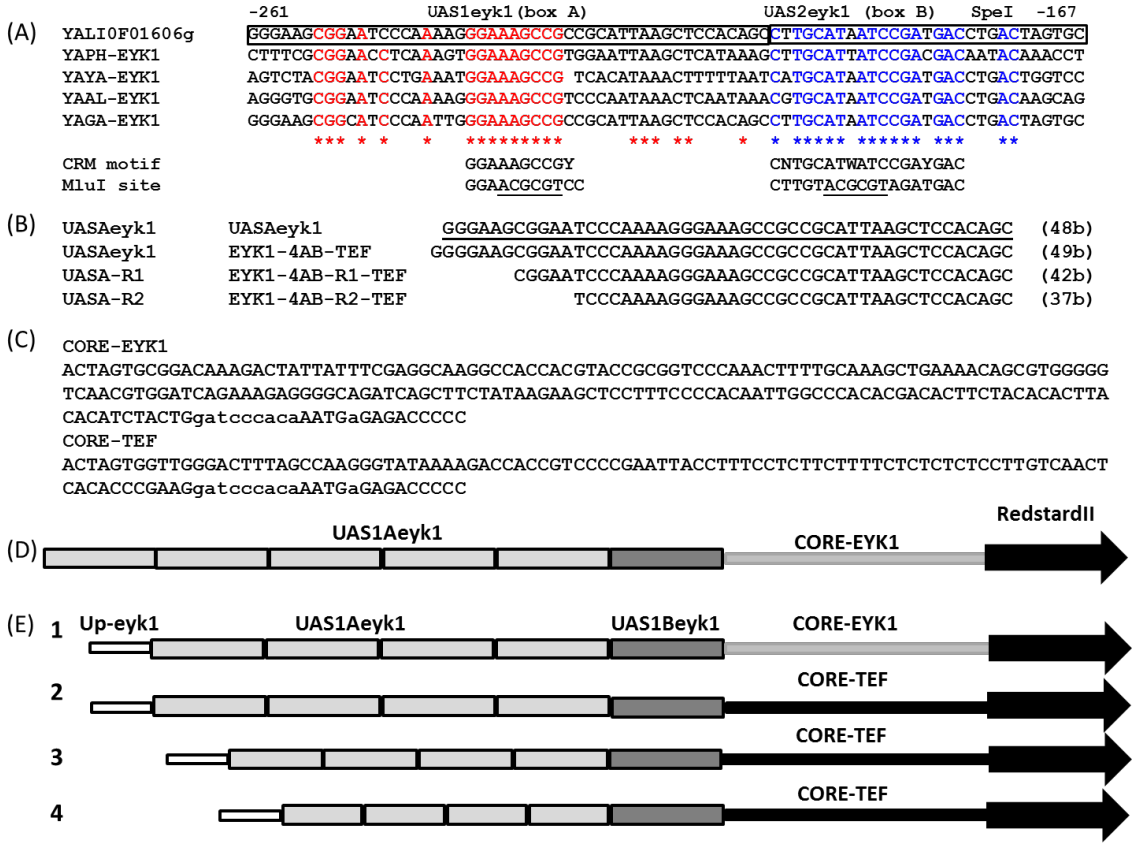


Figure 3.2. Multiple alignment of the *EYK1* promoter containing the two CRMs and a schematic representation of the *EYK1* hybrid promoters.

(A) Alignment of the *Y. lipolytica EYK1* promoter region –261 to – 167 containing the two CRMs (the UAS1-eyk1 and UAS2-eyk1 motifs). The CRMs are indicated with asterisks, and the corresponding CRM consensus sequences are provided. The genomic sequences are from *Y. lipolytica* W29 (YALI; *YALI0F01606g*), *Yarrowia phangngensis* (YAPH), *Yarrowia yakushimensis* (YAYA), *Yarrowia alimentaria* (YAAL), and *Yarrowia galli* (YAGA). The underlined *Mul*I sites used in the mutation of the CRMs are shown in panel (A). The region containing the UAS1-eyk1 motif used for tandem repeat construction is boxed. (B) Sequence of the UAS1-eyk1 motif (48 bp) used for EYK3AB hybrid promoter construction [Trassaert *et al*. 2017] and the reduced regions used in this study. (C) core EYK and core TEF sequences. (D) Schematic representation of the hybrid promoters that contained varying numbers of copies of UAS1-eyk1, which controlled the expression of RedStarII. (E) Schematic representation of the hybrid promoters that control the expression of RedStarII. 1, EYK-4AB promoter; 2, EYK-4AB-coreTEF; 3, EYK4AB-R1-coreTEF; 4, EYK4AB-R2-coreTEF.

N (any base), W (A or T), and Y (C or T) are defined according to IUPAC nucleotide codes [IUPAC, 1970].

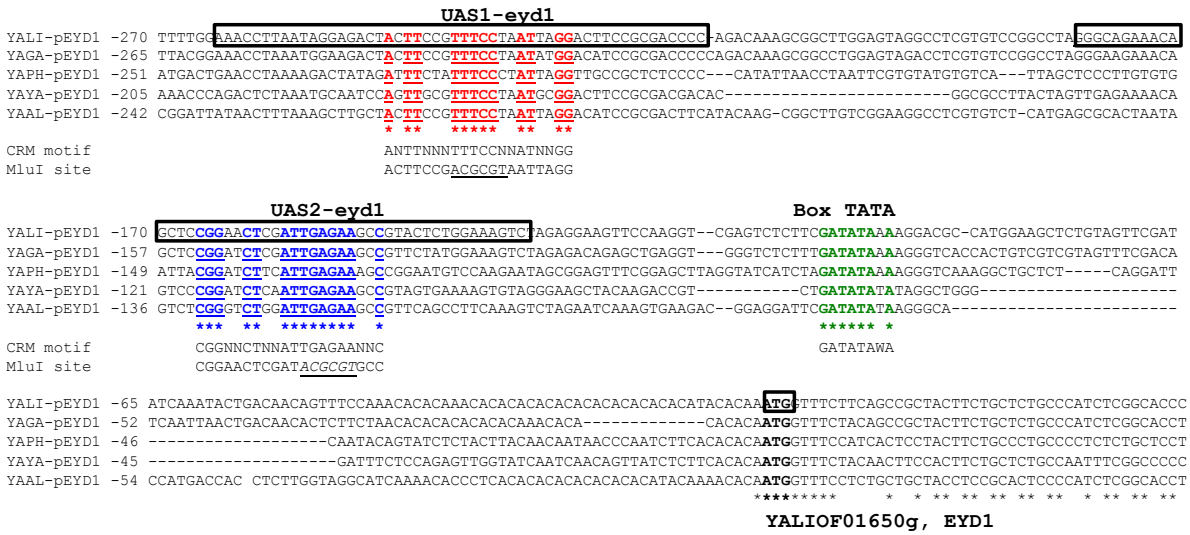


Figure 3.3. Multiple alignment of the *EYD1* promoter.

The alignment of the region between *YALI0F01650g* (*EYD1*) and the upstream gene *YALI0F01672g* in *Yarrowia* clade highlights the putative conserved cis-regulatory modules (CRMs) that represent putative regulatory elements for the expression and regulation of the *EYD1* gene by erythritol and erythrulose. The genomic sequences are from *Y. lipolytica* W29 (YALI-pEYD1), *Yarrowia phangngensis* (YAPH-pEYD1), *Yarrowia yakushimensis* (YAYA-pEYD1), *Yarrowia alimentaria* (YAAL-pEYD1), and *Yarrowia galli* (YAGA-pEYD1). The region containing the UAS1-eyd1 and UAS2-eyd1 motifs used for tandem repeat construction is boxed. The nucleic acids that have been conserved in the five species are indicated with asterisks. The start codon of *EYD1* is indicated as a boxed ATG. The *Mul*I sites used in the mutation of the CRMs are shown. In the CRM sequences, N represents any nucleotide.

3.2.2. Construction of expression cassettes by Golden Gate assembly for promoter analysis

Each promoter biobrick was designed and constructed to be compatible with *Y. lipolytica* GGAS, previously described by Celińska and colleagues [Celińska *et al*. 2017]. The assemblies were designed containing different promoter variants; the ORF encoding fluorescent protein RedStarII; and the *LIP2* terminator, which were all incorporated using the BsaI sites and specific overhangs (Table 2.1). Assembled plasmids were extracted and verified by PCR using the primer pair URA3-internal-FW/ZetaDOWN-internal-RV and *Not*I digestion. The expression cassettes digested by *Not*I were transformed into the *Y. lipolytica EYK1* WT strain (JMY1212) and *eyk1*Δ strain (JMY7126), respectively. All constructed plasmid and *Y. lipolytica* strains are described in Table 3.1.

Table 3.1. Table of promoter construction scheme.

Promoter	Primer		Template used	*E. coli* strain	*Y. lipolytica* strain	
	Forward	Reverse			JMY1212	JMY7126
TEF1	P1 TEF FW	P1 TEF RV	JME2928	GGE085	GGY037	GGY109
EYK1	P1 EYK FW	P1 EYK RV	JME3934	GGE238	JMY7382	JMY7384
EYK1-2AB	P1 EYK FW	P1 EYK RV	synthesized	GGE130	GGY027	GGY056
EYK1-3AB	P1 EYK FW	P1 EYK RV	synthesized	GGE104	JMY7345	JMY7394
EYK1-4AB	P1 EYK FW	P1 EYK RV	synthesized	GGE132	GGY033	GGY068
EYK1-5AB	P1 EYK FW	P1 EYK RV	Synthesized	GGE250	JMY7390	JMY7392
EYD1AB	P1 EYD FW	P1 EYD RV	*Y. lipolytica* genomic DNA	GGE140	JMY7386	JMY7388
EYD1A*B	P1 EYD FW EYD UAS1 MluI FW	EYD UAS1 MluI RV P1 EYD RV	GGE140	GGE172	JMY7398	JMY7349
EYD1AB*	P1 EYD FW EYD UAS2 MluI FW	EYD UAS2 MluI RV P1 EYD RV	GGE140	GGE174	JMY7396	JMY7351
EYK UAS1-4AB-TEF	-	-	Synthesized	JME4417	JMY7325	JMY7400
EYK UAS1-4AB-R1-TEF	-	-	Synthesized	JME4418	JMY7327	JMY7402
EYK UAS1-4AB-R2-TEF	-	-	Synthesized	JME4419	JMY7329	JMY7404
EYK1/EYD1A-EYK1	-	-	Synthesized	JME4420	JMY7430	JMY7432
EYK1/EYD1A-TEF	-	-	Synthesized	JME4421	JMY7335	JMY7337
EYK1/EYD1B-EYK1	-	-	Synthesized	JME4422	JMY7339	JMY7341
EYK1/EYD1B-TEF1	-	-	Synthesized	JME4423	JMY7343	JMY7408

3.2.3. Construction of inducible promoter with tandem repeats of $UAS1_{EYK1}$

It was shown that promoter strength was increased with the hybrid promoter pEYK300A3B, which was composed of three repeats of the 48 bp UAS1-eyk1 [Trassaert *et al*. 2017]. Four new hybrid promoters were generated by fusing two, three, four, and five UAS1-eyk1 tandem elements taken from the *EYK1* promoter, which were named EYK1-2AB, EYK1-3AB, EYK1-4AB, and EYK1-5AB, respectively (Figure 3.2 (D)). The expression levels and strengths of the hybrid *EYK1* promoters were determined by quantifying RedStarII expression. Fluorescence was expressed as mean specific fluorescence rate (SFU/h, SFU: florescence per OD_{600nm}).

We determined the mean specific fluorescence rate (SFU/h) of the *EYK1* WT (JMY1212) grown on erythritol and of the *eyk1*Δ strain (JMY7126) grown on glucose + erythritol (results were compared to glucose-only medium; Figure 3.4 and Table 3.2). In the *EYK1* WT (JMY1212), the expression of RedStarII was increased slightly with UAS1-eyk1 repeats on the glucose medium, ranged from 0.54 to 4.42 SFU/h (Table 3.2). The SFU rate was significantly increased on the erythritol medium, from 2.28 SFU/h for pEYK1 (one copy) to 48.12 SFU/h for EYK1-5AB (five copies). Relative induction was also increased, from 4.3-fold to 19.0-fold. The optimal level of expression was observed for EYK1-4AB. When erythritol was used as an inducer, TEF promoter strength (65.42 SFU/h) was equivalent to that on glucose medium; the strength of EYK1-4AB (48.12 SFU/h) was comparable to that of pTEF.

In *eyk1*Δ strain (JMY7126), the expression was also increased concomitantly with UAS1-eyk1 repeats, ranging from 0.76 to 13.15 SFU/h on glucose medium (Table 3.2). The mean SFU rate was increased significantly on erythritol medium, from 7.13 SFU/h for EYK1 (one copy) to 90.15 SFU/h for EYK1-5AB (five copies). Relative induction was also increased, from 9.4-fold to 45.8-fold. The optimal level was observed for EYK1-2AB. When erythritol was used as an inducer, the TEF promoter displayed slightly reduced strength (17.45 SFU/h), while EYK1-5AB remained strong (90.15 SFU/h). Under such growth conditions and for this strain background (deletion of *EYK1* gene), the performance of the *EYK1* hybrid promoter surpassed that of the *TEF* promoter, shown as 5.16-fold stronger expression.

3.2.4. Reduction of the $UAS1_{EYK1}$region

Promoter strength also depends on the core promoter [Shabbir Hussain *et al*. 2016]. We tested hybrid promoters with a core TEF and examined the effect of reducing the size of the UAS1-eyk1 motif (Figure 3.2 (E)). Synthetic promoters with different UAS lengths were constructed: UAS1-4AB-TEF (four repeats of a 69 bp UAS1-eyk1), UAS1-4AB-R1-TEF (four repeats of a 62 bp UAS1-eyk1r1), and UAS1-4AB-R2-TEF (four repeats of a 57 bp UAS1-eyk1r2). When erythritol was used as an inducer, the strength of the EYK1-4AB-coreTEF promoter was increased by 1.65-fold (80.14 SFU/h *vs.* 45.50 SFU/h for EYK1-4AB) in the *EYK1* WT (JMY1212) and, more surprisingly, that of the EYK1-4AB-coreTEF promoter was increased by 4.04-fold (340.52 SFU/h *vs.* 84.29 SFU/h for EYK1-4AB) in the *eyk1*Δ strain (JMY7126) (Figure 3.4). Although we observed an increase in expression levels, the induction levels were reduced. This result indicates that promoter strength decreases when the length of the UAS1-eyk1 motif shrinks, which shows that CRM1 eyk1 extends to the conserved CGG sequence, yielding a consensus sequence of [CGGNANCNNNANNGGAAAGCCG].

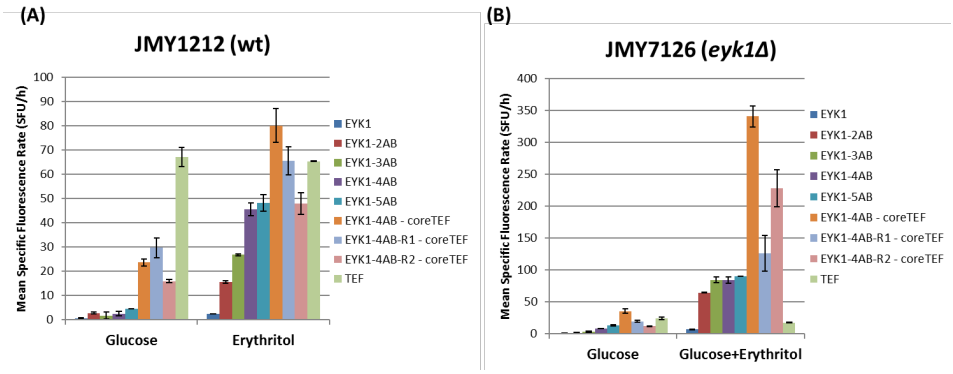


Figure 3.4. Hybrid *EYK1* promoter expression and strength depending on the medium and strain genetic background.

(A) Results for the *EYK1* wild-type (JMY1212), which could use erythritol for growth. (B) Results for the *eyk1*Δ mutant (JMY7126), which could not metabolize erythritol. Promoter strength was determined by quantifying RedStarII expression and comparing the mean rate of specific fluorescence (SFU/h) obtained when the *EYK1* wild-type (JMY1212) was grown on erythritol medium or the *eyk1*Δ (JMY7126) was grown on glucose + erythritol medium *versus* when they were grown on glucose alone.

Table 3.2. Promoter expression and induction levels in the *EYK1* wild-type (WT) and the *eyk1*Δ mutant.

Promoter	*EYK1* WT (JMY1212)			*eyk1*Δ mutant (JMY7126)		
	Glucose[a] (SFU/h)	Erythritol[a] (SFU/h)	Fold change[b]	Glucose[a] (SFU/h)	Glucose + Erythritol[a] (SFU/h)	Fold change[b]
TEF	67.16 ± 3.87	65.42 ± 0.17	1.0	24.11 ± 1.88	17.45 ± 0.39	0.7
EYK1	0.54 ± 0.23	2.28 ± 0.04	4.3	0.76 ± 0.13	7.13 ± 0.51	9.4
EYK1-2AB	2.63 ± 0.38	15.55 ± 0.55	5.9	1.41 ± 0.57	64.48 ± 0.49	45.8
EYK1-3AB	1.68 ± 1.44	26.76 ± 0.38	15.9	3.23 ± 1.39	84.41 ± 4.55	26.1
EYK1-4AB	2.39 ± 0.88	45.50 ± 2.70	19.0	8.18 ± 0.07	84.29 ± 5.21	10.3
EYK1-5AB	4.42 ± 0.09	48.12 ± 3.43	10.9	13.15 ± 0.81	90.15 ± 0.30	6.9
EYK1-4AB - coreTEF	23.57 ± 1.37	80.14 ± 7.06	3.4	35.53 ± 3.73	340.52 ± 16.45	9.6
EYK1-4AB-R1 - coreTEF	29.62 ± 4.01	65.50 ± 5.80	2.2	19.72 ± 1.54	125.94 ± 28.09	6.4
EYK1-4AB-R2 - coreTEF	15.88 ± 0.76	47.89 ± 4.49	3.0	12.06 ± 0.68	227.84 ± 29.20	18.9

[a]SFU: specific fluorescence value (florescence per $OD600_{nm}$), [b]calculated by comparing the results on erythritol to those on glucose.

3.2.5. Construction of EYDBoth $UAS1_{EYD1}$ and $UAS2_{EYD1}$ give rise to an inducible promoter in both the WT (JMY1212) and the *eyk1Δ* mutant (JMY7126)

In order to verify the functional CRMs of the *EYD1* gene, the two conserved motifs, CRMa and CRMb, were mutated by introducing a *Mlu*I site respectively (Figure 3.5 (B)). Then promoter strength and induction levels of the EYD1A*B and EYD1AB* were compared with those of the *EYK1* and *EYD1* promoters using the *EYK1* WT (JMY1212) and the *eyk1*Δ mutant (JMY7126) (Figure 3.6 and Table 3.3).

In the *EYK1* WT (JMY1212) on glucose medium, the RedstarII expression levels by pEYD1 (0.85 SFU/h) were similar to those by pEYK1 (0.54 SFU/h) (Table 3.2 and Table 3.3). The mutation of Box A (EYD1A*B) completely abolished the expression of RedStarII on glucose medium (Figure 3.6). However, RedStarII continued to be slightly expressed on erythritol (0.16 SFU/h), indicating that CRMa is important for expression and induction. In contrast, the mutation of Box B (EYD1AB*) resulted in just a 2-fold reduction of RedStarII expression on glucose medium (0.43 SFU/h). RedStarII expression levels were higher on erythritol (2.57 SFU/h), indicating that CRMb is less important than CRMa for expression and induction (Table 3.3).

In the *eyk1*Δ mutant (JMY7126), unexpected patterns of expression and relative induction were observed on glucose + erythritol medium (Figure 3.6 and Table 3.3). All three promoters, including the mutated ones, showed low levels of expression on the glucose medium (0.5 SFU/h) but higher levels of expression on the glucose + erythritol medium (194.50 to 457.51 SFU/h); a tremendous induction was observed, ranging from 357.6 to 896.1 SFU/h. These

results indicate that both CRMa and CRMb are important for expression and induction under these growth conditions and in this genetic background.

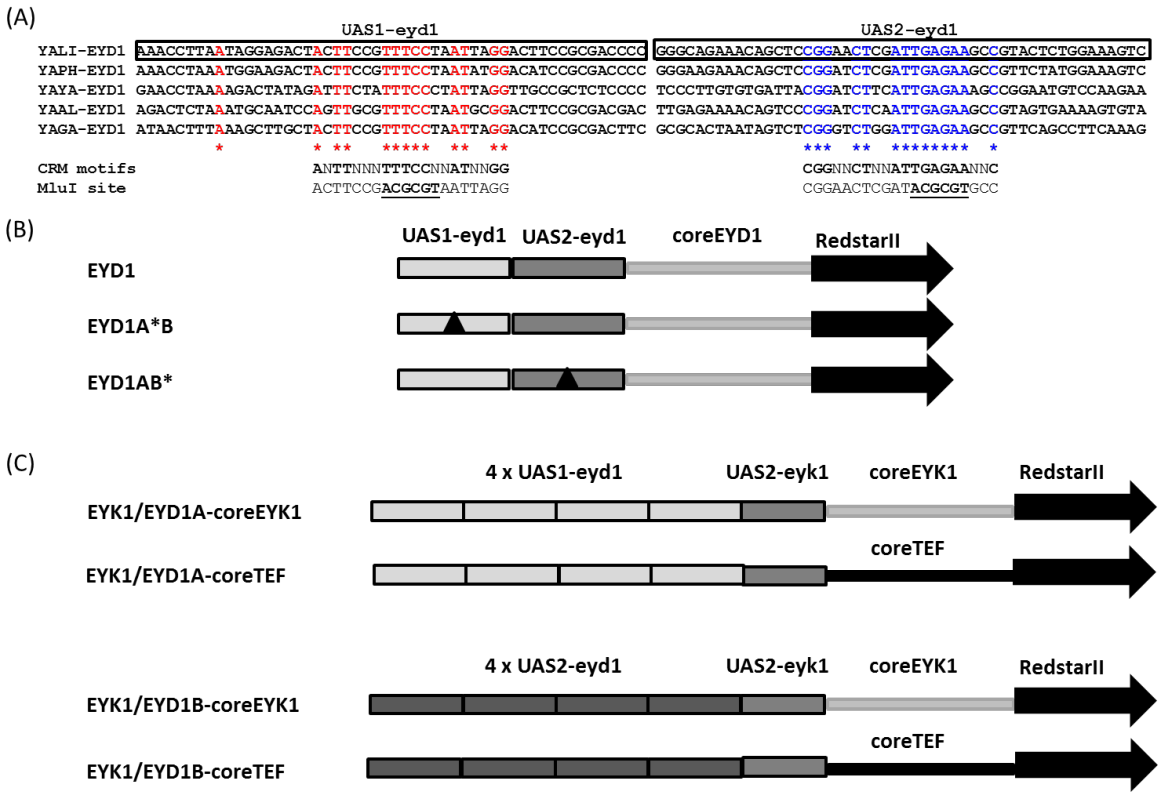


Figure 3.5. Multiple alignment of the *EYD1* UAS and a schematic representation of the mutated and hybrid promoters used in this study.

(A) Multiple alignment of the UAS1-eyd1 and UAS2-eyd1 motifs of the *EYD1* promoter in the *Yarrowia* clade. The CRMs are indicated with asterisks, and the corresponding CRM consensus sequences are provided. The region containing the UAS1-eyd1 and UAS2-eyd1 motifs used in tandem repeat construction is boxed. (B) Schematic representation of the wild-type *EYD1* promoter (EYD1) and the mutated *EYD1* promoters (EYD1A*B and EYD1AB*). (C) Schematic representation of the hybrid EYD1/EYK1 promoters containing either the core EYK1 or the core TEF promoter; EYK1/EYD1A-EYK1, four tandem repeats of UAS1-eyd1 + UAS2-eyk1 + coreEYK1; EYK1/EYD1A-TEF, four tandem repeats of UAS1-eyd1 + UAS2-eyk1 + coreTEF; EYK1/EYD2-EYK1, four tandem repeats of UAS2-eyd1 + UAS2-eyk1 + coreEYK1; or EYK1/EYD1B-TEF, four tandem repeats of UAS2-eyd1 + UAS2-eyk1 + coreTEF.

3.2.6. Characterization of the role of $UAS1_{EYD1}$ and $UAS2_{EYD1}$

To determine the respective role of CRMa and CRMb in erythritol-based expression and induction, four hybrid promoters were constructed (Figure 3.5 (A)). Two hybrid promoters EYK1/EYD1 were designed; they incorporated either four tandem repeats of UAS1-eyd1 or four tandem repeats of UAS2-eyd1 in the place of UAS-eyk1, which gave rise to EYK1/EYD1A-coreEYK1 and EYK1/EYD1B-coreEYK1, respectively (Figure 3.5 (C)). Two additional hybrid promoters were designed using a core TEF, which gave rise to EYK1/EYD1A-coreTEF and

EYK1/EYD1B-coreTEF (Figure 3.5 (C)). These expression cassettes were introduced into the *EYK1* WT (JMY1212) and the *eyk1*Δ mutant (JMY7126). In *EYK1* WT (JMY1212), UAS1-eyd1 allowed efficient expression of RedStarII in erythritol medium (66.94 SFU/h, with a 6.8-fold change between glucose and erythritol media). In contrast, in both media, low expression levels were observed for the promoters containing the four tandem repeats of UAS2-eyd1 (Figure 3.5 (C) and Table 3.2). In the *eyk1*Δ mutant (JMY7126), both UAS1-eyd1 and UAS2-eyd1 allowed expression of RedStarII in erythritol medium (91.15 SFU/h and 52.57 SFU/h, respectively). This result confirmed that both UAS1 and UAS2 are involved in erythritol induction (Figure 3.6 and Table 3.3). In both strains, exchanging the core EYK1 with the core TEF had a drastic effect on erythritol induction (Figure 3.6 and Table 3.4) but did not modify expression levels significantly. This result shows that the use of a more efficient core promoter did not contribute to the development of inducible promoters.

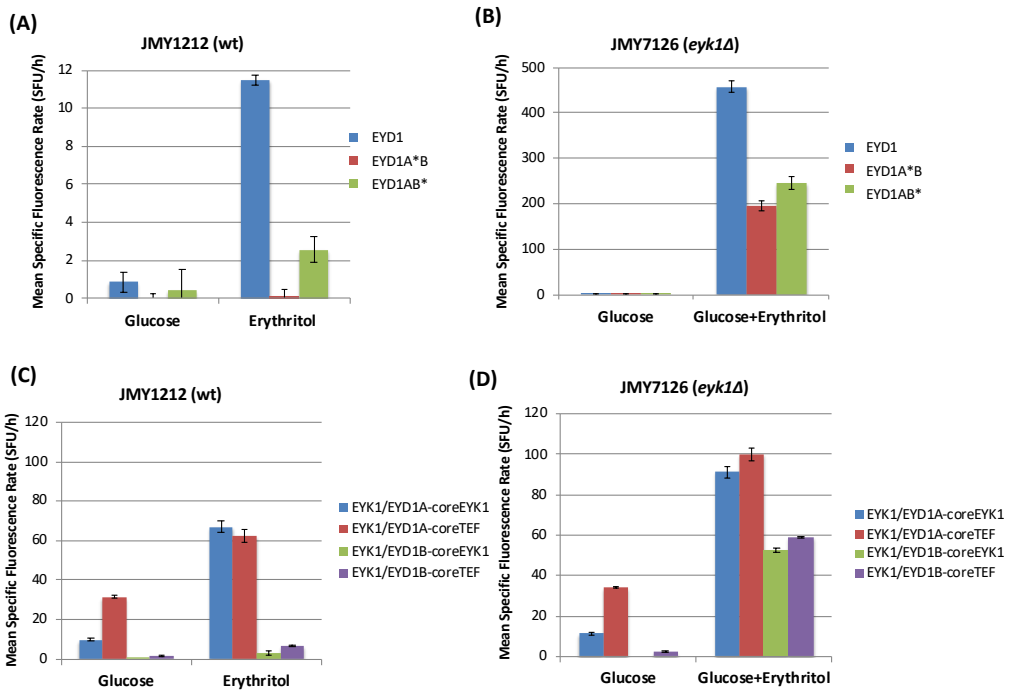


Figure 3.6. Hybrid *EYD1* promoter expression and strength depending on medium and strain genetic background.

(A) and (C) Results for the *EYK1* wild-type (JMY1212), which could use erythritol for growth. (B) and (D) Results for the *eyk1Δ* mutant (JMY7126), which could not metabolize erythritol. Promoter strength was determined by quantifying RedStarII expression and comparing the mean specific fluorescence rate (SFU/h) obtained when the *EYK1* wild-type (JMY1212) was grown on erythritol medium or the *eyk1Δ* transformant (JMY7126) was grown on glucose + erythritol medium *versus* when they were grown on glucose alone.

Table 3.3. Strength of different promoters in the *EYK1* wild-type (WT) and the *eyk1*Δ mutant.

Promoter	***EYK1* WT (JMY1212)**			***eyk1*Δ mutant (JMY7126)**		
	Glucose[a] (SFU/h)	Erythritol[a] (SFU/h)	Fold change[b]	Glucose[a] (SFU/h)	Glucose + Erythritol[a] (SFU/h)	Fold change[b]
EYD1AB	0.85 ± 0.54	11.50 ± 0.25	13.4	0.67 ± 1.52	457.51 ± 11.37	682.5
EYD1A*B	-[c]	0.16 ± 0.32	-	0.54 ± 0.88	194.50 ± 11.50	357.6
EYD1AB*	0.43 ± 1.09	2.57 ± 0.66	5.9	0.27 ± 0.15	245.27 ± 14.56	896.1

[a]SFU: pecific fluorescence value (florescence per $OD600_{nm}$), [b]calculated by comparing the results on erythritol to those on glucose, [c]No fluorescence was detected.

Table 3.4. Promoter strength in the *EYK1* wild-type (WT) and the *eyk1Δ* mutant depending on the *EYD1* upstreaming activating sequence (UAS) and core promoter.

Promoter	***EYK1* WT (JMY1212)**			***eyk1*Δ mutant (JMY7126)**		
	Glucose[a] (SFU/h)	Erythritol[a] (SFU/h)	Fold change[b]	Glucose[a] (SFU/h)	Glucose + Erythritol[a] (SFU/h)	Fold change[b]
EYK1/EYD1A-coreEYK1	9.85 ± 0.78	66.94 ± 2.81	6.8	11.24 ± 1.82	91.15 ± 8.46	8.1
EYK1/EYD1A-coreTEF	31.57 ± 0.50	62.38 ± 3.30	2.0	34.06 ± 1.65	99.70 ± 17.14	2.9
EYK1/EYD1B-coreEYK1	0.10 ± 0.00	3.23 ± 1.62	32.3	-[c]	52.57 ± 0.76	
EYK1/EYD1B-coreTEF	1.51 ± 0.42	6.81 ± 0.42	4.5	2.11 ± 0.29	59.03 ± 6.00	28.0

[a]SFU: pecific fluorescence value (florescence per $OD600_{nm}$), [b]calculated by comparing the results on erythritol to those on glucose, [c]No fluorescence was detected.

During this work, a master internship student (Paulina Korpys, Poznan University of Life Science, Poland) has participated in the part of strain construction and fluorescence measurement.

3.3. Application of the synthetic inducible promoter for recombinant protein production; the expression of CalB

As a proof of concept, the *EYK1*- and *EYD1*-derived hybrid promoters were used for the production of a protein of industrial interest, the lipase CalB from *Candida antarctica*. For that purpose, the pro-CalB sequence was codon-optimized for *Y. lipolytica*, fused with the signal sequence from the *LIP2* gene for the secretion (annotated as *CalB* afterwards), and cloned under the control of promoters pEYD1, pEYK1-3AB, pHU8EYK, and of the strong constitutive promoter pTEF as a control. Under different promoters, CalB gene expression and extracellular activity were monitored during cultures in bioreactor.

3.3.1. Construction of expression vectors and CalB production strains

In most of recombinant protein production processes, protein synthesis occurs in a growth phase-decoupled manner. For that purpose, promoters based on *LIP2* and *POX2* genes strongly induced by oleic acid, and the promoter from *XPR2* gene induced by peptides have been developed [Sassi *et al*. 2016; Nicaud *et al*. 2002; Madzak *et al*. 2000]. Although these regulated promoters delivered strong gene expression, their utilization at industrial scale is impeded by water-insoluble property of inducers (i.e. triglycerides and fatty acids) or the requirements of high peptides concentrations and a pH above six [Trassaert *et al*. 2017]. In that promoters derived from *EYK1* and *EYD1* genes are induced by hydrophilic substances such as erythritol and erythrulose [Trassaert *et al*. 2017; Park *et al*. 2019; unpublished observations], these inducible promoters are more favorable for industrial protein production. Three promoters, pEYK1-3AB (three copies of UAS1-eyk1, one copy of UAS2-eyk1, and core *EYK1*), pHU8EYK (eight copies of UAS1-xpr2 and coreEYK1), and pEYD1, were selected for this study (Figure 3.7). The expression cassettes of CalB under different promoters were transformed into JMY7126 strain (*MATA ura3-302 leu2-270-LEU2-Zeta*, *xpr2-322*, *lip2Δ*, *lip7Δ*, *lip8Δ*, *lys5Δ*, *eyk1Δ*), resulting the integration at zeta-docking platform. The docking system prevents variability caused by random genomic integration, allowing to compare the three selected erythritol/erythrulose-inducible promoters with the constitutive promoter pTEF used as a reference.

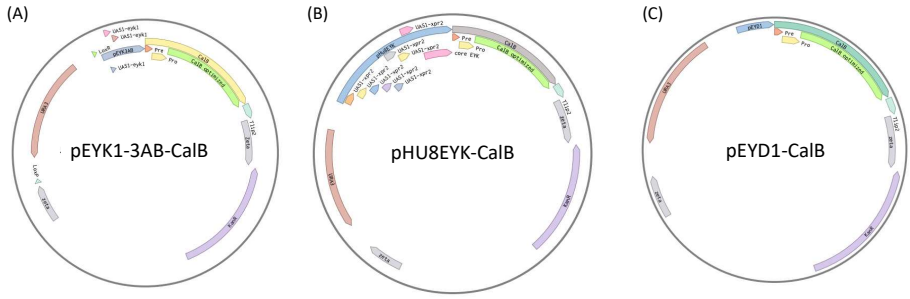


Figure 3.7. Maps of *CalB*-expressing plasmids.
(A) JME4365 containing pEYK1-3AB. (B) JME4243 containing pHU8EYK. (C) JME4590 containing pEYD1.

3.3.2. Comparison of erythritol-inducible promoters on *CalB* gene expression and protein production

The expression levels and enzymatic activities of CalB were analyzed for the four promoters in the bioreactor (Figure 3.8, the detailed condition of fermentation and analysis are described in Appendix E – Park *et al*. Microbial Cell Factories, 2019). A good correlation between promoter strength and lipase activity were observed. A similar expression was obtained with pEYD1 and pTEF, while 2.5- and 2.7-fold higher expression of CalB than pTEF was shown with pEYK1-3AB and pHU8EYK (Figure 3.8 (A)). The specific lipase activities of CalB with pEYK1-3AB and pHU8EYK were 2.8- and 2.5-fold higher than that obtained with pTEF, respectively (Figure 3.8 (B)). The enzymatic productivities obtained with pEYK1-3AB and pHU8EYK were 1.7- and 1.6-fold higher than the lipase productivity obtained from the previous study with *LIP2* promoter [Fickers *et al*. 2005].

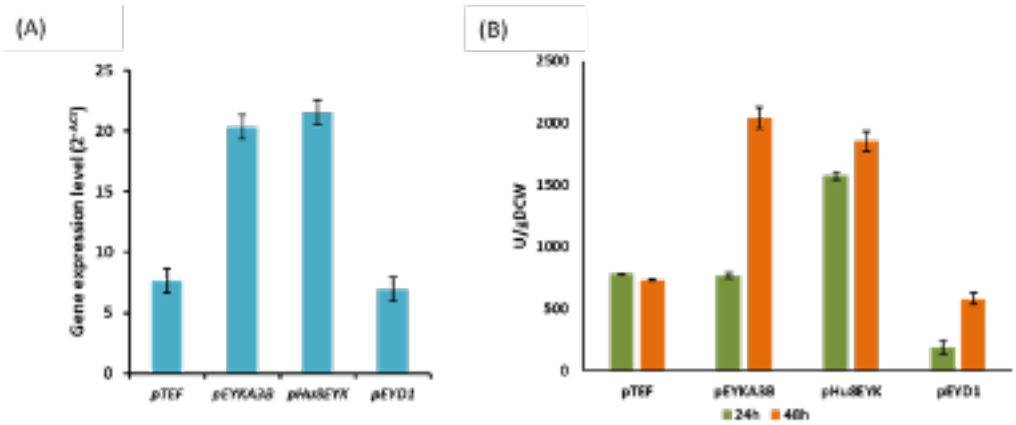


Figure 3.8. Expression levels (A) and enzymatic activities (B) of *CalB* gene under the control of promoters pTEF (strain JMY7536), pEYKA3B (strain JMY7539), pHu8EYK (strain JMY7544), and pEYD1 (strain JMY7548).

Cells were grown at 28 °C in YNBGE medium, in 2Mag mini bioreactors. Gene expression levels were analyzed from the cells grown for 24h, and the expression levels were normalized to that the one of actin. The specific lipase activities were analyzed after 24 h and 48 h of cultivation.

3.3.3. CalB production in bioreactor

From the activity analysis with different promoters, pEYK1-3AB was selected to test process conditions. The strain JMY7989, a prototroph derivative of strain JMY7539 was grown for 48 h in YNBG2E medium in DASGIP bioreactor, with pH and pO2 regulation (more details of fermentation condition are described in Appendix E – Park *et al*. Microbial Cell Factories, 2019). The exponential growth phase lasted for 12 h with a specific growth rate of 0.29 ± 0.00 h^{-1} and final biomass of 6.96 ± 0.04 g L^{-1} (Figure 3.9 (A), Table 3.5). Within the first 24 h, the main carbon source (glycerol) had been entirely consumed, and the inducer (erythritol) assimilated by the cells. Lipase activity reached its highest titer (28,024 ± 743 U mL^{-1}) after 24 h of culture (Figure 3.9 (A)). Then, it decreased slightly after until the end of the culture (20,150 ± 1,060 U mL^{-1}). Analysis of culture supernatant by SDS-PAGE clearly highlighted that

CalB is the only secreted protein in those conditions (Figure 3.9 (B)). During the enzyme production phase (between 3.5 and 24 h of culture), the lipase volumetric productivity was of 1,357 ± 34 U mL^{-1} h^{-1} (Table 3.5). Culture of strain JMY7990 (pTEF-CalB prototroph) in the same experimental conditions yielded to a 6.2-fold lower lipase activity after 24 h (data not shown).

Additional copy of CalB (pEYK1-3AB-CalB) was transformed to JMY7539, resulting JMY7991 strain in order to see if the multi-copy expression is increasing the production of lipase. Cell growth kinetics of strain JMY7991 (multi-copy) was found similar to that of RIY368 (mono-copy) (Figure 3.9 (A)). It seems that the additional expression of heterologous gene does not alter host strain metabolism. As shown in Figure 3.9 and Table 3.5, lipase activity of JMY7991 reached its highest level (45,125 ± 2,144 U mL^{-1}) after 24 h of culture, again after entire consumption of glycerol and assimilation of erythritol. At the maximal value, JMY7991 lipase activity was 1.6-fold higher than the one of JMY7989, which is consistent with the ratios of volumetric and specific production rates.

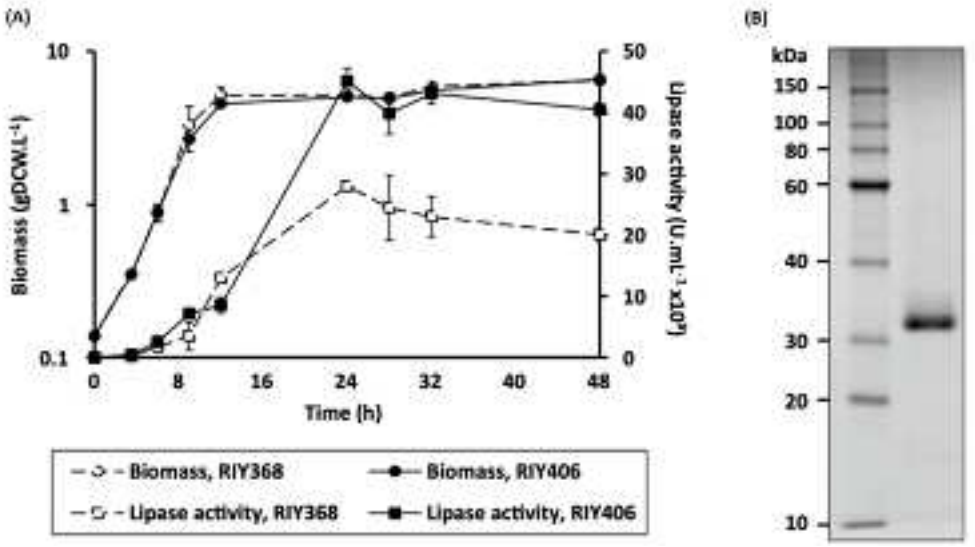


Figure 3.9. Dynamics of culture of strains JMY7989 (mono-copy) and JMY7991 (multi-copy) in DASGIP bioreactors.

Cells were grown for 48 h at 28 °C in YNBG2E medium. (A) Growth curve and lipase activity of strains JMY7989 and JMY7991. Values are means and standard deviations of duplicate experiments. (B) SDS-PAGE gel of 5 µL of supernatant (containing 40 U of lipase CalB, sample taken at 24 h). Protein sizes are indicated on the left-hand side.

Table 3.5. Dynamics of CalB production for DASGIP bioreactor cultures of strains JMY7989 (mono-copy) and JMY7991 (multi-copy).

Cells were grown for 48 h at 28 °C in YNBG2E medium, in DASGIP bioreactors. Displayed means and standard deviations are the result of duplicate experiments.

Parameters	Strains	
	JMY7989	JMY7991
Maximum lipase activity (U mL^{-1})	28,024 ± 743	45,125 ± 2,144
Lipase volumetric production rate (U mL^{-1} h^{-1})	1,357 ± 34	2,179 ± 104

The project of CalB production was a collaborative work with the group of professor Patrick Fickers at the University of Liège - Gembloux Agro - BioTech in Belgium. I have participated in the construction of the plasmids and *Y. lipolytica* strains, and the screening of CalB-expressing strains described in the part 3.3.1. The analysis of gene expression and CalB production during fermentation described in the part 3.3.2. and 3.3.3. were performed by the collaborating group.

3.4. Discussion

Traditionally, promoters and their regulatory elements are studied through deletion or point-mutation and assessment of gene expression, as exemplified by the research in which the regulatory motifs of *XPR2*, *TEF1*, and *POX2* promoters in *Y. lipolytica* were determined [Madzak *et al*. 2000; Blazeck *et al*. 2011; Blazeck *et al*. 2013; Hussain *et al*. 2015]. As the number of available genomes increases and the costs of sequencing decrease, researchers can more frequently employ strategies such as phylogenetic footprinting, which is a powerful tool for identifying CRMs with regulatory functions of interest. In this study, we employed phylogenetic footprinting within the *Yarrowia* clade to explore the *cis*-regulatory modules of the *EYD1* and *EYK1* genes involved in the catabolism of erythritol.

From the mutation of each CRM in *EYD1* promoter, we discovered that both CRM-eyd1 and CRM-eyd2 are important for effective expression and induction, regardless of genetic background. Between the conserved motifs A and B of the *EYD1* promoter, motif A seemed to be more involved in erythritol-based induction. Trassaert and colleagues obtained similar results after introducing a mutation into the conserved motifs A (pEYK300aB) and B (pEYK300Ab) of the inducible *EYK1* promoter [Trassaert *et al*. 2017]. When grown in minimal YNB medium containing 1% erythritol, the strain carrying the pEYK300A*B-YFP cassette with the mutated motif A displayed a decreased level of YFP expression compared to that of the unmutated pEYK300 (683 and 3,536 SFU after 60 h, respectively). In contrast, when motif B was mutated, induction levels were higher under the same conditions (8,389 and 3,536 SFU after 60 h, respectively).

Expression levels have been found to be dependent on UAS copy numbers, which have ranged from 4 tandem copies of UAS1B-xpr2 to as many as 32 copies of UAS1B-xpr2 [Madzak *et al*. 2000; Blazeck *et al*. 2011; Blazeck *et al*. 2012]. However, this relationship was not observed for the *EYK1* and *EYD1* hybrid promoters examined in this study. It was found that an increased number of UAS1-eyk1 copies increased promoter strength when the *EYK1* wild-type strain (JMY1212) was grown on glucose or erythritol (Figure 3.4 and Table 3.2) and that four tandem repeats seemed optimal. Similar results were obtained in the *eyk1*Δ mutant strain (JMY7126), but the optimal expression was reached with 3 tandem repeats. This result reflects that the stronger expression by erythritol induction may lead to a saturation of expression.

For the hybrid promoter in which the core promoter was exchanged (i.e., EYK1-4AB-coreTEF *vs.* EYK1-4AB), expression levels were higher, while induction levels were lower. Indeed, when the coreTEF hybrid promoter was used, expression increased 10- and 2-fold, respectively, in the *EYK1* WT (JMY1212) and *eyk1*Δ mutant (JMY7126) grown on glucose. When erythritol was used as an inducer, hybrid promoter strength increased less than when glucose medium was used (2-fold in the *EYK1* WT (JMY1212), 5-fold in the *eyk1*Δ mutant (JMY7126)). It seems that while the core TEF is able to act in a similar way as the core elements of the erythritol-inducible promoter, the strength of its inducible response is less than that of the native *EYK1* promoter. The hybrid promoter could be further improved by exchanging the core promoters

or by employing a combination of TATA boxes from other inducible promoters [Redden and Alper, 2015; Shabbir Hussain *et al*. 2016].

Promoter strength was improved in the *eyk1*Δ mutant (JMY7126) utilizing erythritol as a free inducer, not as a carbon source. With the presence of erythritol in the medium, a 45.8-fold increase in EYK1-2AB promoter strength was observed in the *eyk1*Δ mutant (JMY7126) while a 5.9-fold increase under same promoter in the EYK1 WT (JMY1212). Under the same condition, the *EYD1* promoter showed a 682.5-fold and a 13.4-fold increase of expression in the *eyk1*Δ mutant (JMY7126) and the *EYK1* WT (JMY1212), respectively. The *EYD1* promoter is a very tight promoter with very low expression levels without an inducer, and a significant increase of expression level (680-fold) with an inducer in the *eyk1*Δ strain.

As a proof of concept, lipase CalB was expressed in mono-copy in strain JMY7126 under the control of three types of erythritol inducible promoters. With hybrid inducible promoters, pEYK1-3AB and pHU8EYK, the expression of CalB was increased by 2.5- and 2.7-fold compared to the constitutive expression with pTEF. However, the similar expression of CalB with pEYK1-3AB and pHU8EYK showed an inconsistent result compared to the expression of the fluorescent reporter protein, which showed a significantly higher fluorescence level with pHU8EYK than pEYK1-3AB [Trassaert *et al*. 2017; unpublished data]. A similar disparity between the promoter strength and heterologous protein production was also observed from a previous study on the expression of xylanase C [Dulermo *et al*. 2017]. It is possible that excessive protein production due to the stronger promoter could lead to the processing burden in protein folding and secretion machinery, resulting in a saturated production level of heterologous protein [Dulermo *et al*. 2017; Ahmad *et al*. 2014]. This underlines the necessity of the separation the growth phase and the production phase to relive the processing burden in heterologous protein production, which can be controlled by the inducible promoter.

In brief, the identification of CRMs, the design of a broad range of hybrid promoters, and application of hybrid promoters for producing the protein of interest were demonstrated in this chapter. These new promoters that respond to erythritol could be very useful in not only fundamental research to understand the architecture of promoter but also the recombinant protein production, as is the case of the *Gal1* promoter in *S. cerevisiae*. Process development shall expand the potentialities of the proposed expression system even further, and the combination may greatly improve the production of recombinant proteins in *Y. lipolytica*.

CHAPTER 4. PRODUCTION OF ODD-CHAIN FATTY ACIDS (OCFAS)

4.1. Improve propionate tolerance

4.1.1. Introduction

Propionate, a key substrate for OCFA production, can be used by *Y. lipolytica* as a sole carbon source in lipid accumulation [Fontanille *et al*. 2012; Kolouchová *et al*. 2015; Gao *et al*. 2017]. However, it is also known that propionate has an inhibitory effect on the cell growth at concentrations above 5 g/L in several previous studies. For this reason, it is necessary to improve microbial resistance to propionate when used as a substrate for lipid accumulation. Very few studies have looked at the tolerance and utilization of propionate by oleaginous yeast for lipid production. Consequently, it is required to identify propionate tolerant genes and have a better understanding of the molecular and regulatory responses of yeast to propionate for utilizing propionate as a substrate for biomass and OCFA production.

Several researches have investigated stress response mechanisms in yeast, and the efforts have been made to engineer strains with enhanced tolerance, mostly to acetic acid. For example, acetic acid tolerance in *S. cerevisiae* was improved when the FA composition of the cell membrane was modified by overexpressing *ELO1* encoding elongase [Zheng *et al*. 2013]. It was also improved by blocking aquaglyceroporin channels [Zhang *et al*. 2010] or by introducing the acetate consumption pathway [Wei *et al*. 2013]. As well as the strategies such as overexpressing or knocking out one or more genes, genomic or global approaches also have been showing the tolerant phenotype [Santos and Stephanopoulos, 2008; Nicolaou *et al*. 2010]. By using genomic library enrichment, butanol-tolerant *Clostridium acetobutylicum* strains were screened [Borden and Papoutsakis, 2007]. In *S. cerevisiae*, key genes involved in acetic acid tolerance were identified by screening deletion or overexpression libraries [Ding *et al*., 2013; Peña *et al*. 2013]. When researchers used transcriptome analysis to study the stress response of *S. cerevisiae* to a variety of weak organic acids (propionate, benzoate, sorbate, and acetate), it was revealed that acetate and propionate had a stronger impact on membrane-associated transport processes [Abbott *et al*. 2007]. However, the mechanisms underlying propionate tolerance have remained elusive, regardless of the species [Guo and Olsson, 2014]. Therefore, it is necessary to carry out further research into the regulatory responses of yeast to propionate to develop more robust strains capable of employing propionate to produce OCFAs.

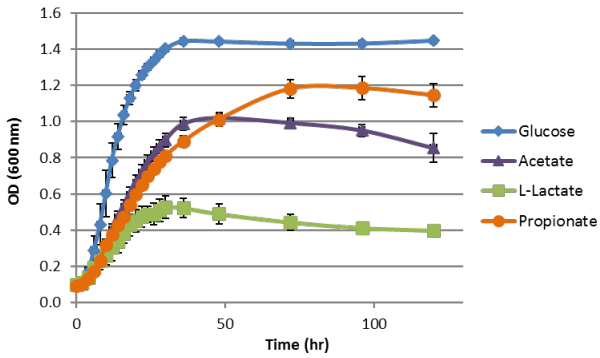
In this part, a genomic library was constructed and used to screen for the propionate-tolerant gene in *Y. lipolytica*. The candidate genes were characterized by deletion and overexpression to verify the tolerance to propionate. Furthermore, the tolerance to other organic acids of the candidate genes was also explored.

4.1.2. Results

4.1.2.1. The growth on propionate as a sole carbon source.

The *Y. lipolytica* strains used in this study were derived from the wild-type *Y. lipolytica* W29 strain (ATCC20460). The auxotrophic derivative, Po1d was previously described by Barth and Gaillardin [Barth and Gaillardin, 1996]. The Po1d prototroph derivative, JMY2900 was used as wild-type reference strain for the comparison with engineered strains derived from Po1d [Dulermo *et al*. 2014]. Growth performance of our reference strain on weak acids with same concentration (5 g/L) was analyzed in microplate (Figure 4.1). *Y. lipolytica* was able to grow on weak acids at similar growth rate, about 0.16 h^{-1}, lower than in glucose (0.25 h^{-1}) (Figure 4.1). As previously shown, *Y. lipolytica* can utilize propionate as a sole carbon source, a substrate that promotes OCFA production. The strain JMY2900 was able to grow on propionate although the growth rate and the final OD were lower than those in glucose (Figure 4.1). This growth inhibition was also shown with other organic acids in the following order: L-lactate > propionate > acetate. In comparison to acetate, growth on propionate showed lower growth rate (0.16 h^{-1}) but higher final OD at same concentration (5 g/L). Although acetate has been regarded as preferable carbon source among VFAs because of its relatively lower growth inhibitory effect in previous studies [Fontanille *et al*. 2012; Gao *et al*. 2017], our results showed that propionate also can be a potential carbon source for biomass and lipid production in *Y. lipolytica* strain.

(A)



(B)

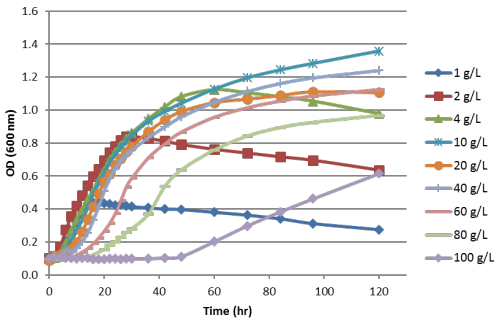
Carbon source	Growth rate (μ_{max}, h^{-1})	maximal OD
Glucose	0.249 ± 0.076	1.447 ± 0.009
Acetate	0.170 ± 0.024	1.020 ± 0.079
L-Lactate	0.177 ± 0.021	0.529 ± 0.028
Propionate	0.161 ± 0.035	1.184 ± 0.066

Figure 4.1. The cell growth of *Y. lipolytica* WT strain in different weak acids and glucose (5 g/L). (A) The growth curves. (B) The growth rate and maximal OD.

In order to explore if *Y. lipolytica* strain could grow on higher concentration of propionate, JMY2900 was cultivated with different concentrations of propionate up to 100 g/L (Figure 4.2). Our strain was able to grow on all concentration of propionate tested in this experiment, and the highest growth rate was observed at 2 g/L of propionate (Figure 4.2). There was no big difference in initial OD trends between 4 g/L and 10 g/L of propionate, but JMY2900 was able to grow at higher cell density at 10 g/L. The inhibitory effect of propionate to the cell growth was observed from 10 g/L. The growth test at 100 g/L of propionate showed longer than 48

hours of lag phase. Our strain appeared to be less sensitive to propionate than *Y. lipolytica* strain used in other studies. For example, *Y. lipolytica* CICC31596 strain showed an inhibitory effect of propionate on growth rate and lag phase at 5 g/L [Gao *et al*. 2017], and *Y. lipolytica* ISA 1834 strain showed higher growth rate, 0.29 h^{-1}, on propionate [Rodriguez *et al*. 2000]. This demonstrates important differences in propionate sensitivity depending on either strain, culture condition, or media composition.

(A)



(B)

Propionate (g/L)	Growth rate (μ_{max}, h^{-1})	maximal OD
1	0.195	0.439
2	0.236	0.838
4	0.195	1.123
10	0.179	1.359
20	0.145	1.111
40	0.161	1.242
60	0.142	1.126
80	0.180	0.968
100	0.029	0.616

Figure 4.2. The cell growth of *Y. lipolytica* WT strain in different concentration of propionate as a sole substrate. (A) the growth curve, (B) the growth rate and maximal OD.

4.1.2.2. Genomic library construction

In order to construct the genomic library, genomic DNA of *Y. lipolytica* W29 was partially digested using the Sau3A restriction enzyme. The fragments up to 5 kb in size were cloned at the BamHI position of the replicative plasmid (pINA240, [Barth and Gaillardin, 1996]) and used to form the library's 10 reference pools (Figure 4.3 (A)). They were then stored in the Microbiology and Molecular Genetics Collection (*Microbiologie et Génétique Moléculaire*, MGM) under the reference codes MGM1221021–MGM1221030 at the INRAE (Thiverval-Grignon, France). The detailed verification of genomic library and optimization of screening condition are described in Appendix E – Park and Nicaud, Yeast, 2019.

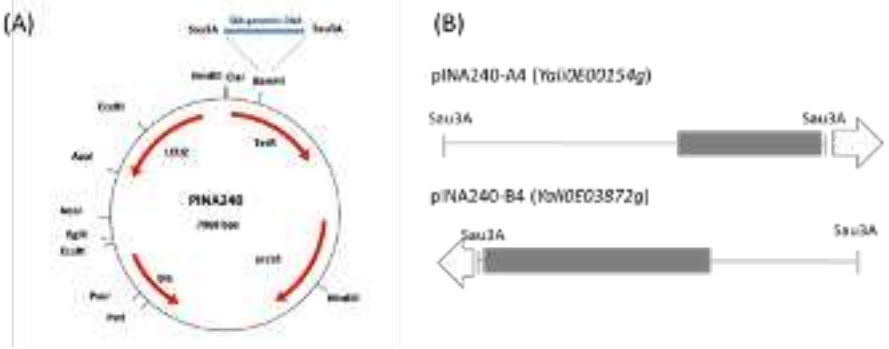


Figure 4.3. The genomic library of *Y. lipolytica*. (A) Schematic map of the replicative plasmid pINA240 used to construct the gDNA library. The partial Sau3A-digested genomic fragments were cloned at the BamH1 dephosphorylated site. (B) Schematic map of the gDNA fragments inserted into JMY7588 (pINA240-A4) and JMY7589 (pINA240-B4), respectively. The gray bars indicate the genes' open reading frames (ORFs) cloned in pINA240 plasmid, and the white arrows show the ORFs of each gene that were not included in pINA240 plasmid.

4.1.2.3. Screening for propionate-tolerant strains

As a control strain to test genomic library, we used *Y. lipolytica* strain JMY7228 (*phd1Δ mfe1Δ tgl4Δ* +pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex) which is unable to use propionate as its sole carbon source because of *PHD1* gene knock-out [Park *et al*. 2018]. By using this propionate-sensitive strain, the propionate-tolerant clones can be found more distinctly. In order to use this strain, glucose should always be supplemented during propionate tolerance test because the propionate-consumption pathway is blocked.

Two reference pools—MGM1221021 and MGM1221030— were transformed to *Y. lipolytica* strain JMY7228 by the Frozen-EZ Yeast Transformation Kit. After transforming the same amount of DNA (308 ng) from each reference pool, around 13,400 transformants from MGM1221021 and 6,700 transformants from MGM1221030 were selected on YNBD medium. Among them, 150 colonies of transformants from each reference pool were transferred to propionate-containing plates (YNBD0.5P4) to screen candidate clones displaying higher propionate tolerance. After 3–5 days of cultivation, 15 clones (7 from MGM1221021, 8 from MGM1221030) displayed better growth on propionate. Among these candidates, two clones JMY7588 and JMY7589 were verified that they contained partial sequences from the genes *YALI0E00154g* and *YALI0E03872g,* respectively (Figure 4.3 (B) and Figure 4.4). In JMY7588, the plasmid contained a 2610 bp genomic fragment that harbored 1584 bp of the promoter region and 1026 bp of *YALI0E00154g* ORF (NCBI XP_503361.1), which codes for a 793 amino acidresidue protein. In JMY7589, the plasmid contained a 2135 bp genomic fragment that harbored 1353 bp of the promoter region and 782 bp of *YALI0E03872g* ORF (NCBI XP_503517.1), which codes for a 448 amino acidresidue protein.

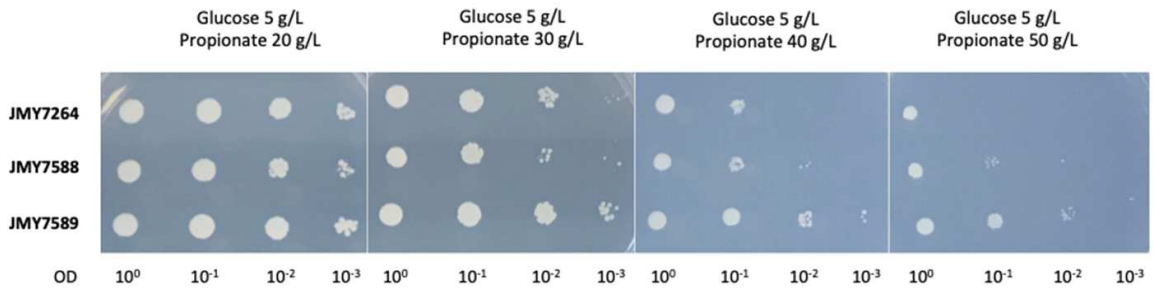


Figure 4.4. Spot assay for the control strain (JMY7264), the RTS1r strain (JMY7588), and the MFS1r strain (JMY7589). Minimal media (YNBD0.5) containing different propionate concentrations were used. Pictures were taken after 3 days of growth at 28 °C.

YALI0E00154g is homologous to *S. cerevisiae RTS1*, which encodes the regulatory subunit of protein phosphatase 2A (PP2A); the latter is involved in cell growth control, cell division control, and the stress response in this yeast [Evangelista *et al*. 1996; Ronne *et al*. 2015]. It has been reported that the deletion of *RTS1* caused sensitivity to temperature, ethanol, sorbate, and osmotic pressure, and increased accumulation of CYC7 RNA, which is involved in

the global stress response in *S. cerevisiae* [Evangelista *et al*. 1996; Shu *et al*. 1997; Mollapour *et al*. 2004]. In *Y. lipolytica*, PP2A was found to act as a regulator of glycogen metabolism [Queiroz-Claret *et al*. 2002]. However, neither PP2A nor its RTS1 subunit have been observed to play a role in the stress response to weak acids (e.g., propionate) in *Y. lipolytica*.

YALI0E03872g is similar to YJR124C in *S. cerevisiae*, but gene function remains unknown in *S. cerevisiae* and *Y. lipolytica*. From the amino acid sequence BLAST, it was found that *YALI0E03872g* was similar (~48%) to a major facilitator superfamily (MFS, pfam07690) transporter found in several fungi (e.g., *Nadsonia fulvescens*, *Metarhizium album*) [Zhang and Madden, 1997]. The MFS transporter facilitates the transport of a variety of substrates, including ions, sugar phosphates, drugs, amino acids, and peptides, across cytoplasmic or internal membranes. In addition, it has recently been shown that the MFS transporter regulates the stress response machinery and controls membrane potential and/or internal pH [Dos Santos *et al*. 2014]. In *Y. lipolytica*, using MultAlin tool [Combet *et al*. 2000], we found three genes coding for a putative MFS transporter: *YALI0E03872g, YALI0C08228g*, and *YALI0A15774g*. They were named *MFS1*, *MFS2*, and *MFS3*, respectively. The three proteins contain the characteristic cd06174 conserved motif of MFS secondary transporters and present nine putative transmembrane domains (Figure 4.5).

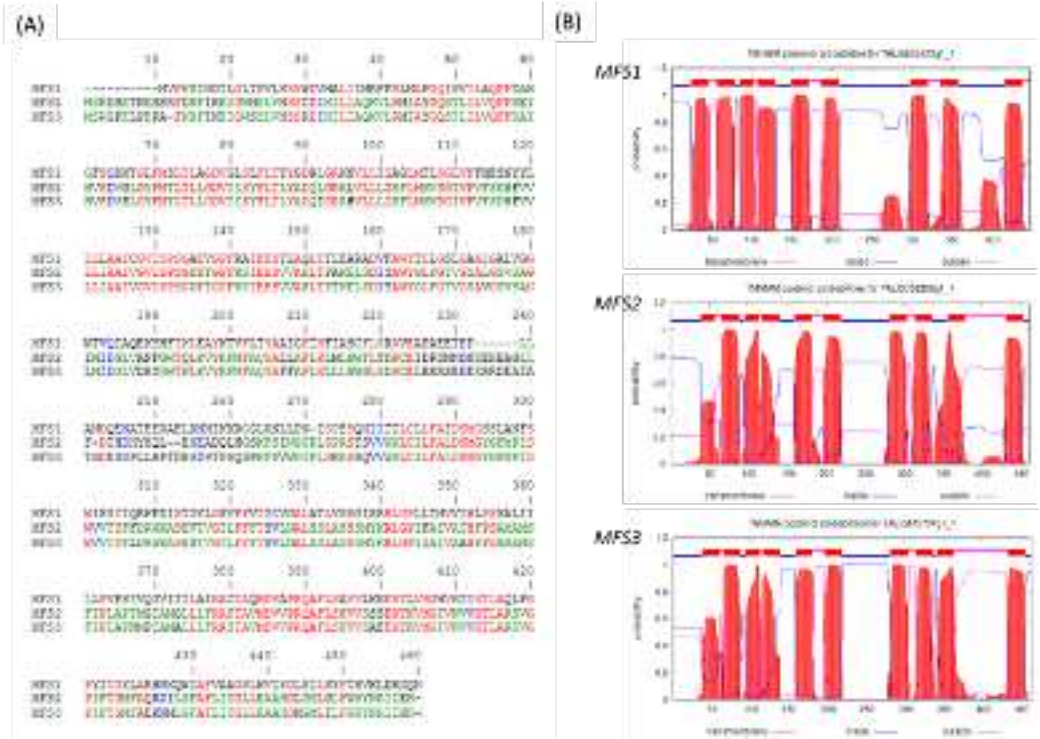


Figure 4.5. Sequence analysis of Mfs1p, Mfs2p, and Mfs3p in *Y. lipolytica*.
(A) Multiple alignments of three proteins, which was performed *via* the following website: https://npsa-prabi.ibcp.fr/cgi-bin/align_multalin.pl. Color codes: red, similarities shared by all genes; green, similarities shared by two of the three genes; blue, amino acids types conserved across the three genes, *MFS1* (*YALI0E03872g*), *MFS2* (*YALI0C08228g*), and *MFS3* (*YALI0A15774g*). (B) Transmembrane predictions of three proteins which were carried out *via* the following website: http://www.cbs.dtu.dk/services/TMHMM/. Color codes: red, transmembrane domains; blue, inner regions; violet, outside domains.

4.1.2.4. Disruption of *RTS1* and *MFS1*

In order to verify the sensitivity to propionate of two identified gene, the disruption of each gene (*RTS1*, *MFS1*, *MFS2*, and *MFS3*) was performed by CRISPR/Cas9 method. The disruption of each gene was verified by sequencing, the part of gene was deleted in all cases; *RTS1* (3 bp, 1 bp from two different clones), *MFS1* (16 bp), *MFS2* (6 bp), and *MFS3* (1 bp). To assess strain phenotype and propionate tolerance, the growth of the deleted strains on propionate media was compared with that of the WT strain by spot assays on agar plate (Figure 4.6). The rts*1*Δ strain showed similar growth on 5 g/L of propionate compared to control strain, but the strain lost the tolerance to propionate at 20 g/L of propionate which the control strain still can grow. In case of *MFS* genes, they showed higher sensitivity to propionate than the *rts1*Δ strain. Two strains (*mfs1*Δ and *mfs2*Δ) did not grow at 5 g/L of propionate, and *mfs3*Δ strain grew a little but much slower than control strain.

It is known that *RTS1* deletion in *S. cerevisiae* showed growth defect and multiple phenotypes (sensitivity to temperature, sensitivity to osmotic stress, increased resistance to different compounds). Thus, the further characterization of *rts1*Δ and *mfs1*Δ such as multiple gene disruption, localization analysis, and additional stressed conditions will explain more and clearer about the propionate response mechanisms and the function of the enzymes in *Y. lipolytica*.

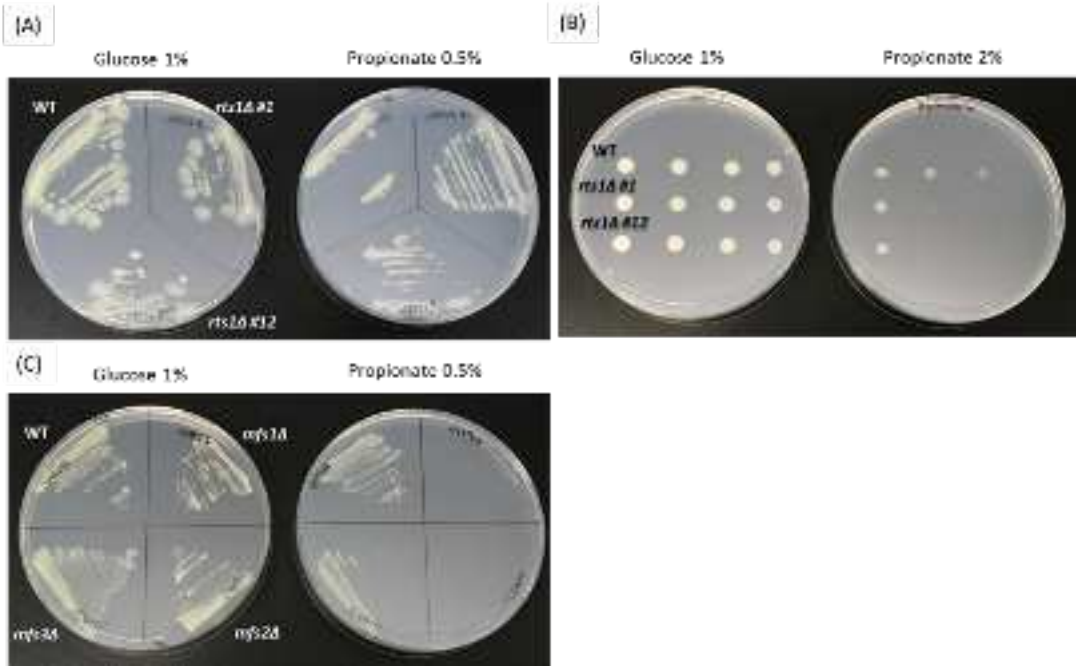


Figure 4.6. Phenotype of *RTS1*- and *MFS*-disrupted strains.

(A) WT strain (JMY195) and *rts1*Δ strains in YNBD1 and YNBP0.5. (B) WT strain (JMY195) and *rts1*Δ strain in YNBD1 and YNBP2. (C) WT strain (JMY195), *mfs1*Δ, *mfs2*Δ, and *mfs3*Δ strains in YNBD1 and YNBP0.5. Pictures were taken after 3 days of growth at 28 °C.

4.1.2.5. Overexpression of *RTS1* and *MFS1*

We simplified the name *RTS1r* to strain JMY7588 and *MFS1r* to strain JMY7589, in reference to the replicative plasmids that expressed the truncated forms of the genes. The strains expressing the full-length genomic genes under pTEF were constructed and named *RTS1i* (JMY7567) and *MFS1i* (JMY7569). To assess strain phenotype and propionate tolerance, the growth in stress condition was performed either in liquid cultivation or by spot test on agar plate (Figure 4.4 and 4.7).

Compared to the control, both *RTS1r* and *MFS1r* displayed greater tolerance to propionate. However, *MFS1r* had higher tolerance than *RTS1r*, and it remained tolerant even at 50 g/L of propionate (Figure 4.7). In the liquid media, the growth curves of the two strains were similar, and their final OD_{600nm} values were slightly higher than that of the control strain in media containing 10 and 20 g/L of propionate (Figure 4.7). When the propionate concentration was 40 g/L, the difference in growth between the control and the two transformants was significantly greater. The final OD_{600nm} values of the two strains were almost twice of that of the control strain in YNBD0.5P4. Both *RTS1r* and *MFS1r* displayed improved growth rates under all the experimental conditions (Table 4.1). The maximal growth rates of *RTS1r* and *MFS1r* at 40 g/L of propionate were 0.065 h^{-1} and 0.054 h^{-1} (increase by 2.2- and 1.8-fold over control), respectively. These results confirmed that the two strains displayed higher propionate tolerance than the control even though they were expressing truncated genes.

The propionate tolerance of *MFS1i* was similar to that of *MFS1r* regarding their growth rates, which means that *MFS1i* had greater propionate tolerance than control (Table 4.1 and Figure 4.7). The propionate tolerance of *RTS1i* showed the different tendency compared to *RTS1r*. *RTS1r* appeared to display greater growth rate than *RTS1i* (0.065 h^{-1} *versus* 0.057 h^{-1}) at 40 g/L of propionate (Figure 4.7 (D) and (I)). This difference between the two strains could be attributable to differences in gene length, promoter type, or plasmid type, as described in another study showing the different expression and secretion of alkaline protease between episomal and integrative expression cassettes [Nicaud *et al*. 1991].

Table 4.1. Growth rates of the different *RTS1*- and *MFS1*-expressing strains in minimum glucose media (YNBD0.5) containing different propionate concentrations (P1 to P4: 10 to 40 g/L). The growth rate was calculated for the exponential phase of each strain under each set of conditions; it is expressed in μ_{max} (h^{-1}).

μ_{max} (h^{-1})		YNBD0.5P1	YNBD0.5P2	YNBD0.5P3	YNBD0.5P4
JMY7264	Control	0.103 ± 0.007	0.079 ± 0.008	0.047 ± 0.014	0.030 ± 0.021
JMY7588	*RTS1r*	0.083 ± 0.011	0.085 ± 0.004	0.063 ± 0.003	0.065 ± 0.002
JMY7567	*RTS1i*	0.098 ± 0.022	0.079 ± 0.005	0.079 ± 0.014	0.057 ± 0.005
JMY7589	*MFS1r*	0.100 ± 0.001	0.077 ± 0.004	0.061 ± 0.002	0.054 ± 0.016
JMY7569	*MFS1i*	0.111 ± 0.012	0.076 ± 0.005	0.069 ± 0.010	0.047 ± 0.004

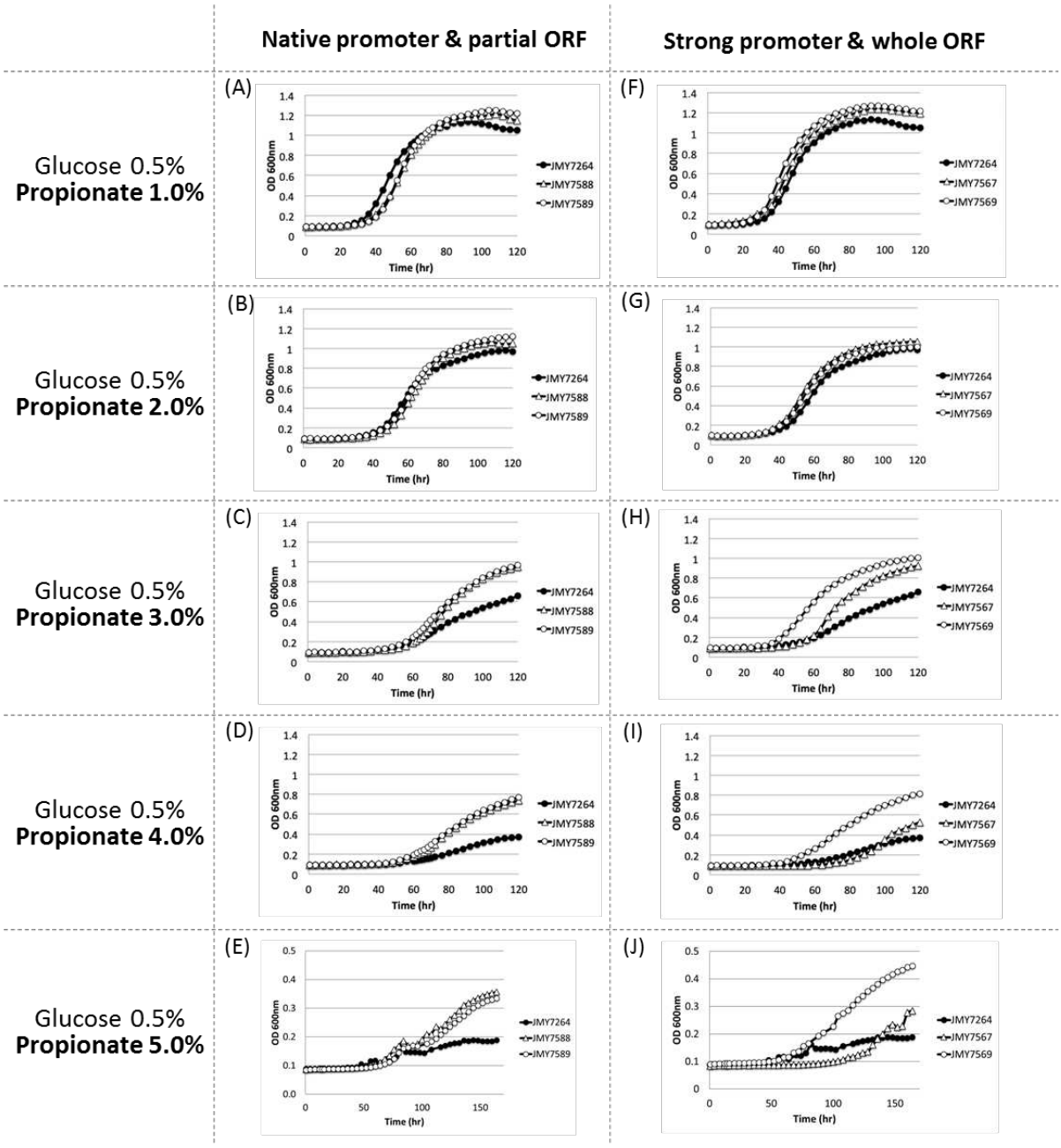


Figure 4.7. Growth test in propionate with four propionate-tolerant strains.
The control strain (JMY7264, ●), the *RTS1r* strain (JMY7588, Δ), and the *MFS1r* strain (JMY7589, ○) from (A) to (E);The control strain (JMY7264, ●), the *RTS1i* strain (JMY7567, Δ), and the *MFS1i* strain (JMY7569, ○) from (F) to (J). The strains were cultivated in YNBD0.5 medium with different concentration of propionate from 1.0% to 5.0%. Average values were obtained from two replicate experiments.

In order to assess the general stress tolerance of *RTS1i* and *MFS1i*, we performed the spot assay with other weak organic acids, namely acetate, lactate, formic acid, succinic acid, and malic acid (Figure 4.8). We observed the same differences in tolerance between *RTS1i* and *MFS1i* that we had already seen for propionate. JMY7567 (*RTS1i*) was more tolerant to lactate,

formic acid, and succinic acid than control strain. In contrast, JMY7569 (*MFS1i*) was less tolerant to formic acid, succinic acid, and malic acid than was control strain. In the case of acetate, there were no differences in growth among strains, even at high concentrations (60 g/L). These findings suggest that *MFS1* is involved in a propionate-specific stress response in *Y. lipolytica*. In comparison, the overexpression of full-length *RTS1* increased tolerance not only to propionate but also to other weak acids. It has been found that the deletion or overexpression of *RTS1* resulted in different levels of tolerance, depending on parental strain and stressor type [Shu and Hallberg, 1995; Evangelinsta *et al*. 1996; Shu *et al*. 1997]. Taken together, our results and previous studies suggest that PP2A probably has a functional role in more than one pathway.

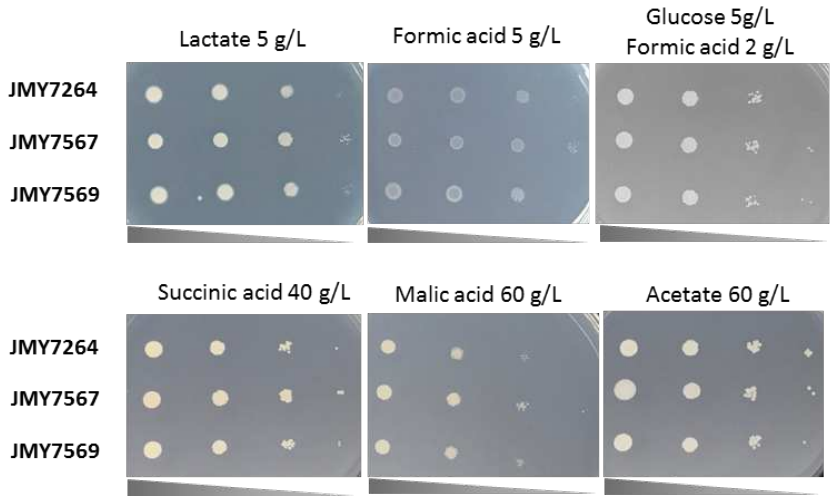


Figure 4.8. Spot assay for the control strain (JMY7264), the *RTS1i* strain (JMY7567), and the *MFS1i* strain (JMY7569) on different weak acids (5 g/L). Pictures were taken after 3 days of growth at 28 °C.

4.1.2.6. Overexpression of *RTS1* and *MFS1* on OCFA production

Propionate is an important substrate for lipid synthesis in *Y. lipolytica*, especially when it comes to the production of OCFAs. To determine whether increased propionate tolerance could improve total lipid accumulation as well as the production of OCFAs, lipid synthesis by the *RTS1*- and *MFS1*-expressing strains was evaluated. After 120 h of cultivation in a minimal glucose medium containing a high concentration of propionate (YNBD0.5P4), the experimental strains had produced less biomass than the control strain (by 6.4–11.5%). Total lipid content was lower in the *RTS1*-expressing strains (by 4.5% and 23.1% for *RTS1r* and *RTS1i*, respectively), while it was higher in the *MFS1*-expressing strains (by 7.1% and 10.4% for *MFS1r* and *MFS1i*, respectively). Despite these low biomass and similar lipid content, the ratio of OCFAs to total lipids was higher for all the experimental strains (Table 4.2).

Table 4.2. Biomass and lipid accumulation in the different *RTS1*- and *MFS1*-expressing strains compared to the control strain. Strains were grown for 120 h in minimal glucose medium (YNBD0.5) supplemented with 40 g/L of propionate. DCW = dry cell weight.

Strain		DCW (g/L)	% of change	Total lipid content % (g/g)	% of change	OCFAs/ Total lipids (%)	% of change
JMY7264	Control	3.29 ± 0.09		8.92 ± 0.46		64.90 ± 1.04	
JMY7588	*RTS1r*	3.08 ± 0.02	-6.4%	8.88 ± 0.73	-4.5%	66.44 ± 1.44	+2.4%
JMY7567	*RTS1i*	2.99 ± 0.07	-9.1%	6.86 ± 0.89	-23.1%	73.12 ± 0.51	+12.7%
JMY7589	*MFS1r*	3.01 ± 0.17	-8.5%	9.55 ± 0.05	+7.1%	67.21 ± 1.40	+3.6%
JMY7569	*MFS1i*	2.91 ± 0.19	-11.5%	9.85 ± 0.35	+10.4%	67.03 ± 1.80	+3.3%

4.1.3. Discussion

The objective of this part was to identify the gene potentially involved in propionate tolerance in *Y. lipolytica*. To this end, we screened a plasmid-based genomic library harboring native promoters and genes under propionate stress condition and identified two genes of potential interest: *RTS1 (YALI0E00154g)* and *MFS1 (YALI0E03872g)*. We discovered that the initial transformants were expressing truncated genes and compared the phenotypes of the strains expressing the partial and full-length genes.

Two strains expressing *RTS1*, which encodes a regulatory subunit of the protein phosphatase 2A (PP2A), showed different growth patterns on propionate depending on gene length and promoter type. Growth was stronger and lipid accumulation was greater in the strain expressing the truncated gene under a native promoter (*RTS1r*) than those in the strain expressing the whole gene under a strong promoter (*RTS1i*). Both strains (*RTS1r* and *RTS1i*) had higher levels of OCFA production than that of the control strain. Further research should focus on whether these phenotype differences stem from differences in expression levels or differences in sequence conservation between the partial and full-length genes. Interestingly, the overexpression of *RTS1* seems to enhance tolerance to other weak acids, such as lactate, formic acid, and succinic acid. Given that *RTS1* encodes a single subunit of PP2A which has another regulatory subunit (encoded by *CDC55*) as well as a catalytic subunit (encoded by four genes), the relationship among these subunits and its contribution to the tolerance to weak acids need to be further explored to better understand the mechanisms at hand.

On propionate media, the *MFS1*-expressing strains (*MFS1r* and *MFS1i*) showed greater propionate tolerance, shorter lag phases, and higher growth rates than the control strain. They also accumulated more lipids and more OCFAs. Based on the sequence alignment results,

we identified two more genes coding for putative MFS transporters (named *MFS2* and *MFS3*) in *Y. lipolytica*. Further research into how these genes function to mediate stress responses would be helpful in clarifying the mechanisms underlying propionate tolerance in *Y. lipolytica*.

The role of these genes in the stress response to propionate and other weak acids remains unclear. Therefore, we need more studies such as expression analysis at the transcriptional level or metabolic flux analysis under stress conditions induced by propionate to gain insight on the regulatory mechanisms. These are also crucial steps to engineer strains with improved tolerance for use in industry. Library enrichment and evolutionary engineering are promising strategies that employ pre-existing libraries or strains [Borden and Papoutsakis, 2007; Wright *et al*. 2011]. In addition, combining computational and experimental approaches may also help to improve tolerance by predicting the effects of expressing or disrupting multiple genes at the same time [Goodarzi *et al*. 2010].

4.2. Increase the production of OCFAs

4.2.1. Introduction

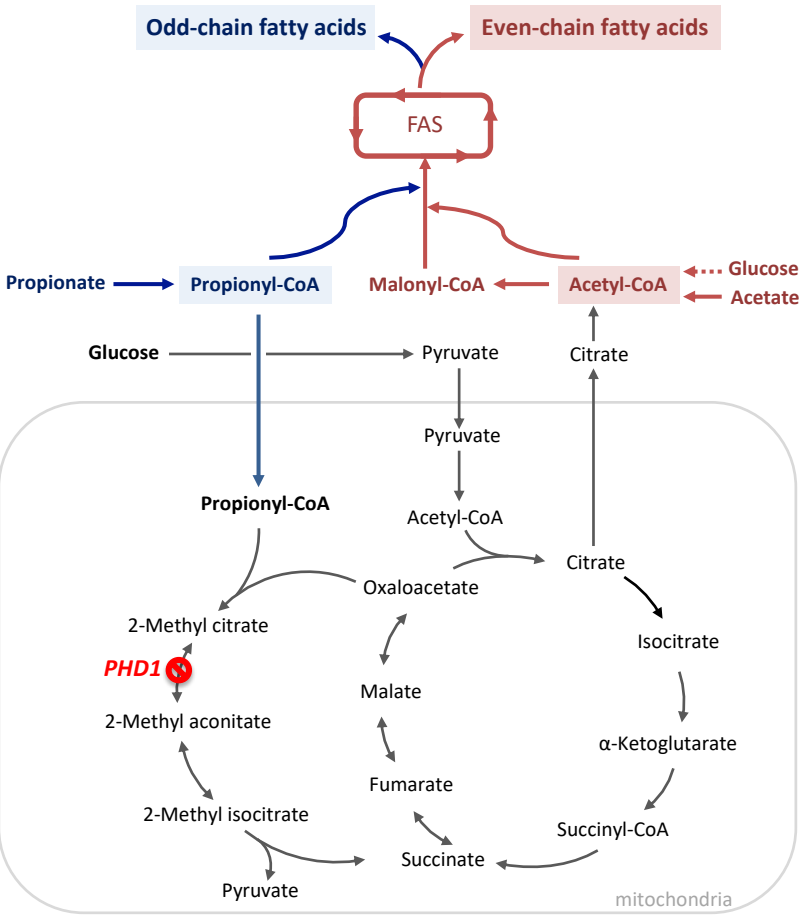


Figure 4.9. The pathways involved in odd- and even-chain fatty acid production including the link with the TCA and methyl citrate cycles in *Y. lipolytica*.

Generally, *de novo* fatty acid synthesis in microorganisms begins with the condensation of acetyl-CoA and malonyl-CoA, which are derived from substrates with its own metabolic pathway (Figure 4.9, The detailed information of lipid synthesis is described in the part 1.2.4.1.1. and Figure 1.3). OCFA synthesis is also going through the same pathway excepting for propionyl-CoA as a key primer of lipid synthesis. The simplest way to provide propionyl-CoA is propionate supplementation. Propionate is activated by the cytosolic acyl-CoA synthase (*ACS1*) to form the propionyl-CoA which is transported into mitochondria to enter the methyl citrate pathway. Propionyl-CoA is condensed with oxaloacetate to form 2-methyl citrate by 2-methyl citrate synthase (probably encoded by *CIT2, YALI0E02684g*). 2-Methyl citrate dehydratase removes an H_2O to form 2-methyl aconitate by 2-methyl citrate dehydratase (encoded by *PHD1*, *YALI0F02497*), then is hydrated to form 2-methyl isocitrate probably by the aconitase (encoded by *ACO1*, *YALI0D09361g*; *ACO2*, *YALI0E14949g*), which is

cleaved by 2-methyl isocitrate lyase (encoded by *ICL2*, *YALI0F31999g*) to give succinate and pyruvate.

In this part, the production of OCFAs from propionate either as a sole carbon and energy source or in combination with glucose was investigated in *Y. lipolytica*. In order to increase propionyl-CoA pool for the synthesis of OCFAs, the inhibition of 2-methrylcitrate cycle by deleting *PHD1* encoding 2-methyl citrate dehydratase was investigated. Further engineering of strain to accumulate more lipids in general by enhancing the synthesis capacity and blocking the degradation of lipids was also performed. Additionally, a fed-batch co-feeding strategy with glucose and propionate was tested to further increase OCFA production.

4.2.2. Results and discussion

4.2.2.1. Accumulation of OCFAs in propionate medium

In order to see whether propionate is a suitable carbon source for OCFA production, flask cultures of wild-type JMY2900 with several compositions of carbon sources (YNBD1, glucose 1%; YNBD1P1, glucose 1% and propionate 1%; YNBP1, propionate 1%; YNBP2, propionate 2%) were carried out. It was revealed that lipid content obtained on YNBP1 (7.48% of DCW) was comparable to that on YNBD1 (7.86% of DCW) with significant difference of lipids composition (Table 4.3, Figure 4.10). In YNBD1 media, oleic acid (C18:1) and linoleic acid (C18:2) were the major FAs with a percentage of 50.72% and 20.26%, respectively. Only 1.75% of OCFAs in total fatty acids were produced in this condition. However, in case of medium without glucose like YNBP1 and YNBP2, the ratio of OCFAs to total lipids increased to around 35%. In these conditions, the percentage of oleic acid and linoleic acid in total lipids decreased in contrast to the increase of heptadecenoic acid (C17:1). In addition to heptadecenoic acid (C17:1), other OCFAs such as pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and nonadecanoic acid (C19:0) were also produced from all propionate-containing medium (Figure 4.10 (A)). These results indicate that propionate can be used as a primer for the synthesis of OCFAs in *Y. lipolytica* as reported in other studies [Kolouchova *et al*. 2015; Zheng *et al*. 2012]. Although the total lipid contents from YNBD1 and YNBP1 are similar, the biomass produced was significantly different (5.37 g/L and 2.60 g/L, respectively, Table 4.3). The difference in biomass production in YNBP1 and YNBP2 was already shown in Figure 4.2, and it might be due to the inhibitory effect of propionate. In spite of lower ratio of OCFAs to total fatty acids in YNBD1P1 than that of in YNBP1, 0.12 g/L of OCFAs was produced which showed the highest amount in this culture condition (Table 4.3). JMY2900 accumulated slightly higher OCFA and total lipids in YNBP2 than YNBP1, but it did not show significantly better performance for OCFA production. In addition, higher concentration of propionate showed inhibitory effect from the beginning of culture (data not shown). From these results, YNBD1P1 is the best condition for the OCFA and total lipids production, and YNBP1 is also a suitable condition for the high ratio of OCFAs to total fatty acids (Figure 4.10 (B)).

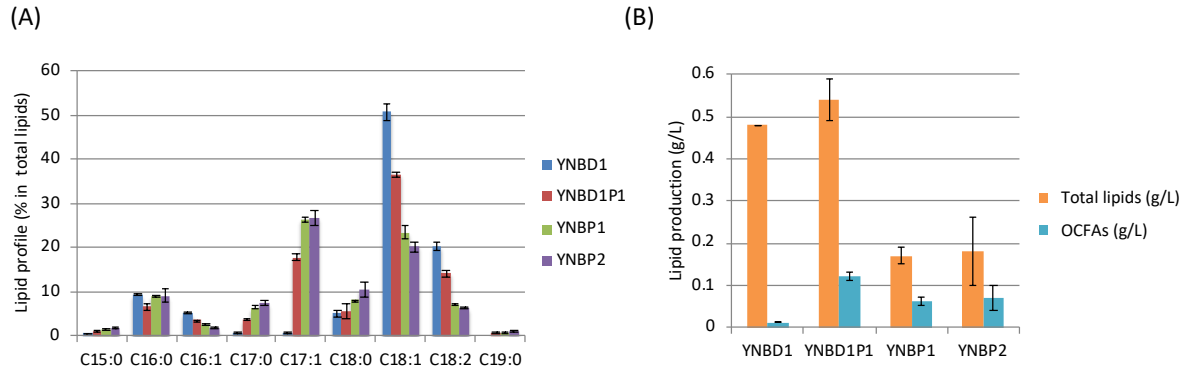


Figure 4.10. Lipid profiles and lipid production of WT strain in different media. JMY2900 was grown in glucose (YNBD1), glucose and propionate (YNBD1P1), and in propionate media (YNBP1 and YNBP2) for 72 h at 28 °C, 180 rpm. The results presented are the mean values and standard deviations for three independent biological replicates. (A) Lipid profiles depending on carbon sources. (B) Total lipid (orange) and total OCFA (cyan) production.

Table 4.3. Biomass and lipid production by WT strain JMY2900 in minimal medium. Strain was grown for 72 h at 28 °C and 180 rpm. The mean value of three independent experiments is shown and the standard deviation is indicated. DCW = dry cell weight.

	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs / Total FAs (%)	Lipids (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
YNBD1	5.37 ± 0.13	7.86 ± 0.13	0.14 ± 0.00	1.75 ± 0.02	0.48 ± 0.00	0.01 ± 0.00
YNBD1P1	6.80 ± 0.08	7.39 ± 0.15	1.70 ± 0.06	22.93 ± 0.68	0.54 ± 0.05	0.12 ± 0.01
YNBP1	2.60 ± 0.37	7.48 ± 0.69	2.61 ± 0.21	34.96 ± 0.45	0.17 ± 0.02	0.06 ± 0.01
YNBP2	2.71 ± 0.58	8.14 ± 1.51	3.01 ± 0.72	36.52 ± 2.18	0.18 ± 0.08	0.07 ± 0.03

4.2.2.2. Inactivation of the propionate catabolic pathway improved OCFAs content and production.

It was previously reported that the methyl citrate cycle is important for glycerol metabolism in *Y. lipolytica* [Papanikolaou *et al*. 2013]. Phd1p, a mitochondrial protein, catalyzes the conversion of 2-methyl citrate to 2-methyl-*cis*-aconitate in the methyl citrate cycle. It has been shown that, in *Y. lipolytica*, the deletion of *PHD1* resulted in the accumulation of 2-methyl citrate, which is the inhibitor of aconitase and therefore could potentially halt the TCA cycle [Papanikolaou *et al*. 2013]. Additionally, deletion of the *PHD1* gene was shown to improved lipid accumulation in *Y. lipolytica*. The methyl citrate cycle can be regarded as competitive pathway for the synthesis of OCFAs because the key precursor of OCFA, propionyl-CoA, can be catabolized through the methyl citrate cycle. We hypothesize here that inhibition of 2-methyl citrate pathway by deleting *PHD1* increases the propionyl-CoA pool that could be used for further synthesis of OCFAs. To prove this, the cultivation of JMY3350 (WT

*phd1*Δ) was performed in YNBD1 and YNBP1. As expected, JMY3350 was not able to grow in YNBP1 since propionate cannot be used as a sole carbon source. This confirmed that propionate cannot be metabolized through methyl citrate cycle to form pyruvate in JMY3350. In glucose media (YNBD1), we observed an increase of the ratio of OCFAs to total fatty acid even without propionate by 1.35 times (Table 4.4). Inactivation of Phd1 blocks the TCA cycle, which might cause growth defects and increase sensitivity of propionate to the cell. Therefore, we added lower amount of propionate (4 g/L) with glucose (10 g/L) after 16 hours of cultivation in YNBD1. In order to compare the capability of the two strains for OCFA production, JMY2900 (WT) and JMY3350 (WT *phd1*Δ), we used an equivalent amount of metabolizable carbon with a C/N ratio 30. Therefore, the glucose amount was adjusted in JMY3350 strain to compensate the lack of use of propionate as carbon source for biomass formation. The lipid content of JMY3350 increased 1.17-fold compared to the wild-type (8.01% and 6.85%). The ratio of OCFAs to total lipids was also higher (46.82%) than the wild-type (28.32%) (Table 4.5). JMY3350 produced 0.17 g/L of OCFAs, increased by 21.4% than control strain despite of its lower biomass. In addition, the deletion of *PHD1* modified the composition of lipids (Figure 4.11 (A)). The percentage of heptadecanoic acid in total lipids increased by 1.4 times. Heptadecenoic acid (C17:1) showed the highest portion (35.56%) of total lipids in WT *phd1*Δ (JMY3350), while JMY2900 produced mostly oleic acid (44.75%), likewise most of other *Y. lipolytica* strains.

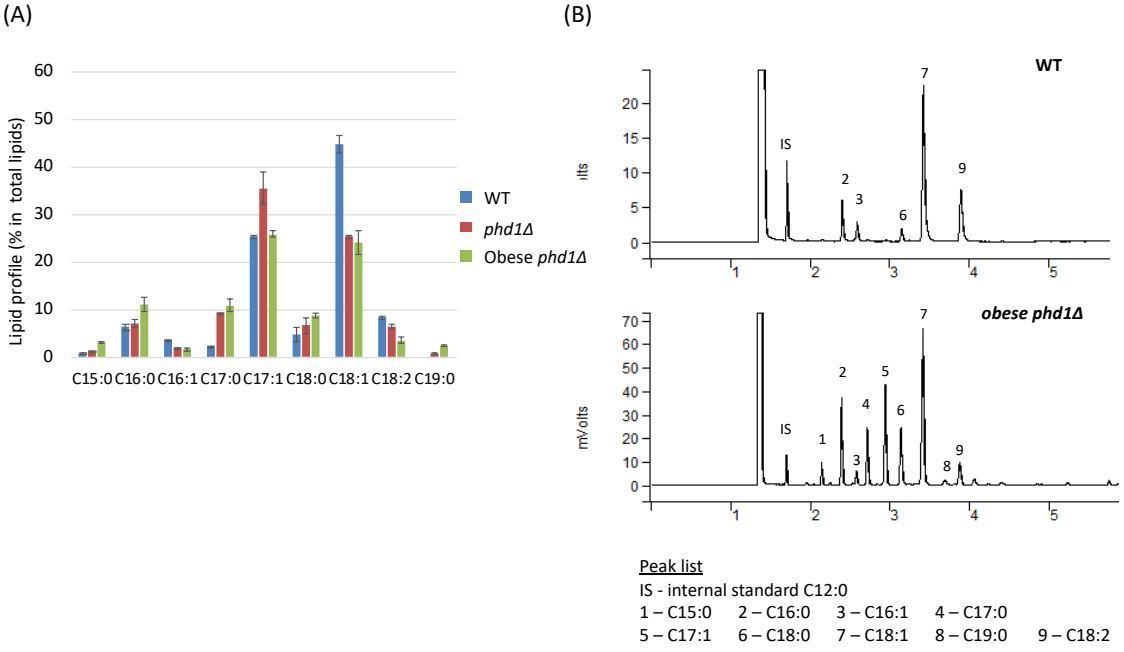


Figure 4.11. Comparison of lipid profiles of JMY2900 (WT), JMY3350 (WT *phd1Δ*), and JMY3776 (obese *phd1Δ*) in glucose and propionate media. Cells were grown for 72 h at 28 °C, 180 rpm. (A) Lipid profiles. (B) GC chromatogram of JMY2900 and JMY3776. The results presented are the mean values for three independent biological replicates.

Table 4.4. Comparison of lipid profiles of JMY2900 (WT) and JMY3350 (WT *phd1*Δ) in glucose media (YNBD1). Cells were grown for 72 h at 28 °C, 180 rpm. The results presented are the mean values for three independent biological replicates. DCW = dry cell weight.

YNBD1	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipids (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
JMY2900 (WT)	5.37 ± 0.13	7.86 ± 0.13	0.14 ± 0.01	1.75 ± 0.02	0.48 ± 0.01	0.008 ± 0.001
JMY3350 (WT *phd1Δ*)	5.35 ± 0.15	7.43 ± 0.24	0.18 ± 0.01	2.36 ± 0.15	0.47 ± 0.01	0.011 ± 0.001

Table 4.5. Biomass and lipid production by WT, WT *phd1*Δ, and obese *phd1*Δ strains in minimal glucose and propionate media. Strain JMY2900 (WT), JMY3350 (WT *phd1Δ*) and JMY3776 (obese *phd1Δ)* were grown for 72 h at 28 °C and 180 rpm. The mean value of three independent experiments is shown and the standard deviation is indicated. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipids (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
JMY2900 (WT)	7.18 ± 0.20	6.85 ± 0.21	1.94 ± 0.03	28.32 ± 0.01	0.49 ± 0.01	0.14 ± 0.01
JMY3350 (WT *phd1Δ*)	4.50 ± 0.50	8.01 ± 0.64	3.75 ± 0.04	46.82 ± 0.03	0.36 ± 0.01	0.17 ± 0.02
JMY3776 (obese *phd1Δ*)	5.53 ± 0.32	24.76 ± 2.51	10.37 ± 0.49	41.90 ± 0.02	1.36 ± 0.06	0.57 ± 0.01

4.2.2.3. Engineering strain for higher accumulation of lipids

Once we verified that *PHD1*-deleted strain was able to produce more OCFAs, the engineering of the strain to accumulate higher amount of OCFA and total lipids was carried out. The strain named obese *phd1*Δ (JMY3776) was constructed by multiple modifications (detailed history of strain construction is described in Appendix E - Park *et al*. Biotechnol for Biofuels, 2018). First, in order to block β-oxidation, *MFE1* encoding the multifunctional enzyme, involved in the second step of β-oxidation, was deleted [Back *et al*. 2016]. To inhibit triacylglycerols (TAG) remobilization, *TGL4* encoding a triglyceride lipase was deleted [Gajdos *et al*. 2016]. In addition, to push and pull TAG biosynthesis, *DGA2* encoding the major acyl-CoA: diacylglycerol acyltransferase [Beopoulos *et al*. 2012; Tai and Stephanopoulos, 2013; Liu *et al*. 2015], and *GPD1* encoding glycerol-3-phosphate dehydrogenase were overexpressed [Back *et al*. 2016]. We then studied lipid production of engineered strain (obese *phd1*Δ, JMY3776)

in the same conditions as before. As expected, OCFA accumulation increased as well as total lipid accumulation, by 3.35 and 3.78 times, respectively (Table 4.5). The ratio of OCFAs to total lipids was slightly decreased in JMY3776, however remained above 40%. The amount of all saturated fatty acids from C15:0 to C19:0 was increased contrary to the decreased unsaturated fatty acids (Figure 4.11). This phenomenon was also shown in our previous study [Dulermo and Nicaud, 2011], the strains optimized for lipid accumulation (called obese strain) produced more C16:0 than wild-type and less unsaturated C16 and C18 commonly in different carbon sources (glucose, fructose, and sucrose).

4.2.2.4. Increase of accumulation of OCFAs by fed-batch co-feeding carbon sources

As described above, propionate is a key carbon source for production of OCFAs meanwhile shows growth inhibitory effect in *Y. lipolytica*. Besides, the engineered strain is more sensitive to propionate allowing only small amount of propionate being used for OCFA synthesis. Several fed-batch fermentation strategies have been used to improve yield and productivity by avoiding high level of inhibitory compounds in culture medium [Dulermo *et al*. 2013; Beopoulos *et al*. 2012]. In order to see whether fed-batch strategy could boost the production of OCFAs while minimizing the inhibitory effect of propionate, we investigated fed-batch co-feeding of carbon sources during cultivation.

The obese *phd1*Δ strain was cultured in YNBD1 with addition of carbon sources (glucose 4 g/L and propionate 0.5 g/L) at four time points (Figure 4.12 (A)). As a result, the production of total lipid and OCFAs content were increased by 50.35% and 12.64%, respectively (Table 4.6). However, the percentage of OCFAs in total lipids was diminished by 60% likely due to the co-feeding with glucose. Nevertheless, the amount of total OCFAs from fed-batch co-feeding reached 0.75 g/L, 1.31-fold higher than in batch (Table 4.7). This represents a 535% increase of OCFA production between wild-type JMY2900 and the obese p*hd1Δ* strain (Figure 4.12 (B), (C)). Fed-batch fermentation has been beneficial for the production of other compounds by *Y. lipolytica*, such as lipids described for the obese strain JMY3501 on synthetic media [Lazar *et al*. 2010] or carotenoids with the concomitant production of lipids on rich media [Palmqvist and Hahn-Hagerdal, 2000]. The fermentation conditions can be further optimized by testing various feeding rate of glucose and propionate, which will allow to improve final biomass, higher lipid content, and OCFAs content.

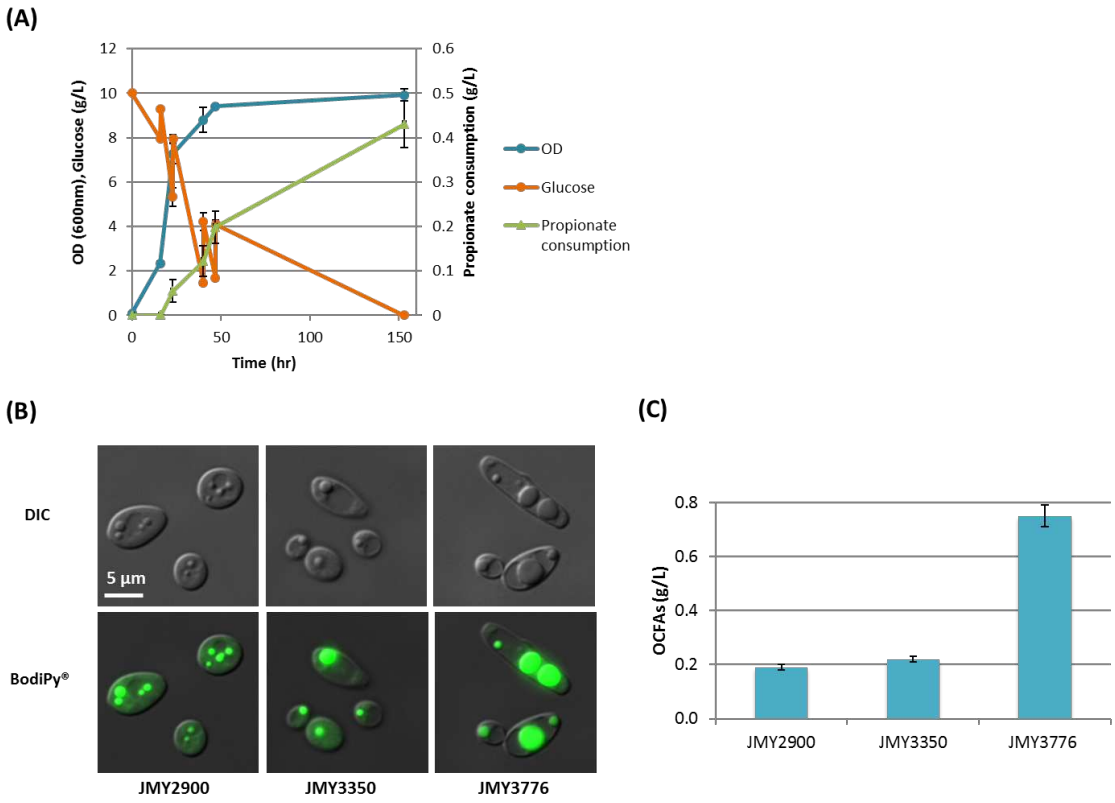


Figure 4.12. Improvement of OCFA production of JMY3776 by fed-batch co-feeding of propionate and glucose.

(A) Increase of the accumulation of OCFAs by co-feeding of propionate and glucose. Cells were grown for 16 hr in glucose media (YNBD1), then pulses of glucose + propionate (4 g/L and 0.5 g/L, respectively) were added at t= 16, 23, 40, 47 hr. The results presented are the mean values for three independent biological replicates. (B) Microscope image of cells (DIC) and lipid body visualization with Bodipy of JMY2900 (WT), JMY3350 (WT *phd1Δ*) and JMY3776 (obese p*hd1Δ*). (C) Increase of OCFAs accumulation by pathway engineering and fed-batch strategy.

Table 4.6. Lipid production by co-feeding of glucose and propionate. The mean value of three independent experiments is shown and the standard deviation is indicated. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs/ Total FAs (%)	Lipids (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
JMY2900 (WT)	8.20 ± 0.05	17.23 ± 0.31	2.30 ± 0.02	13.36 ± 0.01	1.41 ± 0.02	0.19 ± 0.01
JMY3350 (WT *phd1Δ*)	4.83 ± 0.13	17.71 ± 0.70	4.58 ± 0.05	25.90 ± 0.01	0.85 ± 0.01	0.22 ± 0.01
JMY3776 (obese *phd1Δ*)	5.93 ± 0.13	50.35 ± 1.99	12.64 ± 0.45	25.11 ± 0.17	2.99 ± 0.18	0.75 ± 0.04

CHAPTER 5. ENGINEERING PRECURSOR POOLS FOR INCREASING LIPID PRODUCTION

5.1. Introduction

Synthesis of adequate precursor levels for the production of target compounds of interest has been considered one of the crucial strategies to improve the productivity of cell factories in metabolic engineering. The importance of the precursor pool sizes was underlined in several studies for the production of desired compounds [Hong *et al*. 2017]. For example, acetyl-CoA is a precursor to a variety of biotechnology products, including fatty acids, 1-butanol, polyhydroxyalkanoates, polyketides, isoprenoids, *etc*. [Krivoruchko *et al*. 2015; Nielsen, 2014]. A number of studies have focused on increasing acetyl-CoA pools, by engineering the pyruvate dehydrogenase complex (PDH) bypass route for producing amorphadiene in *S. cerevisiae*, or by introducing a cytosolic form of PDH or heterologous PDH for producing 1-butanol, and so on [Shiba *et al*. 2007; Lian *et al*. 2014]. Oleaginous yeast like *Y. lipolytica* has a natural capability of high flux through acetyl-CoA compared to non-oleaginous yeast. Therefore, the studies aiming to boost acetyl-CoA pools have been done later than in *S. cerevisiae*. It has been reported that the engineering of acetyl-CoA pools improved the production of triacetic acid lactone (TAL), squalene, fatty-alcohol, and so on [Markham *et al*. 2018; Huang *et al*. 2018; Wang *et al*. 2016]. In this chapter, the boosting of precursor pools (acetyl-CoA, malonyl-CoA, propionyl-CoA, and β-ketovaleryl-CoA) was investigated individually or simultaneously to see if the production of lipids in general or OCFAs specifically can be improved.

5.2. Increasing malonyl-CoA pool

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step towards lipid biosynthesis, converting cytosolic acetyl-CoA into malonyl-CoA, which is the primary precursor for fatty acid elongation. From extensive studies on ACC, now it is regarded that the overexpression of *ACC1* is an effective approach for the production of fatty acid and fatty acid-derivatives in several bacteria and yeasts [Shi *et al*. 2014]. In *Y. lipolytica*, the overexpression of native *ACC1* under hp4d promoter also showed the increase of lipid accumulation, 2-fold higher lipid content over control (from 8.77% to 17.90% of DCW) [Tai and Stephanopoulous, 2013]. In this part, we tried to increase the activity of ACCase, thus to improve lipid production, through overexpression of *ACC1* whose posttranslational regulation is abolished by site-directed mutagenesis.

5.2.1. Amino acid sequence blast

In *S. cerevisiae*, control of Acc1 activity occurs mainly at two levels, i.e., regulation of transcription and repression at the protein level by Snf1 protein kinase. The posttranslational

regulation of Acc1 was abolished by site-directed mutagenesis in Ser659 and Ser1157, resulting in increased activity of ACC in *S. cerevisiae* [Shi et al. 2014]. In order to determine whether the regulating sites of Acc1 that were conserved between *S. cerevisiae* and *Y. lipolytica*, amino acid sequence alignment was carried out. *S. cerevisiae* has two *ACC1* in different localization (*ACC1* in cytosol, *HFA1* in mitochondria), but *Y. lipolytica* has one *ACC1* (*YALI0C11407g*) in genome database with two ATG codons (Table 5.1). *YlACC1* has 65.2% of sequence identities to *ScACC1*, and two mutation sites (Ser659 and Ser1157) were conserved in *YlACC1* (position 1: Ser653 and position 2: Ser1163).

Table 5.1. Sequence blast of *ACC1* between *S. cerevisiae* and *Y. lipolytica*.

	S. cerevisiae	*Y. lipolytica*	Sequence identity
Cytoplasmic	ACC1 (YNR016C)	ACC1 (*YALI0C11407g*)	65.17 %
Mitochondrial	HFA1 (YMR207C)		50.39%

5.2.2. Cloning of *YlACC1* with two mutation

Cloning of *YlACC1* was started from JME2408 plasmid harboring cytosolic form of *YlACC1*. As described, *YlACC1* (*YALI0C11407g*) has two ATG codons and we constructed JME2408 plasmid from second ATG without intron and named as *YlACC1*cyto. Cytosolic *YlACC1* was cloned to TOPO plasmid, and then mutation of each position was performed with overlap PCR. Each mutated *YlACC1* was cloned to expression plasmid (JMP62-*URA3*ex-pTEF) respectively, and then double mutated plasmid was cloned by enzyme digestion (SphI) and ligation (Figure 5.1). The sequences of *YlACC1*, *YlACC1* 1*, *YlACC1* 2*, and *YlACC1* 1*2* were verified by sequencing.

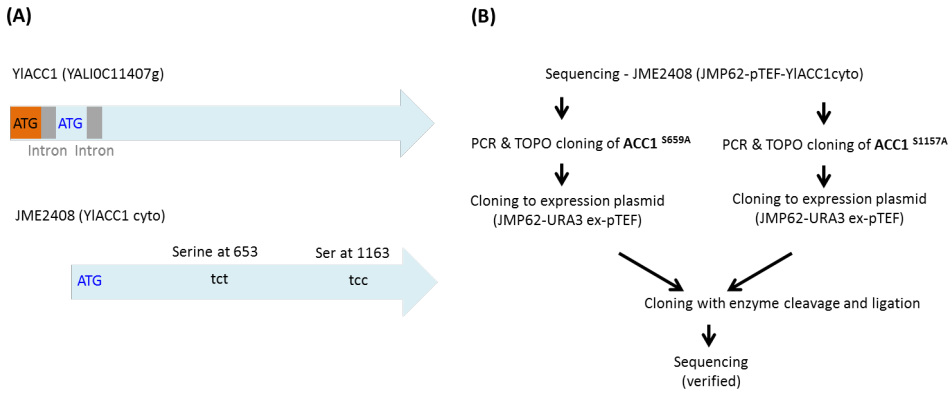


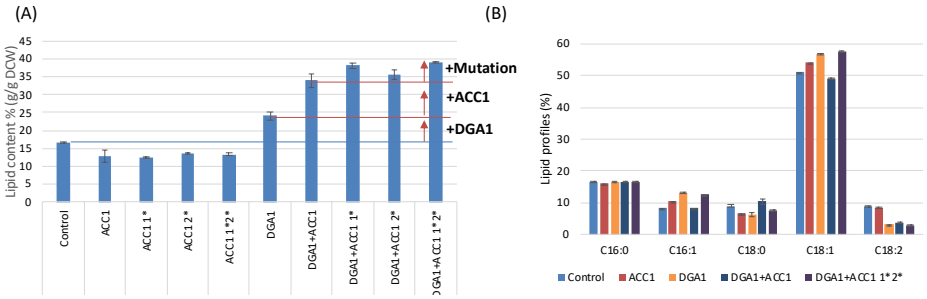
Figure 5.1. Cloning scheme of *YlACC1* 1*2*.

(A) The sequence map of *YlACC1* and *YlACC1cyto*. (B) The construction step of the plasmid of *YlACC1* 1*2*.

5.2.3. Overexpression of mutated *YlACC1* in *Y. lipolytica*

All types of *YlACC1* expression cassettes were transformed respectively to different genotype of *Y. lipolytica* to see the effect of mutation. The *Y. lipolytica* strains used in transformation were wild-type (JMY195, prototrophe JMY2900), *YlDGA1*-overexpressing strain (JMY7590, prototrophe JMY7593), and obese strain (JMY3371, prototrophe JMY7608).

For lipid synthesis test, YNB medium containing 0.08% (w/v) NH_4Cl and 50 mM phosphate buffer (pH 6.8) with 60 g/L of glucose (C/N=90) was used. This high C/N ratio condition is similar to ones used in the study showning the improvement of lipids production by overexpressing *YlACC1 in Y. lipolytica* [Tai and Stephanopoulos, 2013]. As a result, *ACC1* overexpression (including mutated *ACC1*s) in WT strain did not show any improvement of lipid synthesis (in terms of total lipid content %, g/g DCW) but increase of biomass (Figure 5.2), which is not consistent with the previous report.



(C)

		DCW (g/L)	Lipid content % (g/g DCW)	Lipids (g/L)
JMY2900	**Control**	**13.57**	**16.62**	**2.26**
JMY7509	**ACC1**	**15.12**	**12.91**	**1.92**
JMY7512	ACC1 1*	14.95	12.65	1.89
JMY7514	ACC1 2*	14.53	13.75	2.00
JMY7517	ACC1 1*2*	15.00	13.28	1.99
JMY7593	**DGA1**	**19.53**	**24.03**	**4.69**
JMY7596	DGA1+ACC1	16.48	34.00	5.58
JMY7599	DGA1+ACC1 1*	17.57	38.09	6.69
JMY7602	DGA1+ACC1 2*	17.63	35.59	6.26
JMY7605	DGA1+ACC1 1*2*	17.37	39.03	6.78

Figure 5.2. Lipid accumulation of *ACC1*-overexpressing strains.

(A) Total lipid content % (g/g DCW). (B) Lipid profiles (% in total lipids). (C) Table of biomass (g/L), lipid content % (g/g DCW), and lipids (g/L). The strains were cultivated in YNBD6 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments. DCW = dry cell weight.

As explained in introduction (part 1.2.4.2.1.), Tai and Stephanopoulos showed the metabolic engineering strategy to create a driving force by sequestering product formation into lipid bodies [Tai and Stephanopoulos, 2013]. Co-expression of *ACC1* and *DGA1* showed 4.7-fold improvement of lipid content (41.4% (g/g DCW)) due to a better balance between the fatty acid and TAG synthesis by pushing the carbon flux of the upstream pathway (*ACC1*) and the pull the downstream pathway (*DGA1*).

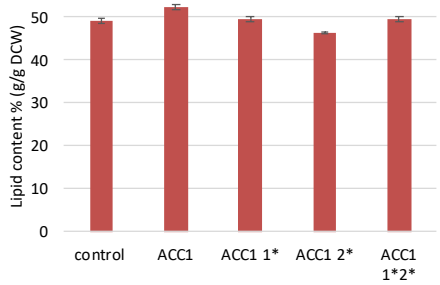
In order to see if the overexpression of *DGA1* together with *ACC1* could increase the lipid accumulation in our strain, the expression cassette of native *DGA1* was transformed into the

ACC1-overexpressing strains (Figure 5.2). Coexpression of *DGA1* and *ACC1* increased total lipid content from 24.0% to 34.0% of DCW compared the strain overexpressing only *DGA1*. Mutations of *ACC1* showed further increase of total lipid content up to 39.0% of DCW. The strain overexpressing *DGA1* and *ACC1* with double mutation (JMY7605, *YlACC1* 1*2*) produced the highest amount of lipids 6.78 g/L (1.44-fold and 1.21-fold higher than those of negative control (JMY7593, without *ACC1*) and positive control (JMY7596, *ACC1*), respectively). It is confirmed that the overexpression of *ACC1* and *DGA1* stimulates the carbon flux to lipid synthesis. Also, the inhibition of the posttranslational regulation of Acc1 of *Y. lipolytica* resulted in the increase of lipid synthesis, consistent with the results obtained in *S. cerevisiae*.

In order to investigate the effect of *ACC1* overexpression in the obese strain (JMY7608, *pox1-6Δ tgl4Δ* +pTEF-*DGA1*-*LEU2*ex + *URA3*) engineered to accumulate large amounts of lipids deleting the *POX* genes (*POX1-6*) and *TGL4* for inhibiting lipid degradation and remobilization, respectively, as well as overexpressing *DGA1* for enhancing TAG synthesis, the expression cassettes of *ACC1* was transformed to the obese strain [Beopoulos *et al*. 2008; Dulermo *et al*. 2013; Lazar *et al*. 2014]. As a result, the difference on lipid synthesis among the strains was not significant as *DGA1*-overexpressing strains (Figure 5.3). When native *ACC1* is overexpressed in obese strain, biomass (g/L) and lipids production (g/L) were slightly increased (1.13-fold and 1.21-fold, respectively). The mutation of *ACC1* didn't show improvement in terms of lipid production.

The enhanced lipid accumulation observed only in the strains co-expressing *ACC1* and *DGA1*, which seems the best balance between the fatty acid and TAG synthesis pathway in our strain. The discrepancy of the effect of *ACC1* overexpression between the present study and previous studies probably came from the difference of genotype, which was already observed in other studies [Friedlander *et al*. 2016]. The apparent metabolic flexibility implies the need for further experimentation to optimize the balance between precursors on genotype-by-genotype basis [Kamineni and Shaw, 2020].

(A)



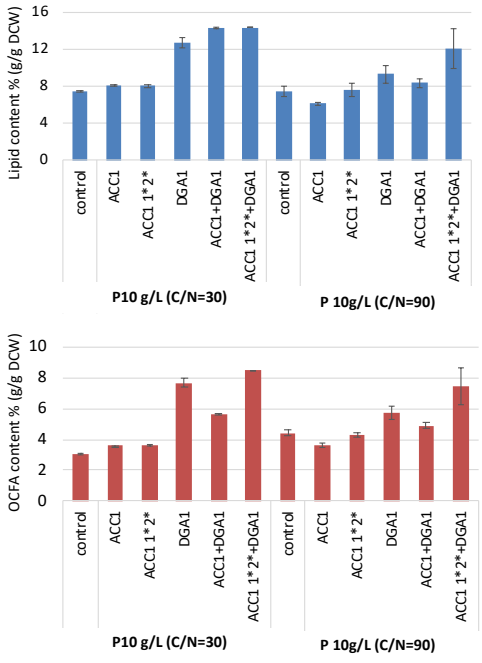
(B)

		DCW (g/L)	Lipid content % (g/g DCW)	Lipids (g/L)
JMY7608	Control (Δ*pox1-6* Δ*tgl4* +pTEF-*DGA1*)	14.90	49.12	7.32
JMY7609	ACC1	16.93	52.34	8.86
JMY7612	ACC1 1*	15.80	49.34	7.79
JMY7615	ACC1 2*	15.02	46.40	6.97
JMY7618	ACC1 1*2*	14.20	49.66	7.05

Figure 5.3. Lipid accumulation of *ACC1*-overexpressing obese strains.
(A) Lipid content % (g/g DCW). (B) Biomass (g/L), lipid content % (g/g DCW), and lipids (g/L). The strains were cultivated in YNBD6 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments. DCW = dry cell weight.

Regarding OCFA production, malonyl-CoA is also an important precursor since it merges with propionyl-CoA to synthesize the primer of OCFA, β-ketovaleryl-CoA (3-*oxo*-valeryl-CoA). Thus, the overexpression of *ACC1* also can be favorable for increasing the synthesis of OCFAs. In order to verify this, the lipid accumulation was assessed by flask culture on propionate (Figure 5.4). Two different carbon to nitrogen (C/N) ratios were tested with 1% (w/v) of propionate to investigate the effect of C/N ratio on OCFA production. The overexpression of *ACC1* showed the increase of lipid synthesis in both WT and *DGA1*-overexpressing strain (1.08-, 1.12-fold, respectively). The double mutation of *ACC1* did not show an additional positive effect on lipid accumulation in both WT and *DGA*-overexpressing strain. When we compared the accumulation of OCFAs, *ACC1* overexpression in WT showed the improved OCFA (from 40.77% to 43.95% in total lipids), which highly decreased in *DGA1*-overexpressing strain. When the strains were cultivated in a higher C/N environment (the C/N ratio=90), the lipid contents were decreased in all strains compared to those from the C/N ratio=30. Only in the strains overexpressing double-mutated *ACC1*, the lipid content was increased compared to their control in the same condition. This implies that the optimization of C/N ratio is necessary for OCFA production especially when using propionate as a substrate. Since it seems that the effect of *ACC1* overexpression affects very differently depends on the genotype, substrate, and carbon to nitrogen ratio, further studies to see if these results are from the mechanism of *ACC1* regulation or diverting the malonyl-CoA pools to other competitive pathways are necessary.

(A)



(B)

		DCW (g/L)	Total lipid content % (g/g DCW)	OCFA/Total lipid (%)
Propionate 10g/L (C/N=30)	Control	2.70	7.51	40.77
	ACC1	2.81	8.16	43.95
	ACC1 1*2*	2.90	8.07	44.64
	DGA1	3.06	12.77	60.06
	DGA1+ACC1	2.60	14.33	39.41
	DGA1+ACC1 1*2*	2.80	14.41	59.02
Propionate 10g/L (C/N=90)	Control	1.99	7.48	59.23
	ACC1	2.28	6.13	59.41
	ACC1 1*2*	2.24	7.70	55.82
	DGA1	1.92	9.37	61.02
	DGA1+ACC1	1.92	8.38	58.70
	DGA1+ACC1 1*2*	1.77	12.10	62.17

Figure 5.4. Lipid accumulation of *ACC1*-overexpressing strains in propionate-containing media.

(A) Total lipid content % (g/g DCW). (B) Table of biomass (g/L), lipid content % (g/g DCW), and lipids (g/L). The strains were cultivated in YNBP1 medium with different C/N ratio for 120 hr. Averages and standard deviations were obtained from two replicate experiments. DCW = dry cell weight.

5.3. Increasing acetyl-CoA pool

Acetyl coenzyme A (acetyl-CoA) is a particularly important precursor of fatty acids, especially ECFAs. The boosting of acetyl-CoA pools is crucial for ensuring high yields in production of lipids. Acetyl-CoA synthetase (Acs2p) is catalyzing the reaction from acetate to acetyl-CoA. We investigated if the overexpression of *ACS2* can improve the production of lipids in WT strain. Two different forms of *ACS2*, cytosolic and peroxisomal, were introduced and assessed their lipid accumulation (Figure 5.5). The overexpression of *ACS2* under pTEF promoter in both forms showed the decreased lipid accumulation. The lipid profiles are different depending on the localization of *ACS2*, cytosolic *ACS2* resulted a decrease of C18:1 and an increase of C16:0 and C18:2 compared to the control strain, while peroxisomal *ACS2* did not show a big difference compared to the control strain.

In order to see whether the enhancement of acetyl-CoA together with malonyl-CoA can improve lipid accumulation, the co-expression of *ACS2* and *ACC1* was carried out (Figure 5.6). As a result, the lipid accumulation of the strain expressing cytosolic *ACS2* and *ACC1*** was increased from 10.19% to 10.89% (g/g DCW) compared to the strain overexpressing *ACC1***. In case of peroxisomal *ACS2*, lipid accumulation was slightly decreased from 10.19% to 9.09% (g/g DCW). The increase of C16:0 and the decrease of C18:1 in cytosolic *ACS2*-expressing strain was consistent with the results shown in Figure 5.5. Co-expression of *ACS2* and *ACC1*** showed somehow the synergistic effect on this modification of the profiles. The results did not show a remarkable progress on lipid accumulation in our experimental condition.

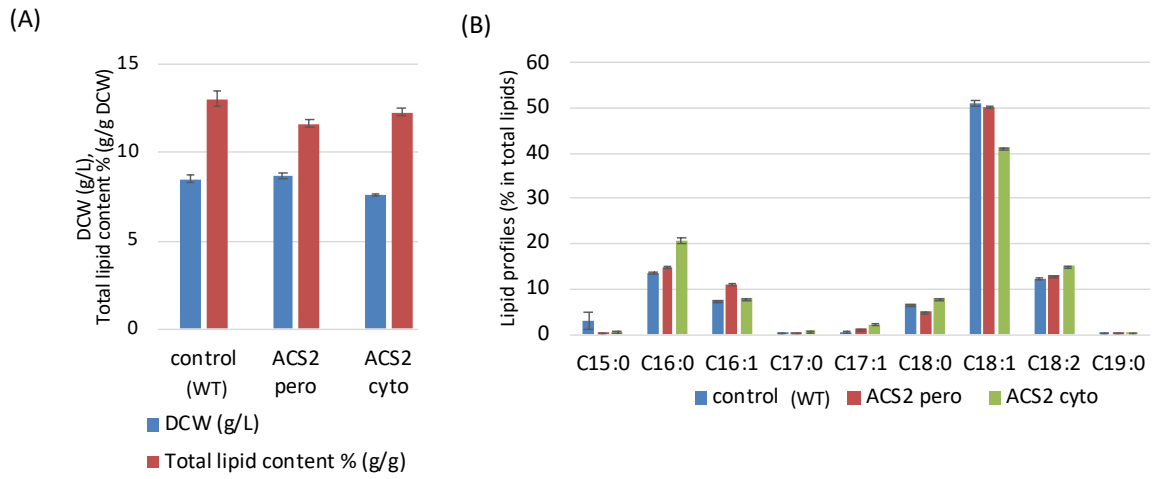


Figure 5.5. Lipid accumulation of *ACS2*-overexpressing strains in YNBD2 (Glucose 20 g/L, C/N=30).

(A) Biomass (g/L) and total lipid content % (g/g DCW). (B) Lipid profiles (% in total lipids). Samples were harvested at 72 hours of cultivation. Averages and standard deviations were obtained from two replicate experiments.

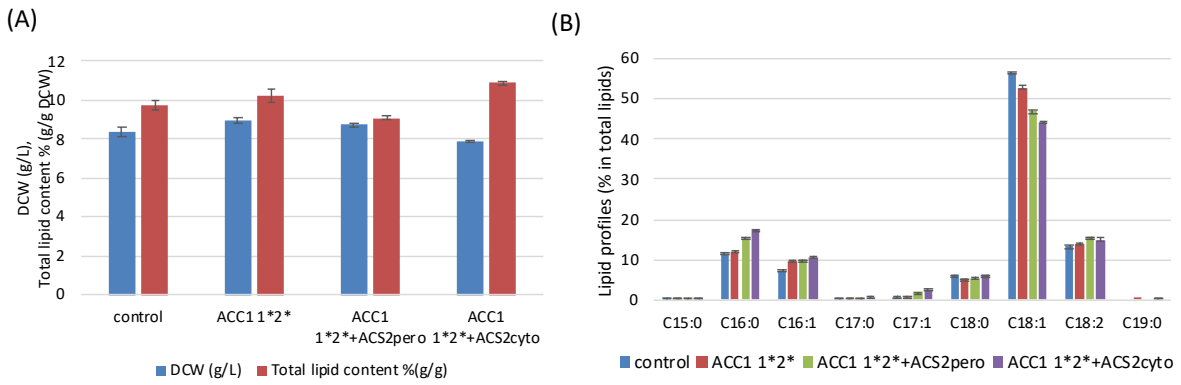


Figure 5.6. Lipid accumulation of *ACS2* and *ACC1* co-overexpressing strains in YNBD2 (Glucose 20 g/L, C/N=30).

(A) Biomass (g/L) and total lipid content % (g/g DCW). (B) Lipid profiles (% in total lipids). Samples were harvested at 72 hours of cultivation. Averages and standard deviations were obtained from two replicate experiments.

5.4. Increasing propionyl-CoA pool for the OCFA production

Studies on improving the propionyl-CoA pool are relatively less abundant than one for the acetyl-CoA pool. It was reported that propionyl-CoA was enhanced by introducing propionate-activating enzymes (*prpP* and *prpE*) to produce PHBV in *E. coli* [Liu *et al*. 2008]. For the production of OCFAs, Wu and San introduced heterologous *prpE* in *E. coli* and showed the superior production of OCFA compared to native *prpE* as described in the part 1.2.5.5 [Wu and San, 2014a]. In yeast, it is known that acetyl-CoA synthetase can catalyze the reaction from propionate to propionyl-CoA. However, the studies of improving propionyl-CoA pool by introducing heterologous enzyme or identifying native enzyme are still limited in yeast.

5.4.1. Evaluating propionate-activating enzymes for OCFA production

In Chapter 4, the production of OCFAs with propionate supplementation was described in wild-type *Y. lipolytica*, which indicated the presence of an endogenous propionyl-CoA synthetase activity in *Y. lipolytica* [Fontanille *et al*. 2012; Park *et al*. 2018]. We evaluated heterologous and native enzymes (CoA transferase and CoA synthetase) involved in propionate activation to see if they could boost propionyl-CoA pools for the production of OCFAs (Figure 5.7 (a)).

Propionyl-CoA transferase (Pct) catalyzes the transfer reaction of the CoA moiety from the donor (generally acetyl-CoA) to the acceptor through ping-pong mechanism; the range of substrate is broad and diverse depending on the origin [Volodina *et al*. 2014]. Propionyl-CoA

synthetase (PrpE) and Acetyl-CoA synthetase (Acs) are both acyl-CoA synthetase enzymes (EC 6.2.1) and share sequence similarities as well as common reaction features. PrpE is a bacterial propionate-activating enzyme, the overexpression of *prpE* from *Salmonella enterica* showed the increase of propionate activation in *E. coli* [Wu and San, 2014]. In yeast, it is known that acetyl-CoA synthetase can catalyze the formation of propionyl-CoA from propionate and coenzyme A [Jones and Lipmann, 1955; Pronk *et al*. 1994], *Y. lipolytica* has one gene (*ACS2*, *YALI0F05962g*) encoding acetyl-CoA synthetase while there are two genes (*ACS1*, *ACS2*) in *S. cerevisiae*. The impact of seven genes, *pct* (from *Ralstonia eutropha, Clostridium propionicum, E. coli,* and *Emiricella nidulans)*, *prpE* (from *E. coli, S. enterica*) and native *ACS2*, on propionate activation ability was in *Y. lipolytica* wild-type strain. The heterologous genes were synthesized with codon optimization for *Y. lipolytica*, all genes were expressed under the constitutive pTEF promoter.

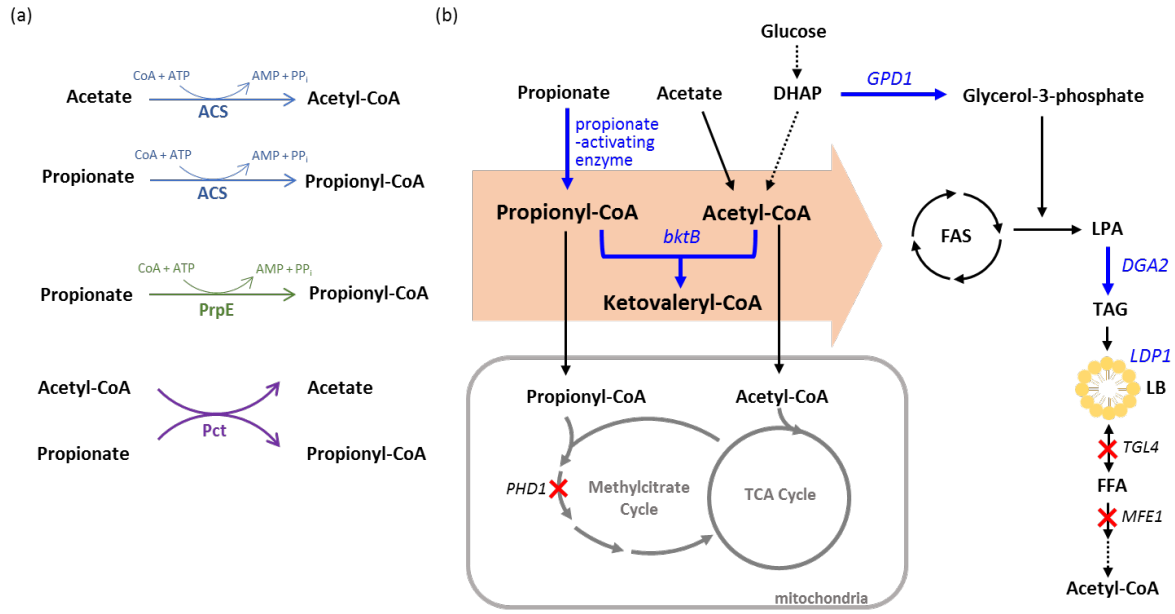


Figure 5.7. Overview of synthesis of lipids including OCFAs in *Y. lipolytica*. (A) Reaction of propionate activating enzymes used in this study. (B) Overall engineering for OCFA production in this study.

Engineered steps by overexpression are indicated by blue arrows and the corresponding genes are written in blue. Inactivated steps are indicated by red crossed cross and the corresponding genes are written in black. Multiple steps are shown as dashed arrows. *bktB*, β-ketothiolase; *GPD1*, glycerol-3-phosphate dehydrogenase; *DGA2*, acyl-CoA, diacylglycerol acyltransferase; *LDP1*, lipid droplet protein; *PHD1*, 2-methyl citrate dehydratase; *TGL4*, triglyceride lipase; *MFE1*, multifunctional enzyme; LB, lipid body.

Y. lipolytica strains expressing individual propionate activating enzymes were grown on minimal medium with various carbon sources (Figure 5.8). The growth of all strains on glucose in YNBD0.5 was mostly similar, while the growth on propionate in YNBP0.5 highly depended on the gene being overexpressed (Figure 5.8 (a) and (b)). *Enpct*-, *Ecpct*-, *EcprpE*- and *SeprpE*-expressing strains grew similarly to wild-type on propionate as a sole carbon source. Three

strains showed impaired growth, the *Cppct-, Repct-* and *ACS2*-expressing strains. The overexpression of native *ACS2* resulted in a 30% decrease of final OD_{600nm} compared to wild-type despite the similar growth rate at the beginning (μ_{max} *YlACS2*=0.67 h^{-1} *vs.* μ_{max} wild-type=0.76 h^{-1}, Table 5.2). In case of *Cppct*-expressing strain, the growth was slower than wild-type, but reached similar final OD_{600nm}. The growth of *Repct*-expressing strain was the most impacted as it grew much slower on propionate than *Cppct*-expressing strain. The growth rate was decreased by 80% compared to wild-type (μ_{max} *Repct*=0.16 h^{-1} *vs.* μ_{max} wild-type=0.76 h^{-1}), and the final OD_{600nm} was lower than wild-type by 26%. The lower growth of *Cppct-* and *Repct*-expressing strains could have different explanations, as the reaction consumes acetyl-CoA and propionate and produces acetate and propionyl-CoA, it could be due to the shortage of acetyl-CoA or the toxicity of released acetate or propionyl-CoA.

In order to verify this hypothesis, we tested cell growth on propionate with co-substrate as glucose or acetate (Figure 5.8 (c) and (d)). When glucose was added to the propionate medium, the lower growth of the *Cppct*-expressing strain on propionate was restored. For *ACS2*-expressing strain, the final OD_{600nm} was almost completely recovered compared to wild-type (93.5% of the final OD_{600nm}). *Repct*-expressing strain, remained the most impacted, even if the addition of glucose slightly improved growth compared to the one on propionate as a sole carbon source, the growth rate is still 80% lower than that of the wild-type (μ_{max} *Repct*=0.022 *vs*. μ_{max} wild-type=0.113 h^{-1}), and still showed a slightly lower final OD_{600nm} than wild-type in YNBD0.5P1. Thus, the addition of glucose to the propionate medium helped the cell growth but differently depending on the strain. When acetate is added to propionate instead of glucose, all strains showed similar growth, including *Repct*-expressing strain (Figure 5.8 (d)).

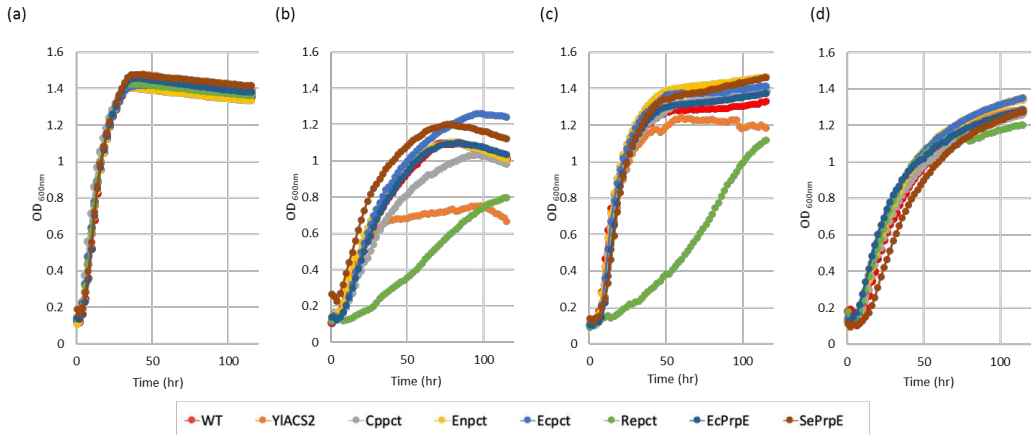


Figure 5.8. Growth of strains harboring propionate activating genes in different carbon sources.

(A) YNBD0.5, (b) YNBP0.5, (c) YNBD0.5P1, and (d) YNBP1A0.5. Averages were obtained from two replicate experiments.

Table 5.2. Maximum growth rate and maximal OD_{600nm} of the strains overexpressing propionate activating genes. Strains were grown in YNBD0.5, YNBP0.5, YNBD0.5P1, and YNBP1A0.5, respectively. Average values were obtained from two replicate experiments.

Medium	YNBD0.5		YNBP0.5		YNBD0.5P1		YNBP1A0.5	
Overexpressed gene	μ_{max} (h^{-1})	Maximal OD_{600nm}	μ_{max} (h^{-1})	Maximal OD_{600nm}	μ_{max} (h^{-1})	Maximal OD_{600nm}	μ_{max} (h^{-1})	Maximal OD_{600nm}
wild-type	0.122	1.406	0.076	1.099	0.113	1.329	0.118	1.275
YlACS2	0.111	1.458	0.067	0.753	0.155	1.245	0.114	1.286
Cppct	0.092	1.434	0.081	1.031	0.144	1.386	0.107	1.260
Enpct	0.124	1.383	0.093	1.104	0.119	1.466	0.108	1.346
Ecpct	0.099	1.420	0.093	1.261	0.143	1.414	0.109	1.350
Repct	0.103	1.409	0.016	0.799	0.022	1.119	0.093	1.202
EcprpE	0.128	1.438	0.083	1.099	0.165	1.375	0.092	1.284
SeprpE	0.113	1.463	0.062	1.203	0.128	1.462	0.080	1.272

In order to see if the overexpression of propionate-activating genes improves the accumulation of OCFAs, the strains were cultivated in nitrogen-limited conditions (0.15% (w/v) NH_4Cl) with different substrates and the lipid accumulation was quantified. The OCFA accumulation of the strains was also variable depending on the substrate as it was observed for cell growth (Figure 5.9). The overexpression of native *ACS2* exhibited a negative effect on OCFAs accumulation compared to wild-type in all conditions. The overexpression of *Cppct*, *Repct*, and *SeprpE* showed an increase of OCFAs (% of total FAs) compared to control for all culture conditions (with an exception for *Repct*-expressing strain in YNBD0.5P1). When we added acetate to YNBD0.5P1, the ratio of OCFAs to total FAs decreased in all strains including wild-type except for *Cppct*-, *Repct*-, and *SeprpE*-expressing strain. Especially, the *Repct*-expressing strain accumulated 53.2% of OCFAs of total FAs, the highest ratio of OCFAs in this experiment. Therefore, the three genes (*Cppct*, *Repct*, and *SeprpE*) showing a significant improvement of OCFAs were selected for subsequent strain engineering.

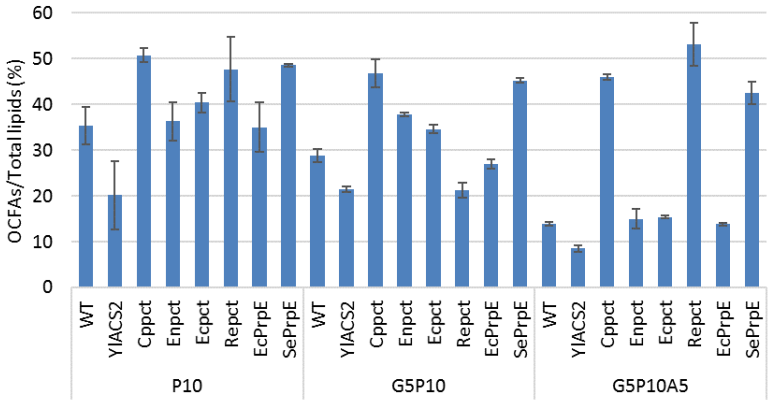


Figure 5.9. Accumulation of OCFAs in *Y. lipolytica* strains harboring propionate activating genes (*ACS*, *pct*, and *prpE*).

The strains were cultivated in YNBP1, YNBD0.5P1, and YNBD0.5P1A0.5 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments.

5.4.2. Improving OCFA production by engineering obese strains

Continued from the chapter 4, we engineered the strain from obese (JMY3776) by overexpressing *LDP1* encoding lipid droplet protein to enhance the storage of TAG and named the strain as JMY7228 (obese-L) strain [Bhutada *et al*. 2018; Park and Nicaud, 2019]. All the modifications are described in Figure 5.7 together with newly introduced modifications in this chapter.

The three selected genes (*Cppct*, *Repct*, and *SeprpE*) were separately overexpressed in obese-L strain, obtaining obese-LP strains. To assess the lipid accumulation of the strains, flask culture was performed in YNBD2P0.5A1 (2% (w/v) glucose, 0.5% (w/v) propionate, 1% (w/v) acetate with a C/N ratio = 30) (Figure 5.10). All obese-LP strains accumulated OCFAs more than 54% of total FAs (Figure 5.10 (b)). The obese-LP (*Repct*) strain (JMY7780) exhibited the highest amount of OCFAs, 61.7% of total lipids. Whereas the obese-L strain (Figure 5.10 (c)) accumulates a majority of ECFA (16:0 and C18:1), C17:1 was the most abundant OCFA in all the obese-LP strains consistently with our previous study (Figure 5.10 (d)). The highest ratio of C17:1 to total lipids (45.6%) was obtained in obese-LP (*SeprpE*) strain. The second major FA from obese-LP strain was oleic acid (C18:1) (between 15% and 20% depending on the strain), and other FAs are lower than 10% of total FAs (Figure 5.11).

All three obese-LP strains showed a higher propionate consumption rate compared to the control strain at the beginning of the culture (until 50 hours) (Figure 5.12). Among them, the obese-LP (*SeprpE)* strain consumed the highest amount of propionate, 2.4 g/L, at a constant rate. The obese-LP (*Repct)* and the obese-LP (*Cppct)* strains also utilized more propionate (1.8 g/L, 1.2 g/L, respectively) than the control strain (1.1 g/L).

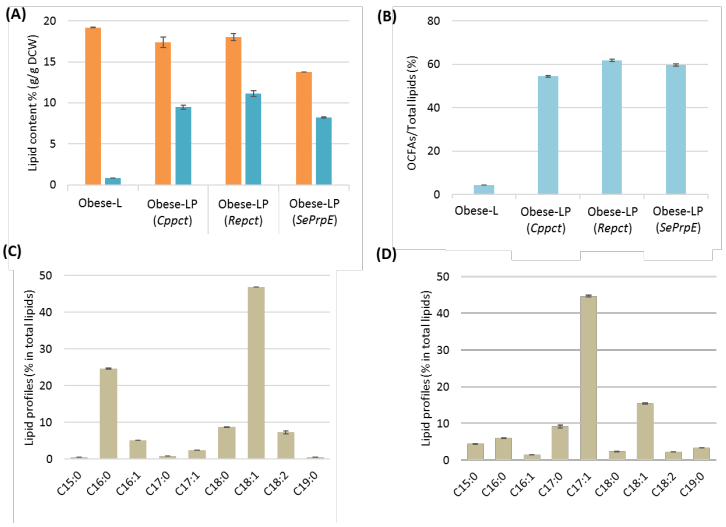


Figure 5.10. Lipid accumulation of obese strains harboring *pct* and *prpE* genes.
(A) The percentage of fatty acids in the DCW, total FAs are indicated in orange and OCFAs are in blue. (B) The ratio of OCFAs to total lipids (%). (C) Lipid profiles (% in total lipids) of obese-L strain. (D) Lipid profiles of obese-LP (*Repct*) strain. The strains were cultivated in YNBD2P0.5A1 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments.

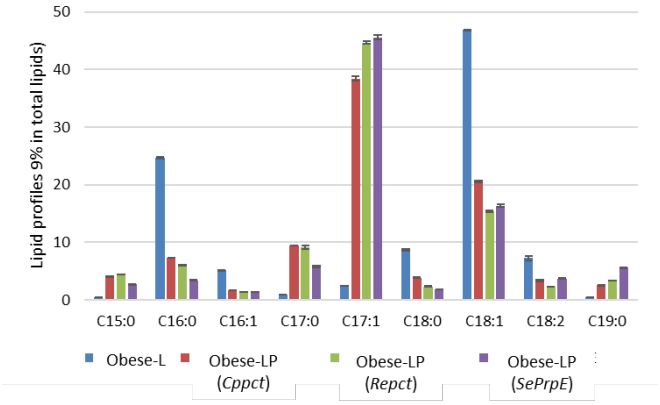


Figure 5.11. Lipid profiles (% in total lipids) of obese-L (JMY7228) and obese-LP (*RePCT*) strains (JMY7780).

The strains were cultivated in YNBD2P0.5A1 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments.

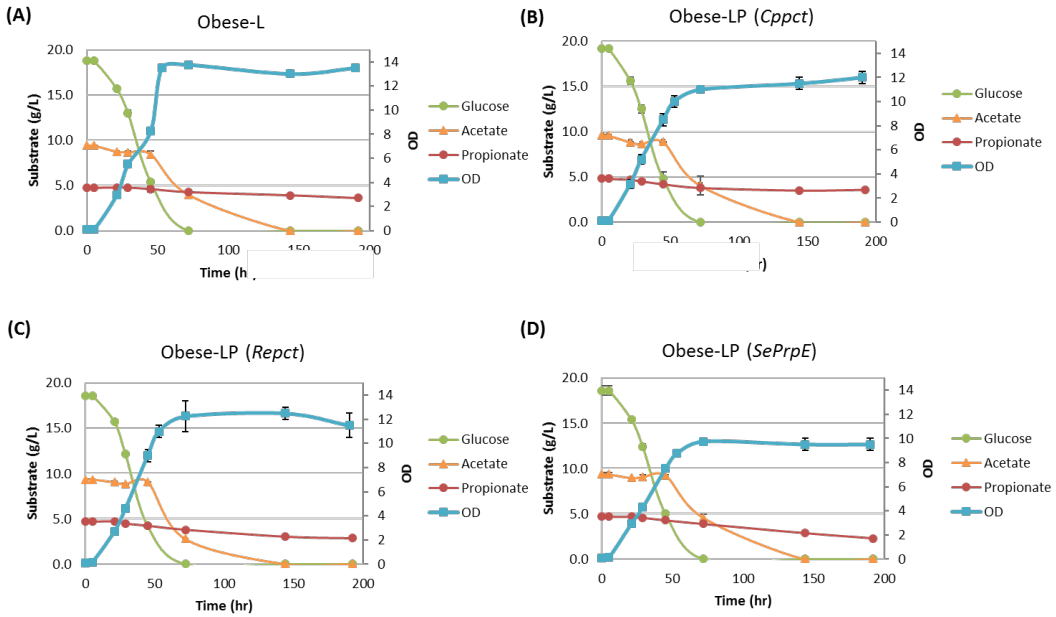


Figure 5.12. Substrate consumption of obese strains.

(A) Control, obese-L, (B) obese-LP (*Cppct*), (C) obese-LP (*Repct*), and (D) obese-LP (*SeprpE*). Averages and standard deviations were obtained from two replicate experiments.

5.4.3. Supplementing acetate in OCFA production

We assume that the addition of acetate boosts acetyl-CoA pools (C2 precursor pool) and will improve the level of ECFA at the expense of OCFA. In order to investigate how the addition of acetate impacts the ratio of OCFA and ECFA, we first cultured the obese-L strain with and without acetate. The ratio of OCFA to total lipids significantly dropped from 28.9% to 3.8% when the acetate (1%) was added to the medium (Table 5.3). We then explored the lipid

accumulation with different amounts of acetate to identify the optimal ratio of propionate and acetate for OCFA production. The JMY7780 (obese-LP (*Repct*)) strain was used for this test, glucose was also added to provide a carbon source for biomass as the previous study showed a beneficial effect of this strategy [Park *et al*. 2018]. Different ratios in the concentration of propionate and acetate (from 2:1 to 1:4) were applied with a constant propionate concentration (0.5% (w/v)) and glucose was adjusted to meet the same total carbon amount in all conditions. When more acetate was added, a lower ratio of OCFAs to total FAs (from 66.3% to 50.7%) was obtained as expected (Figure 5.13). However, increasing acetate resulted in higher biomass and total lipid content (%, g/g DCW). The best combination for OCFA production was 0.5% (w/v) propionate and 1% (w/v) acetate. Heptadecenoic acid (C17:1), the major FA in OCFAs, was decreased while oleic acid (C18:1) was increased with increasing acetate amount. The other OCFAs, pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), showed steady levels regardless of acetate amount.

Table 5.3. Production of OCFAs in obese-L strain with or without acetate.

The strains were cultivated in YNBD2P0.5 and YNBD2P0.5A1 medium for 120 hr. Averages and standard deviations were obtained from two replicate experiments. DCW = dry cell weight.

Media	DCW (g/L)	Lipid content % (g/g DCW)		OCFA/total lipids (%)
		Total lipids	OCFAs	
YNBD2P0.5	5.8	18.81	5.48	28.94
YNBD2P0.5A1	9.2	17.27	0.65	3.79

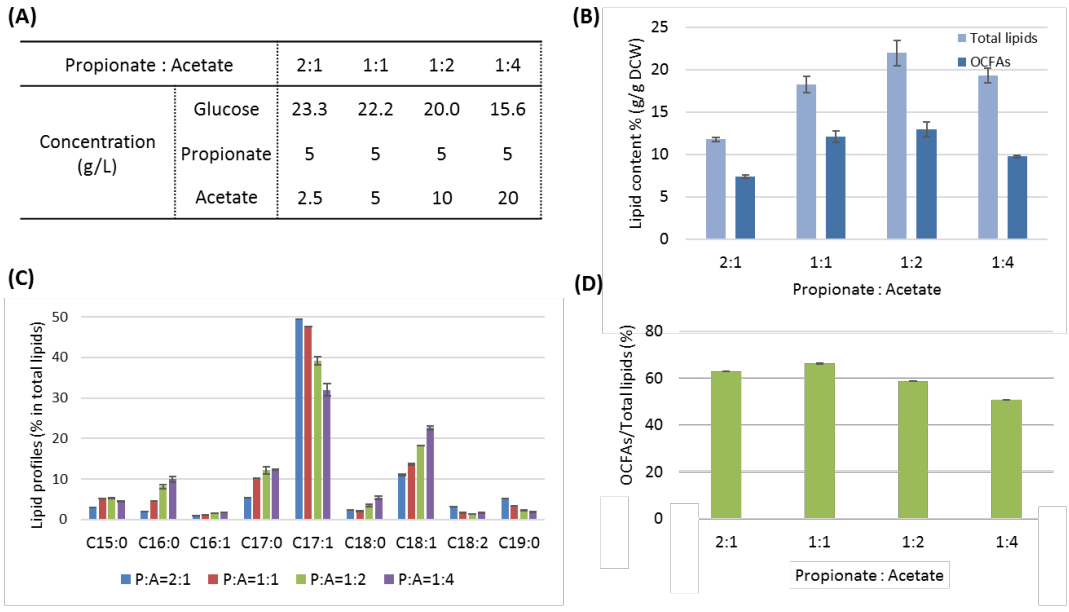


Figure 5.13. Lipid accumulation of JMY7780 (obese-LP (*Repct*)) in the different ratio of propionate and acetate.

(A) the concentration of the substrates (glucose, propionate, and acetate). (B) The percentage of fatty acids in the DCW, total FAs are indicated in light blue and OCFAs are in blue. (C) Lipid profiles (% in

total lipids). (D) The ratio of OCFAs to total lipids (%). The strains were cultivated for 168 hr. Averages and standard deviations were obtained from two replicate experiments.

5.4.4. Boosting precursor pools by overexpression of *bktB*

To further improve precursor pools of OCFAs, lipid accumulation from five-carbon chain compounds was explored. It is reported that valerate (pentanoate, C5) can be utilized as a carbon source for biomass and OCFA production in *Y. lipolytica* [Chakraborty, 2015]. It is explained that the activated form of valerate (valeryl-CoA) goes through the β-oxidation to produce acetyl-CoA and propionyl-CoA, resulting in the accumulation of OCFAs. However, the β-oxidation pathway of the obese-LP strain is blocked by *MFE1* deletion, thus the cleavage of C5 compounds to C2 and C3 compounds is inhibited in this strain. We investigated whether *de novo* synthesis of the C5 precursor, β-ketovaleryl-CoA could boost the precursor pools for OCFA production instead of valerate supplementation to provide acetyl-CoA and propionyl-CoA.

β-Ketothiolase catalyzes the condensation of acetyl-CoA and propionyl-CoA to form β-ketovaleryl-CoA. This enzyme encoded by the *bktB* gene has been used for the synthesis of PHB and PHBV [Mitsky *et al*. 1998; Tseng and Prather, 2012]. The *bktB* gene from *R. eutropha*, well known as a representative microorganism of the PHBV producer, was selected as a candidate enzyme for synthesizing β-ketovaleryl-CoA in *Y. lipolytica*. The sequence of *bktB* from *R. eutropha* H16 was codon-optimized to *Y. lipolytica* and overexpressed under the constitutive pTEF promoter.

To evaluate the impact of *bktB* overexpression on OCFA production, the obese-LP (*Repct*) and obese-LPB (*Repct-RebktB*) strains (JMY7780 and JMY8438, respectively) were grown in YNBD2P0.5A1 medium for 7 days. The production of OCFAs was increased by 33% from 0.99 g/L to 1.32 g/L by overexpressing *bktB*. (Figure 5.14). Total lipid production was also increased by 36% which resulted in a similar ratio of OCFAs to total lipids between obese-LP and obese-LPB strains.

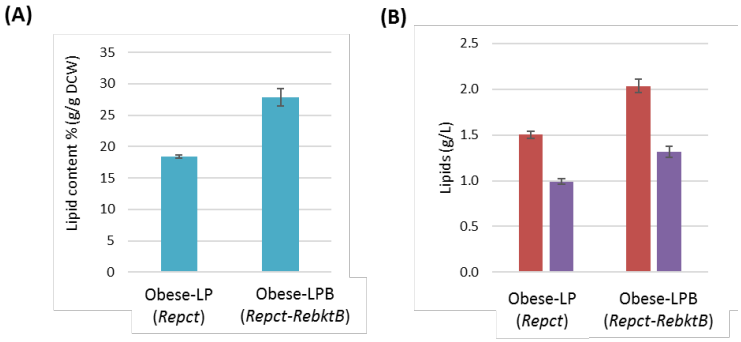


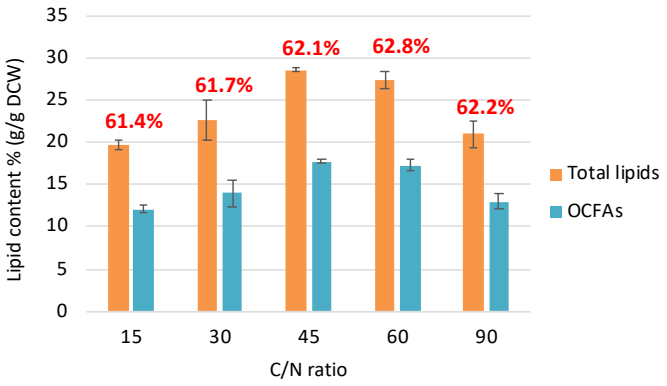
Figure 5.14. Lipid accumulation of obese strains harboring *pct* and *bktB* genes.

(A) The percentage of fatty acids in the DCW. (B) Lipid titer (g/L), total lipids are indicated in red and OCFAs are in violet. The strains were cultivated for 168 hr. Averages and standard deviations were obtained from two replicate experiments.

5.4.5. Optimizing C/N ratio

The C/N ratio significantly affects lipid accumulation in *Y. lipolytica* by inducing nitrogen starvation. Nitrogen-limited media with a high C/N ratio have been used to achieve high levels of lipid accumulation in *Y. lipolytica* [Beopoulos *et al*. 2009; Lazar *et al*. 2014; Blazeck *et al*. 2014]. It is known that the optimal C/N ratio is variable depending on the carbon source and the genotype of the strain [Lazar *et al*. 2015; Ledesma-Amaro *et al*. 2016], thus the optimal C/N ratio for OCFA production in the obese-LPB strain was investigated (Figure 5.15). Different C/N ratios were achieved by adjusting NH_4Cl concentration while holding the initial carbon source concentration constant (YNBD4P1A2). Lipid accumulation was improved up to C/N=45, and it decreased when the C/N ratio was above 45 (Figure 5.15 (a)). The ratio of OCFAs to total lipids is similar in all conditions around 62%, the optimal C/N ratio was 45 producing 1.87 g/L of OCFAs (Figure 5.15 (b)). This is, to date, the highest OCFA titer obtained among bacteria and yeast (Table 1.2).

(A)



(B)

C/N ratio	Total lipid content % (g/g DCW)	OCFAs/Total lipids (%)	Total lipids (g/L)	OCFAs (g/L)
15	19.70	61.41	2.58	1.58
30	22.65	61.71	2.79	1.72
45	28.56	62.07	3.01	1.87
60	27.47	62.82	2.55	1.60
90	20.95	62.15	0.97	0.60

Figure 5.15. Lipid production by JMY8438 (obese-LPB (*Repct-RebktB*)) strain with different C/N ratios.

(A) The percentage of fatty acids in the DCW, total FAs are indicated in orange and OCFAs are in cyan. The percentage of OCFAs to total lipids are written in red. (B) Total lipids and OCFA production. Lipid production was analyzed after 192 hr of cultivation. Different C/N ratios were obtained by adjusting NH_4Cl concentration. Averages and standard deviations were obtained from two replicate experiments.

5.5. Discussion

It is well known that acetyl-CoA and malonyl-CoA are starting precursor of lipid synthesis. Therefore, many attempts to boost the precursor pools for increasing lipid production have been reported. Acc1p catalyzing the reaction from acetyl-CoA to malonyl-CoA is one of main target enzyme in lipid production, the overexpression of *ACC1* in *Y. lipolytica* was assessed in this chapter. In other study, it was revealed that *ACC1* overexpression improved the lipid synthesis, and further increase was obtained when it was combined with *DGA1* overexpression by push and pull carbon flux to lipid synthesis. In this chapter, the co-expression of *ACC1* and *DGA1* showed the increase of lipid synthesis from 24.0% to 34.0% (lipid content %, g/g DCW), while strain overexpressing *ACC1* did not show the increase of lipid synthesis. The mutation for inhibiting the posttranslational regulation showed further increase of lipid synthesis up to 39.0%, reached to 6.78 g/L in *ACC1* and *DGA1* coexpressing strain. It is confirmed that the positive effect of *ACC1* overexpression is highly dependent on the genotype of the strain and the condition of cultivation (substrate concentration, C/N ratio, and so on).

Increasing acetyl-CoA was also explored by overexpressing *ACS2* in *Y. lipolytica*. A slight increase of lipid accumulation was obtained only when *ACS2* was co-expressed with mutated *ACC1*. The results did not show a remarkable progress on lipid accumulation in our experimental condition. Similar strategy was applied on the production of naringenin in *Y. lipolytica,* obtaining 62.6% higher production by overexpressing *ACS2* and *ACC1* [Lv *et al*. 2019]. The exact comparison is not possible due to the different genotype which can lead to a big difference, but it seems that the regulation of these enzymes is dependent on the engineering of carbon flux to different target compounds. The more thorough understanding of regulation of the two enzymes, acetyl-CoA synthetase and acetyl-CoA carboxylase are necessary.

Regarding OCFAs, the synthesis of OCFA *versus* classical ECFA depends only on the first cycle of fatty acid elongation, in which cytosolic propionyl-CoA or acetyl-CoA are condensed with malonyl-CoA, respectively. For the OCFA initiation step, it is thus necessary to have a relatively large propionyl-CoA pool compared to acetyl-CoA. But for the subsequent step of elongation, the high availability of acetyl-CoA is necessary as the malonyl-CoA building block for fatty acid synthesis. Thus, the dual role of acetyl-CoA for the synthesis of OCFA should be taken into consideration.

As the synthesis of OCFAs is started from propionyl-CoA, being synthesized from propionate, we first investigated how propionate activating enzymes of different origins could boost the propionyl-CoA pool, therefore, influence the synthesis of the OCFA. Acetyl-CoA synthetases of different origins have shown a promiscuous propionyl-CoA synthetase activity [Luong *et al*. 2000; Ingram-Smith and Smith, 2006]. However, the detailed studies on the activity of Acs on

propionate or identification of other propionate-activating enzymes in yeast are still limited. In this chapter, native *Y. lipolytica ACS2* was overexpressed to examine its effect on OCFA production. The overexpression of native *ACS2* exhibited a strong negative effect on OCFAs accumulation for any substrate compositions in our experimental condition, suggesting the reaction catalyzed by acetyl-CoA synthetase has a stronger preference for acetate than propionate, thus resulting in a higher amount of ECFAs than OCFAs.

Two PrpEs (propionyl-CoA synthetase) from *E. coli* and *S. enterica* were overexpressed. The overexpression of *EcprpE* did not show any difference in either growth or OCFA production compared to the wild-type, suggesting an inactive or low active enzyme. A higher OCFA production from *SeprpE*-overexpressing strain than wild-type was obtained in all substrate conditions used in this experiment, showing that our strategy of increasing the propionyl-CoA pool for increasing OCFA production was relevant.

Another class of propionate activating enzyme was tested in this experiment, Pct (propionyl-CoA transferase) catalyzing the transfer of CoA moiety from the acetyl-CoA (donor) to the propionate (acceptor). The *pct*-expressing strains showed different growth on propionate. On this medium, the proportion of OCFA in *Ecpct*- and *Enpct*-expressing strains is comparable with the one of the wild-type, whereas the *Cppct*- and *Repct*-expressing strains presented a better accumulation of OCFA than wild-type despite slower growth. We assume that the negative effect on growth for *Cppct*-and *Repct*-expressing strains could be due to a cytosolic acetyl-CoA shortage resulting from a high Pct activity. This correlates with the observed higher accumulation of OCFA as a result of improved propionyl-CoA pool. In order to verify our hypothesis, supplementation of glucose or acetate as an acetyl-CoA supplier on the propionate-containing medium was assessed. The total recovery of growth in the *Repct*-expressing strain was possible only with acetate supplementation, while the growth of the *Cppct*-expressing strain was recovered similar to wild-type in both media. It suggests that the supply of acetyl-CoA from glucose is still limited in *Repct*-expressing strain, representing the highest Pct activity among Pct enzymes tested in this experiment as confirmed by the highest OCFA accumulation (53.2% of OCFAs in total FAs) on medium supplemented with acetate.

The effect of acetate supplementation on OCFA production was explored in different strains. In non-obese strain including wild-type, the ratio of OCFAs to total FAs was decreased when we added 1% acetate in all strains except for *Repct*-expressing strains. This effect was even more pronounced in the Obese-L strain in which the addition of 1% acetate resulted in only 3.8% of OCFA of total lipids. These results indicate the importance of balancing the two precursor pools, propionyl-CoA and acetyl-CoA for OCFA production. The optimization of the OCFA titer has thus to deal with multiple parameters such as the metabolism of the chassis strain, the propionate activating enzyme to be expressed, and the propionate/acetate ratio in the culture medium. For the *Repct*-expressing strain, the best compromised condition for cells growth, lipids content, and lipid composition was 2% (w/v) glucose, 0.5% (w/v) propionate, and 1% (w/v) acetate.

Using acetate as a substrate has been described in several studies taking advantage of its relatively shorter conversion pathway to acetyl-CoA. The lipid production by utilizing acetate has been reported in oleaginous yeasts such as *Y. lipolytica, R. toruloides,* and *Lipomyces starkeyi* [Fontanille *et al*. 2012; Huang *et al*. 2016; Xavier *et al*. 2017]. Acetate as well as propionate, substrates used in this chapter, can easily be found in volatile fatty acids (VFAs) being obtainable from agro-industrial wastes or biodegradable organic wastes. VFAs as a substrate for microbial oil production has recently been gaining a lot of interest due to its low cost [Liu *et al*. 2017; Llamas *et al*. 2019]. Thus, the utilization of VFAs for the production of OCFAs in *Y. lipolytica* will be a feasible and sustainable strategy for the scale-up process. To maximize biomass and OCFA production in *Y. lipolytica*, the co-feeding of substrates as well as C/N ratio need to be optimized during fermentation.

An original strategy consisting of the supply of the C5 precursor, β-ketovaleryl-CoA, was investigated, to determine if it can be incorporated into lipid synthesis pathway. β-Ketothiolase (BktB) from *R. euthropha*, which has been used for the synthesis of PHBV, was selected because of its activity on propionyl-CoA [Mitsky *et al*. 1998; Tseng and Prather, 2002; Yang *et al*. 2012]. This strategy has never been explored to improve the OCFA production and the co-expression of *Repct* and *RebktB* resulted in an increase of lipid synthesis by 36%, with a constant ratio of OCFAs to total lipids. This increase of total lipids probably results from a similar increase of acetoacetyl-CoA (C4) and β-ketovaleryl-CoA (C5) due to the broad substrate specificity of β-ketothiolase [Mitsky *et al*. 1998], thus leading to constant ratio of OCFAs to total lipids.

CHAPTER 6. *DE NOVO* PRODUCTION OF OCFAS FROM GLUCOSE

6.1. Introduction

Most studies on OCFA production have been focused on processes that involve propionate supplementation. However, due to the high cost [Poirier *et al*. 1995; Aldor *et al*. 2002] and toxic effects of propionate [Fontanille *et al*. 2012; Park *et al*. 2019], it is crucial to find alternative pathways for generating propionyl-CoA to be able to produce OCFAs on large scales. There have been a few studies in which OCFAs have been produced using glucose as described in the part 1.2.5.5. and Figure 1.8. Tseng and Prather have shown that, in *E. coli*, the production of very short OCFAs (i.e., propionate, *trans*-2-pentenoate, and valerate) through the upregulation of threonine biosynthesis [Tseng and Prather, 2012]. Another study demonstrated the production of OCFAs in *E. coli* by overexpression of threonine biosynthesis and replacement of β-ketoacyl-ACP synthase [Lee *et al*. 2013]. However, to date, no one has reported the *de novo* production of OCFAs in *Y. lipolytica* without propionate supplementation. Therefore, we aimed to biosynthesize OCFAs *de novo* from glucose without propionate supplementation. A modular metabolic pathway for synthesizing propionyl-CoA from oxaloacetate was constructed in *Y. lipolytica* and confirmed as functional for OCFAs synthesis. The disruption of the potential competitive pathway for OCFAs *via* methyl citrate pathway was explored whether it could be beneficial to produce OCFAs. Additionally, the pyruvate dehydrogenase (PDH) complex was overexpressed in cytosol to see if it could improve the conversion of α-ketobutyrate to propionyl-CoA.

6.2. Results

6.2.1. Modular pathway engineering for OCFAs synthesis

In order to synthesize OCFAs without propionate addition, the synthesis of propionyl-CoA should be investigated. It is known that propionyl-CoA can be synthesized using β-oxidation from direct precursors, propionate, or long-chain FAs. It can also be created from other metabolites *via* several metabolic pathways, such as the citramalate/2-ketobutyrate pathway, the aspartate/2-ketobutyrate pathway, the methylmalonyl-CoA pathway, the 3-hydroxypropionate pathway, and the isoleucine or valine degradation pathway as described in Figure 1.7 [Lee *et al*. 2013; Han *et al*. 2013]. Here, we tested if the overexpression of the α-ketobutyrate pathway—which produces threonine as an intermediate—could increase levels of propionyl-CoA in *Y. lipolytica*. As shown in Figure 6.1 (A), the pathway eventually forms the amino acids aspartate, homoserine, and threonine from oxaloacetate. Then, threonine is deaminated to generate α-ketobutyrate, a reaction catalyzed by threonine dehydratase. Alpha-ketobutyrate is directly or sequentially converted into propionyl-CoA by the pyruvate dehydrogenase (PDH) complex or pyruvate oxidase, respectively. The upregulation of

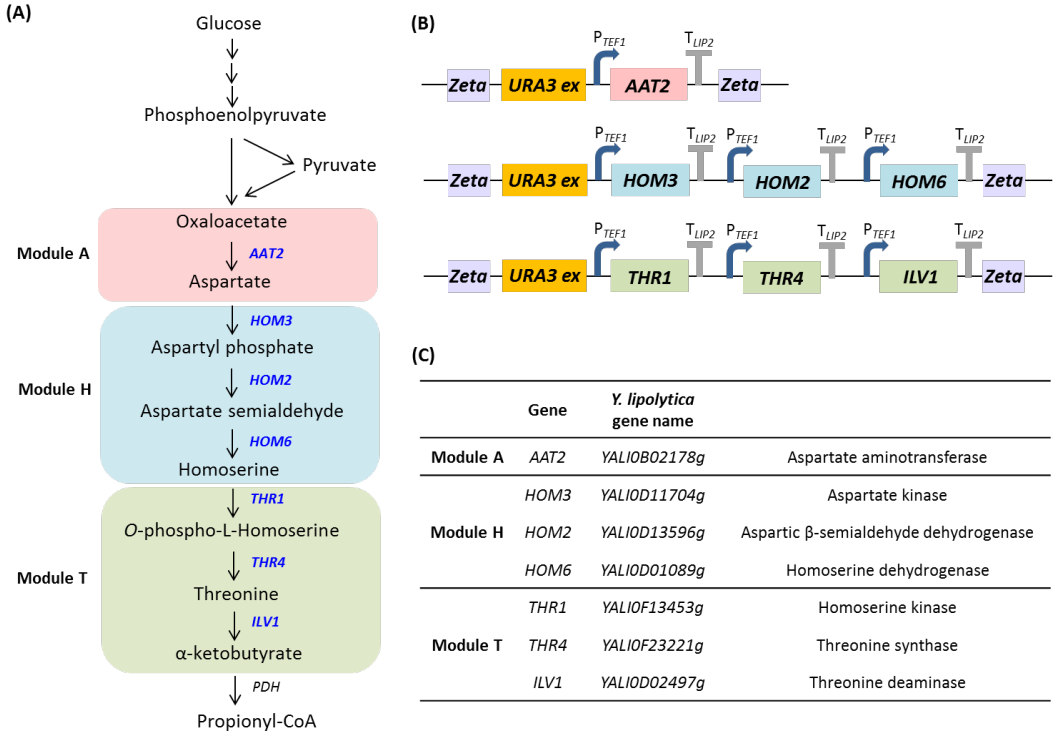
threonine has previously been used to boost propionyl-CoA availability in *E. coli*. Lee *et al*. showed that levels of OCFAs could be increased by introducing the threonine biosynthesis pathway (which creates α-ketobutyrate from aspartate semialdehyde), especially when mutated homoserine dehydrogenase (thrA*, reduced feedback inhibition) was also expressed [Lee *et al*. 2013]. The percentage of OCFAs out of total FAs increased from less than 1% to 18% by overexpressing the threonine pathway in *E. coli*.

In this chapter, we enhanced the extended threonine biosynthesis pathway (the aspartate/α-ketobutyrate pathway)—from oxaloacetate to α-ketobutyrate—by overexpressing seven genes (Figure 6.1 (A)). There were three modules (Figure 6.1 (A) and (C)): the aspartate synthesis module (module A), which included *AAT2*; the homoserine synthesis module (module H), which included *HOM3*, *HOM2*, and *HOM6*; and the threonine synthesis module (module T), which included *THR1*, *THR4*, and *ILV1*. While threonine and α-ketobutyrate (module T) are synthesized in the mitochondria in *S. cerevisiae*, the same may not be true in *Y. lipolytica*. While the locations of the relevant enzymes are as yet unknown in *Y. lipolytica*, predictive analyses suggest enzyme location may differ between *S. cerevisiae* and *Y. lipolytica* (Table 6.1). Because the module T enzymes were predicted to occur in the cytoplasm in *Y. lipolytica*, we used the original sequence of each gene in this study, as described in Table 6.1.

Table 6.1. Prediction of protein localization in *S. cerevisiae* and *Y. lipolytica*.

	Prediction program	CELLO		MitoProt	
S. cerevisiae		Localization	Localization score	Localization	Probablity of export to mitochondria
	THR1	cytoplasm	cyto 1.422 mito 0.517	cytoplasm	0.0758
	THR4	cytoplasm	cyto 1.496 mito 0.899	mitochondria	0.8364
	ILV1	mitochondria	cyto 0.384 mito 3.104	mitochondria	0.9754
	Prediction program	CELLO		MitoProt	
Y. lipolytica		Localization	Localization score	Localization	Probablity of export to mitochondria
	THR1	cytoplasm	cyto 1.542 mito 0.718	cytoplasm	0.0072
	THR4	cytoplasm	cyto 1.165 mito 0.674	cytoplasm	0.1128
	ILV1	cytoplasm	cyto 2.897 mito 0.353	cytoplasm	0.046

The genes in the A, T, and H module were obtained *via* PCR using the genomic DNA of *Y. lipolytica* W29. The genes in each module were cloned into one plasmid using Golden Gate assembly (Figure 6.1 (B)). They were expressed under the constitutive promoter pTEF1, and the expression cassette of each module was randomly integrated into the genome. Each module (A, T, and H) in the pathway was overexpressed in *Y. lipolytica* both individually and in tandem. The strain with the full modular pathway (ATH) was constructed by removing and reusing the *URA3* marker (the detailed strain construction is described in Appendix E – Park *et al*. Frontiers in Bioengineering and Biotechnology, 2020).



(C)

	Gene	*Y. lipolytica* gene name	
Module A	*AAT2*	*YALI0B02178g*	Aspartate aminotransferase
Module H	*HOM3*	*YALI0D11704g*	Aspartate kinase
	HOM2	*YALI0D13596g*	Aspartic β-semialdehyde dehydrogenase
	HOM6	*YALI0D01089g*	Homoserine dehydrogenase
Module T	*THR1*	*YALI0F13453g*	Homoserine kinase
	THR4	*YALI0F23221g*	Threonine synthase
	ILV1	*YALI0D02497g*	Threonine deaminase

Figure 6.1. The biosynthetic pathway for propionyl-CoA.

(A) The metabolic pathway by which propionyl-CoA is synthesized from glucose. The genes overexpressed in this study are shown in blue. Pyruvate produced by glycolysis is converted to oxaloacetate by pyruvate carboxylase, and oxaloacetate is converted to threonine through the biosynthesis of aspartate and homoserine. Threonine deaminase subsequently generates α-ketobutyrate from threonine. Then, α-ketobutyrate is converted to propionyl-CoA by the pyruvate dehydrogenase (PDH) complex. (B) Structure of the multigene modules encoding enzymes in the aspartate/α-ketobutyrate pathway that were constructed in this study. Each gene expression cassette included the native *TEF1* promoter and *LIP2* terminator. (C) The genes included in the modules and the enzymes they encode.

6.2.2. OCFA production from glucose in the engineered strain

To determine whether the modular metabolic pathway was effective in producing OCFAs, we evaluated the engineered strains overexpressing the individual modules and the entire pathway. The strains were cultivated in YNBD6 medium under nitrogen limitation conditions (C/N=60), which have been found to positively influence lipid synthesis [Beopoulos *et al*. 2012; Ledesma-Amaro *et al*. 2016]. For the WT-A strain, which overexpressed *AAT2*, and the WT-T strain, which overexpressed *THR1*, *THR4*, and *ILV1*, total lipid content (%, g/g DCW) was lower than in the wild-type (WT) strain; the percentage of OCFAs out of total FAs was similar (Table 6.2). For the WT-H strain, which overexpressed *HOM3*, *HOM2*, and *HOM6*, this percentage was slightly greater (1.91%) than that in the WT strain (0.84%) (Table 6.2 and Figure 6.2 (A)). For the WT-ATH strain, which overexpressed the entire pathway, OCFAs synthesis was significantly greater, OCFAs content (%, g/g DCW) was 3.8 times higher, and OCFAs titers (g/L) were 3.6 times higher than in the WT strain; the percentage of OCFAs out of total FAs was

3.86%, which was 4.6 times higher than the value seen in the WT strain. These results indicate that the engineered aspartate/α-ketobutyrate pathway can supply propionyl-CoA; they also show that the full pathway is needed for effective OCFAs synthesis.

WT-ATH primarily produced C17:1 FAs (Figure 6.2 (B)). This profile resembles that of an engineered *Y. lipolytica* strain from propionate supplementation [Park *et al*. 2018]. This finding implies that enhancing carbon flux through the α-ketobutyrate pathway can boost propionyl-CoA availability and OCFAs synthesis the same way that propionate supplementation can.

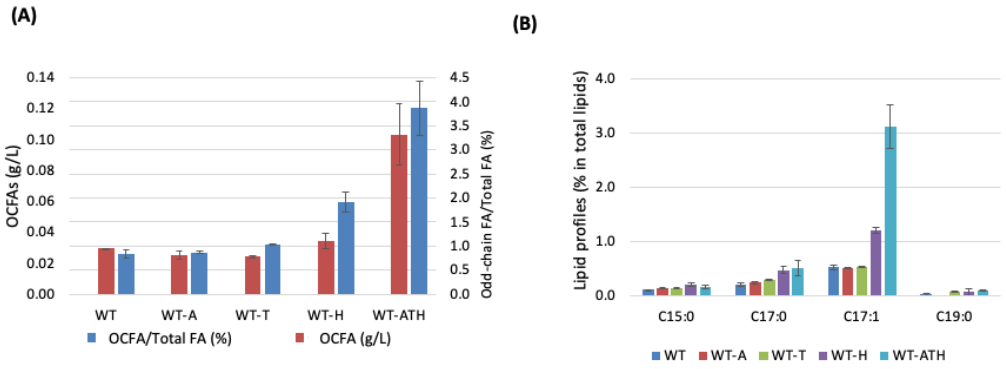


Figure 6.2. OCFA production and profiles for the wild-type and engineered strains.

(A) OCFAs titers (g/L) and the percentage of OCFAs relative to total FAs in the wild-type (WT) and engineered strains. (B) OCFAs profiles (percentage of each FA out of total FAs) of the WT strain and engineered strains. WT: control strain; WT-A: strain expressing module A; WT-T: strain expressing module T; WT-H: strain expressing module H; WT-ATH: strain expressing the full pathway (all three modules). The results represent the means and standard deviations for two independent experiments.

Table 6.2. Fatty acid (FA) production in the wild-type (WT) strain and the engineered strains after growth in YNBD6 medium for 120 hr. The values represent the means and standard deviations for two independent experiments. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipid titer (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
WT	18.65 ± 0.15	19.13 ± 2.22	0.16 ± 0.00	0.84 ± 0.09	3.571 ± 0.442	0.029 ± 0.000
WT-A	17.30 ± 0.15	16.60 ± 1.05	0.14 ± 0.01	0.87 ± 0.03	2.873 ± 0.207	0.025 ± 0.003
WT-T	16.50 ± 0.00	14.41 ± 0.21	0.15 ± 0.00	1.03 ± 0.01	2.378 ± 0.035	0.024 ± 0.001
WT-H	16.30 ± 0.55	10.96 ± 0.09	0.21 ± 0.03	1.91 ± 0.21	1.788 ± 0.075	0.034 ± 0.005
WT-ATH	16.90 ± 0.25	15.73 ± 0.95	0.61 ± 0.13	3.86 ± 0.57	2.656 ± 0.121	0.103 ± 0.020

6.2.3. Engineering strain for higher accumulation of lipids

Once we had determined that the strain overexpressing the full modular pathway produced more OCFAs, the further engineering of the strain was carried out to boost the lipid

accumulation. Previously, we had constructed an obese strain (JMY3501) by inhibiting TAG degradation and remobilization and strengthening TAG biosynthesis *via* push and pull strategy [Lazar *et al*. 2014; Beopoulos *et al*. 2008; Dulermo *et al*. 2013; Delermo and Nicaud, 2011; Tai and Stephanopoulos, 2012]. Therefore, using JMY3501, we built a new obese strain that overexpressed our full modular pathway. It was called the obese-ATH strain. We then studied lipid production under the same conditions as before. As expected, total lipid accumulation was 2.29-fold greater in the obese-ATH strain than in the WT-ATH strain (Table 6.2 and 6.3). Interestingly, the obese-ATH strain also accumulated more OCFAs: the percentage of OCFAs out of total FAs was 5.64% in the obese-ATH strain *versus* 3.86% in the WT-ATH strain. The obese-ATH strain produced 0.36 g/L of OCFAs, which is 7.2 times greater than the amount produced by the regular obese strain. The obese-ATH strain and the WT-ATH strain differed in their even-chain FA profiles (Table 6.4). The obese-ATH had slightly higher levels of C16:0 and slightly lower levels of C18:1, a common pattern seen in strains with the obese background regardless of the carbon source [Lazar *et al*. 2014; Ledesma-Amaro *et al*. 2016].

Table 6.3. Fatty acid (FA) production in the obese strain and the obese-ATH strain after growth in YNBD6 medium for 120 hr. The values represent the means and standard deviations for two independent experiments. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipid titer (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
Obese	19.20 ± 0.04	37.11 ± 0.14	0.25 ± 0.00	0.68 ± 0.01	7.125 ± 0.012	0.049 ± 0.001
Obese-ATH	17.62 ± 0.03	36.02 ± 0.39	2.03 ± 0.05	5.64 ± 0.06	6.347 ± 0.058	0.358 ± 0.009

Table 6.4. Comparison of the lipid profiles (% of each FA) of the WT-ATH strain and the obese-ATH strain. The values represent the means and standard deviations for two independent experiments.

Strain	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C19:0
WT-ATH	0.16 ± 0.03	8.31 ± 0.75	6.13 ± 0.00	0.50 ± 0.14	3.11 ± 0.40	5.96 ± 0.68	61.31 ± 2.43	11.02 ± 0.26	0.10 ± 0.00
Obese-ATH	0.40 ± 0.00	13.07 ± 0.01	7.25 ± 0.11	0.76 ± 0.00	4.26 ± 0.06	5.00 ± 0.17	50.12 ± 0.27	14.11 ± 0.16	0.22 ± 0.13

6.2.4. The disruption of *PHD1* for increased OCFA production

In a previous study (part 4.2), we inactivated *PHD1*, the gene that encodes 2-methyl citrate dehydratase, which catalyzes the conversion of 2-methyl citrate to 2-methyl-*cis*-aconitate in the methyl citrate cycle; we showed that the resulting higher levels of propionyl-CoA could be used to synthesize greater amounts of OCFAs [Park *et al*. 2018]. To investigate whether the inhibition of the methyl citrate cycle—*via* the deletion of *PHD1*—could further improve the accumulation of OCFAs, we disrupted the *PHD1* gene in both the WT-ATH strain and the obese-ATH strain. To disrupt *PHD1*, the cassettes were constructed to include a promoter

(pPHD1), a marker (*URA3* or *LEU2*), and a terminator (TPHD1), which allowed the ORF gene to be removed *via* homologous recombination [Fickers et al. 2003; Papanikolaou *et al*. 2013].

The two *phd1Δ* strains displayed higher total lipid content compared to their relative controls (Table 6.5), a result that was also demonstrated in previous study [Papanikolaou *et al*. 2013]; however, they also displayed lower percentages of OCFAs out of total FAs (Table 6.5). This latter negative effect was significantly more pronounced in the obese-ATH *phd1Δ* strain than in the WT-ATH *phd1Δ* strain. For the obese-ATH *phd1Δ* strain, the ratio of OCFAs to total lipids dropped by 50%, and levels of OCFAs were 67% of those seen in the relative control (0.24 g/L *versus* 0.36 g/L). These results suggest that disrupting the methyl citrate cycle does not provide the benefits seen previously when strains are already overexpressing the aspartate/α-ketobutyrate pathway.

The action of 2-methyl citrate dehydratase is restricted to the mitochondria. Consequently, disabling this enzyme does not directly improve cytosolic levels of propionyl-CoA. In the previous studies showing that the disruption of *PHD1* boosted OCFAs synthesis [Papanikolaou *et al*. 2013; Park *et al*. 2018], there was also propionate supplementation. Taken together, these findings suggest that, when the methyl citrate cycle is disrupted, the overexpression of the aspartate/α-ketobutyrate pathway has a weaker effect on propionyl-CoA levels than does propionate supplementation.

Table 6.5. Comparison of fatty acid (FA) production in the *PHD1*-disrupted strains and their relative controls after growth in YNBD6 for 120 hr. The values represent the means and standard deviations for two independent experiments. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipid titer (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
WT-ATH	14.03 ± 0.47	17.96 ± 0.16	0.65 ± 0.01	3.59 ± 0.02	2.519 ± 0.106	0.091 ± 0.004
WT- ATH *phd1*Δ	13.46 ± 0.07	18.46 ± 0.39	0.57 ± 0.01	3.10 ± 0.11	2.484 ± 0.065	0.077 ± 0.001
Obese-ATH	17.62 ± 0.03	36.02 ± 0.39	2.03 ± 0.05	5.64 ± 0.20	6.347 ± 0.058	0.358 ± 0.009
Obese-ATH *phd1*Δ	19.33 ± 0.02	43.37 ± 0.05	1.22 ± 0.02	2.81 ± 0.05	8.384 ± 0.018	0.236 ± 0.004

6.2.5. Overexpression of the cytosolic pyruvate dehydrogenase (PDH) complex for increased OCFA production

The PDH complex consists of three main catalytic components: E1 (pyruvate dehydrogenase, encoded by *PDA1* and *PDB1*), E2 (dihydrolipoamide acetyltransferase, encoded by *LAT1*), and E3 (dihydrolipoamide dehydrogenase, encoded by *LPD1*). There is a fourth component (protein X, encoded by *PDX1*) that binds to positions E3 relative to E2. In *Y. lipolytica*, the PDH complex is found in mitochondria and catalyzes the conversion of pyruvate to acetyl-CoA. A few studies have examined the functional expression of the PDH complex in *Y. lipolytica*. One study attempted to overexpress the direct pathway from pyruvate to acetyl-CoA in a coordinated manner [Markham *et al*. 2018], and another study showed that the individual

overexpression of *PDA1* (shared subunits with α-ketoglutarate dehydrogenase) improved α-ketoglutarate production [Guo *et al*. 2014].

Here, we wanted to redirect the aspartate/α-ketobutyrate pathway to produce propionyl-CoA *via* the PDH complex, which has already been shown to be possible in *E. coli* [Danchin *et al*. 1984; Lee *et al*. 2013; Tseng and Prather, 2012]. The PDH complex was built with three plasmids, so it could be associated with the full modular pathway (ATH) and could provide a pool of propionyl-CoA in the cytosol where lipid synthesis takes place. The mitochondrial targeting sequences (MTSs) of each gene in PDH complex were predicted using MitoProt and removed to construct the cytosolic PDH complex [Claros and Vincens, 1996]. All genes were synthesized and cloned in the plasmid pUC57 by GeneScript Biotech (New Jersey, US). Cytosolic *PDX1* was cloned into the expression plasmid (JME2563) using the BamHI and AvrII restriction sites. The other four genes were cloned into two plasmids (JME4774 and JME4775) using Golden Gate assembly.

We then created the obese-ATHP strain by overexpressing the cytosolic PDH subunits in the obese-ATH strain. Next, to explore lipid accumulation dynamics, the strain was grown in YNBD6 with lipoic acid, which is required for cytosolic PDH activity in yeast [Kozak *et al*. 2014]. Compared to the control strain, the obese-ATHP strain had significantly lower levels of OCFAs (a 3.8 times lower percentage of OCFAs out of total FAs); however, total lipid levels were higher (Table 6.6). The increase in lipid production might have resulted from the increased levels of cytosolic acetyl-CoA coming from the overexpression of the PDH complex. A similar strategy was utilized in *S. cerevisiae*, it was found to increase levels of acetyl-CoA [Kozak *et al*. 2014; Lian *et al*. 2014] and of the target compound of interest. Since acetyl-CoA is a key precursor in the production of both ECFAs and OCFAs, increasing levels of acetyl-CoA promotes lipid synthesis in general. However, the substantially lower levels of OCFAs in the obese-ATHP strain implies that the PDH complex shows greater specificity for pyruvate than for α-ketobutyrate. The higher Km value of α-ketobutyrate compared to that of pyruvate has been seen elsewhere, such as in *E. coli* [Biswanger, 1981], *Neurospora crassa* [Harding *et al*. 1970], and mammalian cells [Bremer, 1969]. Therefore, it is important to explore enzyme engineering strategies that modify substrate specificity or that introduce heterologous enzymes that can convert acetyl-CoA to propionyl-CoA with a view to further improving OCFA production *via* a threonine-based upregulation strategy.

Table 6.6. Fatty acid (FA) production in the obese-ATH strain and the obese-ATHP strain after growth in YNBD6 medium for 120 hr. The values represent the means and standard deviations for two independent experiments. DCW = dry cell weight.

Strain	DCW (g/L)	Lipid content % (g/g DCW)		OCFAs /Total FAs (%)	Lipid titer (g/L)	
		Total FAs	OCFAs		Total FAs	OCFAs
Obese-ATH	15.68 ± 0.02	29.23 ± 0.01	1.59 ± 0.03	5.44 ± 0.10	4.582 ± 0.010	0.249 ± 0.005
Obese-ATHP	15.25 ± 0.25	33.89 ± 1.06	0.48 ± 0.03	1.42 ± 0.03	5.171 ± 0.246	0.073 ± 0.005

6.3. Discussion

In this chapter, a synthetic biological strategy for the *de novo* production of OCFAs in *Y. lipolytica* was applied. It is important to note that the wild-type *Y. lipolytica* strain produces only negligible amounts of OCFAs even though it has an excellent capacity to accumulate large quantities of lipids. Several studies have shown that propionate supplementation can increase the production of OCFAs [Koluchova *et al*. 2015; Fontanille *et al*. 2012; Park *et al*. 2018]. However, research has yet to explore the *de novo* production of OCFAs from sugars in *Y. lipolytica*.

The overexpression of the aspartate/α-ketobutyrate pathway (from oxaloacetate to homoserine and threonine) *via* Golden Gate assembly resulted in higher levels of OCFAs being produced from glucose. The best strain generated a level of OCFAs, 0.36 g/L in flask that is the highest to date to be achieved in *Y. lipolytica* without propionate supplementation. Furthermore, it is comparable to the levels seen in our study (Chapter 4) that employed propionate supplementation, where OCFAs titers were 0.14 g/L and 0.57 g/L in the wild-type strain and the obese strain, respectively [Park *et al*. 2018]. To further increase the amount of propionyl-CoA produced *via* the overexpression of threonine synthesis, we constructed a cytosolic pyruvate dehydrogenase (PDH) complex. Because of the lower specificity of the PDH complex for α-ketobutyrate *versus* pyruvate, the engineered strain generated lower levels of propionyl-CoA than did the relative control; however, the increased levels of acetyl-CoA in the engineered strain led to larger amounts of total FAs. This study is the first to describe the functional expression of the native PDH complex in the cytosol in *Y. lipolytica*, an approach that could also be employed to produce acetyl-CoA-derived compounds, such as polyhydroxybutyrates, isoprenoids, sterols, polyketides, polyphenols, alkanes, and alkenes [Nielsen, 2014].

In order to achieve the considerable progress on OCFA production, certain issues must be resolved. It is necessary to perform researches on the identification of bottlenecks and the analysis of metabolic fluxes to propionyl-CoA and OCFA by combinatorial pathway analysis [Lütke-Eversloh and Stephanopoulous, 2008], targeted-proteomics analysis [Redding-Johanson *et al*. 2011], and genome-scale metabolic network modeling [Xu *et al*. 2011]. The results of the PDH overexpression experiment suggest that acetyl-CoA is a competitive precursor to propionyl-CoA in OCFAs synthesis and is also a precursor in lipid synthesis which is consistent with the result in acetate supplementation experiment in the part 5.4 and Figure 5.13. Therefore, better balancing the pools between acetyl-CoA and propionyl-CoA could be a key strategy in further increasing OCFAs content. One way to improve OCFAs synthesis is to introduce enzymes—such as CoA transferase—that redirect CoA moieties from acetyl-CoA to propionyl-CoA [Yang *et al*. 2012] or that have greater specificity for 3-oxovaleryl-ACP than for acetoacetyl-CoA [Slater *et al*. 1998].

CHAPTER 7. CONCLUSION AND PERSPECTIVES

In order to make *Y. lipolytica* as a promising chassis for biotechnological applications, various efficient genetic tools are necessary. In this thesis, the identification and characterization of inducible promoters were explored. The CRMs for *EYK1* and *EYD1* were identified and a set of inducible promoters was constructed as a form of biobricks that is compatible to Golden Gate assembly system of *Y. lipolytica*. The series of hybrid promoters composed of multiple repeat of UAS, mutated UAS, and hybridized with different core promoter was shown the various induction level by erythritol with a range from 2.2- to 32.3–fold in WT strain and from 2.9- to 896.1–fold in the *eyk1Δ* strain. These new promoters that respond to erythritol will improve the capability of modulating gene expression in metabolic engineering and protein production. As a proof of concept, the newly constructed inducible promoters were validated by the expression of lipase CalB. Through comparison of gene expression and protein production of each promoters, pEYK1-3AB was shown to be the most appropriate promoter for lipase production in our experimental condition. A high amount of lipase, 45,000 U mL^{-1}, was obtained in batch bioreactor, which represents to date the most efficient process identified for CalB production in yeast.

The production of odd-chain fatty acids (OCFA) in *Y. lipolytica* was investigated in this thesis. OCFAs are a type of valuable lipids with various applications: biomarkers, pharmaceuticals, and intermediates in the production of flavor and fragrance compounds, fuels, and plasticizers. The utilization of propionate is very important for OCFA synthesis in that it provides a key precursor of OCFA, propionyl-CoA. The toxicity of propionate in *Y. lipolytica* was investigated and the propionate-tolerant genes were identified through genomic library screening. Two genes, *RTS1* and *MFS1*, were characterized and shown to confer higher tolerance to propionate.

Metabolic engineering strategy to increase OCFA production from propionate was explored in this thesis. The inhibition of propionyl-CoA catabolism by deleting *PHD1* showed the increase in OCFA accumulation from 28.3% to 46.8% (OCFA in total lipids) in WT strain. We further engineered the strain to accumulate more lipids in general which also make an increase of OCFA accumulation, the production of OCFAs reached to 0.57 g/L. The co-feeding of substrates, glucose and propionate, was shown to be effective for OCFA production by alleviating the toxicity of propionate. The engineering of precursor pools, specifically propionyl-CoA for OCFA synthesis, was explored by introducing heterologous propionate-activating enzymes. The best performing strain (*Repct* overexpression) was able to accumulate up to 53.2% of total lipids as OCFAs, 3.8 times higher than WT strain, in acetate supplemented condition. It was revealed that balancing two precursors, propionyl-CoA and acetyl-CoA, is crucial criteria to improve OCFA production. The further engineering to accumulate high amounts of TAG and to boost the five-carbon precursor, β-ketovaleryl-CoA, by overexpressing *RebktB* promoted OCFA production up to 1.87 g/L representing 62% of

total lipids, which is the highest recombinant OCFA titer reported in bacteria and yeast, to date.

De novo production of OCFAs without providing propionate was also explored in this thesis. The overexpression of threonine up-regulating pathway resulted in the increase of OCFA production by 12 times in the engineered strain compared to WT strain from glucose as a sole carbon source (0.36 *vs.* 0.03 g/L). This result highlights the possibility of using low-cost substrate for the production of OCFAs in *Y. lipolytica*. (All metabolic engineering approaches for OCFA production investigated in this thesis are summarized in Figure 7.1).

Not only for OCFAs, boosting precursors for lipids in general was also investigated. The most general and important precursor, acetyl-CoA, can be synthesized through several pathways. In this study, *ACS2* encoding acetyl-CoA synthetase was overexpressed, but it did not show a significant improvement on lipid production. Similarly, the co-expression of *ACS2* with *ACC1* encoding acetyl-CoA carboxylase to improve malonyl-CoA neither showed the difference on lipid accumulation in our experimental condition. It was confirmed that the effect of *ACC1* overexpression on lipid accumulation was highly dependent on the genotype of the strain and the condition of cultivation such as substrate concentration and C/N ratio. Thorough studies on the regulation of these enzymes systemically with different strains and conditions are necessary in order to engineer the strain to produce lipid with high yield.

In brief, *Y. lipolytica* has been successfully developed as a promising chassis, especially for the production of OCFAs. This study paves a way for the microbial production of OCFAs and its derivatives having high potential in the pharmaceutical, cosmetics, food, and chemical industry.

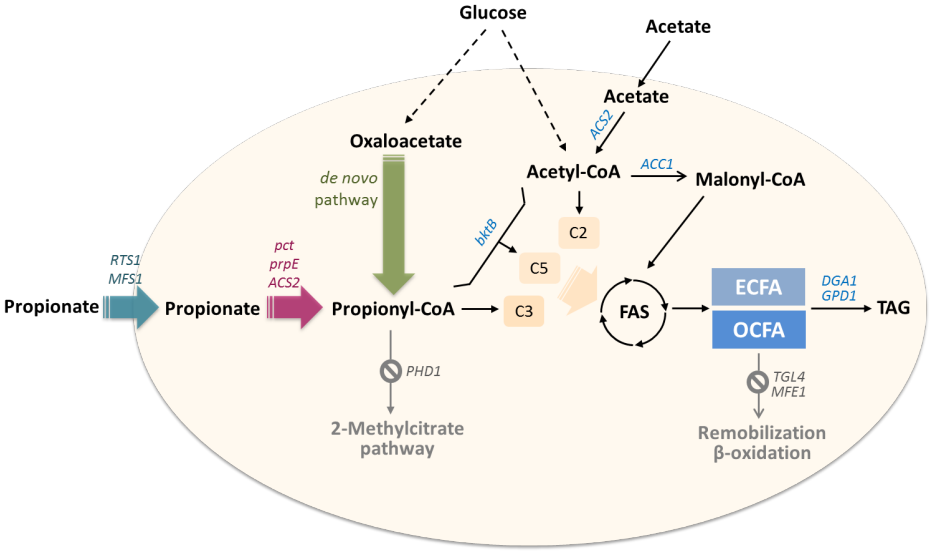


Figure 7.1. Summary of metabolic engineering strategies used in this study.

In order to advance this study and develop the microbial production of OCFAs at industrial level, several different strategies are necessary to be applied. The precursors of lipids are indicated as C2, C3, and C5 in light orange boxes. ECFA, even-chain fatty acid; OCFA, odd-chain fatty acid; TAG, triacylglycerol.

From metabolic engineering, the C17:1-rich OCFAs are produced in *Y. lipolytica* in this thesis. The effect of OCFAs on cell metabolism and morphology need to be further studied which is necessary for identifying the limitation of OCFA titer and applying to next strain engineering and fermentation optimization. The structure of OCFAs and composition of TAG produced in the engineered *Y. lipolytica* strain together with the anlaysis of their biochemical and rheological properties also required to broaden their applications. These fundamental researches on OCFA will help to establish novel and efficient strategy for OCFA production at higher level.

Since the knowledge of propionate and propionyl-CoA metabolisms in *Y. lipolytica* is still limited, the identification of the relative pathways and the genes which are involved by system biological approach may provide more understandings and clues to improve the chassis strain. This will be helpful not only to identify rate-limiting steps of OCFA synthesis but also to understand the regulation mechanism by intermediates or products and the balancing mechanism between the precursor pools in OCFA production phase. From genomic library screening in this thesis, three genes (*MFS1*, *MFS2*, and *MFS3*) which have homolog to major facilitator superfamily were identified as the propionate-tolerant gene. Further study on MFS family such as biochemical analysis, site-specific mutation, and adaptive evolution will be helpful to understand the function of these enzymes and the propionate metabolism in *Y. lipolytica*.

As shown in our result of obese strains engineered to accumulate more lipids, total lipid production also should be improved in order to increase OCFA production. For this, balancing redox metabolism as described in part 1.2.4.2.4. or higher expression of target genes which are known to be effective on lipid accumulation by multi-copy expression or synthetic/inducible promoters developed in this thesis will be potential strategies.

In this thesis, it is verified that propionyl-CoA can be used as a priming subunit of FAS for OCFA synthesis in *Y. lipolytica*. This is in line with the previous studies showed that acyl-chain of different chain lengths as well as functionalized intermediates (keto, hydroxy, or enoyl) could serve as priming substrates of FAS [Pirson et al. 1973; Ingram et al. 1977]. It is plausible that using substrates with odd-numbered, branched, and hydroxy carbon chains might be used for the synthesis of various FA-derivatives. However, detailed studies on fatty acid synthesis with different substrates in *Y. lipolytica* which can prove the incorporation of unusual substrates into native FAS system are still limited. More specific biochemical and structural studies on FAS domains with β-ketovaleryl-CoA or valeryl-CoA are necessary for better understanding [Lomakin et al. 2007; Leibundgut et al. 2008].

Also, the production of OCFA derivatives by metabolic engineering is expected based on previous studies. The chain length of OCFAs can be modified by engineering FAS or elongase to produce medium-chain OCFAs or very long-chain OCFAs [Rigouin *et al*. 2018]. The unusual OCFAs also can be produced by engineering FAS termination enzyme or introducing heterologous enzyme catalyzing acyl-CoA to specific compound as described in part 1.2.4.3. For this, the study on biochemical and physical properties of OCFAs should be investigated to

specify the target compound of interest. Though some specific OCFAs are already known to have positive effects on pharmaceutical purpose or chemical industry, the information is still limited to a few compounds, the more thorough studies on thermal, rheological, and biochemical properties are required for generating the platform of OCFA derivatives can be produced by metabolic engineering.

Even when bioconversion yields are high, biotechnology processes may be economically unviable because of the high and/or unstable cost of common substrates [Ledesma-Amaro and Nicaud, 2016a]. It is analyzed that the percentage of lipid content and the substrate's cost significantly influence the overall yield, productivity, and fermentation cost [Kamineni and Shaw, 2020]. Thus, the utilization of low-cost substrates is necessary to develop the OCFA production as economically feasible process. One possible way is the utilization of raw materials composed of sugars, for example, lignocellulose, starch, molasses, inulin, and so on. Because there are several successful studies on engineering *Y. lipolytica* to broaden substrate ranges as described in part 1.2.1.5, the OCFA production from low-cost substrates will be possible by combining the metabolic pathway of utilizing specific substrate and propionate supplementation. It should be noted that there will be several issues to be investigated such as the inhibition between each substrate, the ratio of substrates, and the feeding strategies of the substrates. These issues should be considered together with the optimization of fermentation conditions. Since the lipid production is highly depended on fermentation condition (e.g. medium composition, C/N ratio, oxygen supply, *etc*.), the optimization of fermentation condition is essentially necessary to improve OCFA production. Apart from sugar-based substrates, volatile fatty acids (VFAs) obtainable from agro-industrial wastes will be promising substrates for OCFA production. VFAs are mostly composed of acetate, propionate, and butyrate with various ratio depending on the origin and the production process, which are well matched with the substrate combination for OCFA production used in this thesis. Besides, the utilization of VFAs for microbial lipid production was recently shown to be successful in several studies which make the OCFA production from VFAs more promising [Vajpeyi *et al*. 2015; Bhatia *et al*., 2019b; Gao *et al*.2017; Llamas *et al*. 2019].

These multidisciplinary approaches on OCFAs from development of chassis strain to optimization of production process and characterization of properties will accelerate the industrialization of microbial OCFA production.

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APPENDIX A. STRAIN LIST

Chapter 3. Development of synthetic biological tools – synthetic inducible promoter

Strain	Genotype or description	Reference
E. coli		
DH5**α**	Φ80*lacZ*Δm15 Δ(*lacZ*YA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Promega
pUC57	GeneScript Biotech donor vector	GeneScript Biotech
GGE114	pSB1A3-ZetaUP-URA3-RFP-ZetaDOWN	Celińska *et al*. 2017
GGE077	pCR4Blunt-TOPO-G1-RedStarII	Celińska *et al*. 2017
GGE020	pCR4Blunt-TOPO-T1-3Lip2	Celińska *et al*. 2017
GGE085	pCR4Blunt-TOPO-pTEF1	Celińska *et al*. 2017
GGE238	pCR4Blunt-TOPO-pEYK1	This study
GGE0130	pCR4Blunt-TOPO-pEYK1-2AB	This study
GGE0104	pCR4Blunt-TOPO-pEYK1-3AB	This study
GGE0132	pCR4Blunt-TOPO-pEYK1-4AB	This study
GGE250	pCR4Blunt-TOPO-pEYK1-5AB	This study
GGE140	pCR4Blunt-TOPO-pEYD1AB	This study
GGE172	pCR4Blunt-TOPO-pEYD1A*B	This study
GGE174	pCR4Blunt-TOPO-pEYD1AB*	This study
JME547	php4d-Cre_Hyg	Fickers *et al*. 2003
JME1046	JMP62-*URA3* ex-pTEF	Nicaud *et al*. 2002
JME3267	PUT *lys5*::*URA3ex*	This study
JME3739	JMP62-*URA3 ex-pTEF*-CalB	This study
JME4001	JMP62-URA3 ex-pHU8EYK-YFP	Vandermies *et al*. 2017
JME4056	PUT of EYK1 (RIE124)	Vandermies *et al*. 2017
JME4123	pUC57-*pEYK1-3AB*	Trassaert *et al*. 2017
JME4230	JMP62-*URA3 ex-pHU8EYK*	This study
JME4243	JMP62-*URA3 ex-pHU8EYK* CalB	This study
JME4265	pTEF-EYK1_hp4d-Cre (RIE132)	Vandermies *et al*. 2017
JME4266	JMP62-*URA3 ex-pEYK1-3AB*	This study
JME4365	JMP62-*URA3 ex-pEYK1-3AB*-CalB	This study
JME4417	pUC57-EYK1-4AB-coreTEF	This study
JME4418	pUC57-EYK1-4AB-R1-coreTEF	This study
JME4419	pUC57-EYK1-4AB-R2-coreTEF	This study
JME4420	pUC57-EYK1/EYD1A-coreEYK1	This study
JME4421	pUC57-EYK1/EYD1A-coreTEF	This study
JME4422	pUC57-EYK1/EYD1B-coreEYK1	This study
JME4423	pUC57-EYK1/EYD1B-coreTEF	This study
JME4579	JMP62-*LYS5 ex-pEYK1-3AB*-CalB	This study
JME4590	JMP62-*URA3 ex-pEYD1*-CalB	This study

Strain	*Genotype or description*	Reference
Y. lipolytica		
JMY195	*Po1d, MATA ura3-302 leu2-270 xpr2-322*	Barth and Gaillardin, 1996
JMY2900	Po1d, *URA3 LEU2*	Barth and Gaillardin, 1996
JMY1212	Po1d *lip2Δ lip7Δ lip8Δ LEU2*-ZETA	Emond *et al*. 2010
JMY5207	JMY1212 *lys5::URA3 ex*	Soudier *et al*. 2019
JMY7121	JMY1212 *lys5Δ*	Soudier *et al*. 2019
JMY7123	JMY1212 *lys5Δ eyk1::URA3 ex*	Soudier *et al*. 2019
JMY7126	JMY1212 *lys5Δ eyk1Δ*	Soudier *et al*. 2019
JMY7536	JMY7126 + *pTEF*-CalB-*URA3 ex*	This study
JMY7539	JMY7126 + *pEYK1-3AB*-CalB-*URA3 ex*	This study
JMY7544	JMY7126 + *pHU8EYK*-CalB-*URA3 ex*	This study
JMY7548	JMY7126 + *pEYD1*-CalB-*URA3 ex*	This study
JMY7989	JMY7539 + *LYS5 ex*	This study
JMY7990	JMY7536 + *LYS5 ex*	This study
JMY7991	JMY7126 + *pEYK1-3AB*-CalB-*URA3 ex* + *pEYK1-3AB*-CalB-*LYS5 ex*	This study

Chapter 4. Production of odd-chain fatty acids (OCFAs)

Strain	Genotype or description	Reference
E. coli		
MGM	pINA240	Barth and Gaillardin, 1996
JME4010	pINA240-Pool 1	Fournier *et al*. unpublished
JME4014	pINA240-Pool 2	Fournier *et al*. unpublished
JME4018	pINA240-Pool 3	Fournier *et al*. unpublished
JME4022	pINA240-Pool 4	Fournier *et al*. unpublished
JME4026	pINA240-Pool 5	Fournier *et al*. unpublished
JME4030	pINA240-Pool 6	Fournier *et al*. unpublished
JME4034	pINA240-Pool 7	Fournier *et al*. unpublished
JME4038	pINA240-Pool 8	Fournier *et al*. unpublished
JME4042	pINA240-Pool 9	Fournier *et al*. unpublished
JME4046	pINA240-Pool 10	Fournier *et al*. unpublished
JME2563	JMP62-*LEU2* ex-pTEF	Dulermo *et al*. 2017

JME4569	JMP62-*LEU2* ex-pTEF-*MFS1i*	This study
JME4596	JMP62-*LEU2* ex-pTEF-*RTS1i*	This study
Y. lipolytica		
JMY2900	Po1d *URA3 LEU2*	Dulermo *et al*. 2014
JMY3776	Po1d *phd1Δ mfe1Δ tgl4Δ* +pTEF-*DGA2-LEU2* ex + pTEF-*GPD1-URA 3*ex	Park *et al*. 2018
JMY7228	Po1d *phd1Δ mfe1Δ tgl4Δ* +pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex	This study
JMY7264	Po1d *phd1Δ mfe1Δ tgl4Δ* +pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex + *LEU2*	This study
JMY7588	JMY7228 + pINA240-*RTS1r*	This study
JMY7589	JMY7228 + pINA240-*MFS1r*	This study
JMY7567	JMY7228 pTEF-*RTS1i-LEU2* ex	This study
JMY7569	JMY7228 pTEF-*MFS1i-LEU2* ex	This study

Chapter 5. Engineering precursor pools for increasing lipids production

Strain	Genotype of description	Abbreviation	Reference
E. coli			
JME1046	JMP62-*URA3 ex*-pTEF		Nicaud *et al*. 2002
JME1841	JMP62-*LEU2 ex*-pTEF-*DGA1*		Gajdos *et al*. 2016
JME2408	JMP62-*URA3 ex* + pTEF-*YlACC1cyto*		unpublished
JME2563	JMP62-*LEU2 ex*-pTEF		Dulermo *et al*. 2014
JME4476	pTOPO-*YlACC1*		This study
JME4551	pTOPO-*YlACC1* 1*(S659A)		This study
JME4553	pTOPO-*YlACC1* 2*(S1157A)		This study
JME4552	JMP62-*URA3* ex-pTEF-*YlACC1* 1*(S659A)		This study
JME4554	JMP62-*URA3* ex-pTEF-*YlACC1* 2* (S1157A)		This study
JME4560	JMP62-*URA3* ex-pTEF-*YlACC1* 1*2*(S659A, S1157A)		This study
JME4066	JMP62-*URA3* ex-pTEF-*ACS2* cyto		This study
JME4666	JMP62-*LEU2* ex-pTEF-*ACS2* cyto		This study
JME1046	JMP62-*URA3* ex		Nicaud *et al*. 2002
JME2563	JMP62-*LEU2* ex		Dulermo *et al*. 2017
JME4066	JMP62-*URA3* ex-pTEF-*YlACS2*		Dusseaux *et al*. 2017
JME4067	JMP62-*URA3* ex-pTEF-*CpPCT*		Dusseaux *et al*. 2017
JME4068	*JMP62-URA3* ex-pTEF-*EnPCT*		Dusseaux *et al*. 2017
JME4069	JMP62-*LEU2* ex-pTEF-*EcPCT*		Dusseaux *et al*. 2017
JME4070	JMP62-*URA3* ex-pTEF-*RePCT*		Dusseaux *et al*. 2017
JME4174	JMP62-*LEU2* ex-pTEF-*EcPrpE*		This study
JME4175	JMP62-*URA3* ex-pTEF-*SePrpE*		This study
JME4667	JMP62-*LEU2* ex-pTEF-*CpPCT*		This study
JME4669	JMP62-*LEU2* ex-pTEF-*RePCT*		This study

JME4670	JMP62-*LEU2* ex-pTEF-*SePrpE*		This study
JME5219	JMP62-*Hygro* ex-pTEF-*ReBktB*		This study
Y. lipolytica			
JMY195	*MATa ura3-302 leu2-270 xpr2-322*		Barth and Gaillardin. 1997
JMY2900	JMY195 *URA3 LEU2*		Dulermo *et al*. 2014
JMY3371	*pox1-6Δ tgl4Δ* +pTEF-*DGA1-LEU2* ex		Unpublished
JMY7497	JMY195 + pTEF-*ACC1*cyto-*URA3* ex		This study
JMY7500	JMY195 + pTEF-*ACC1* (1*) cyto-*URA3* ex		This study
JMY7502	JMY195 + pTEF-*ACC1* (2*) cyto-*URA3* ex		This study
JMY7505	JMY195 + pTEF-*ACC1* (1*2*) cyto-*URA3* ex		This study
JMY7512	Y7500 + *LEU2*	WT-*ACC1* 1*	This study
JMY7514	Y7502 + *LEU2*	WT-*ACC1* 2*	This study
JMY7517	Y7505 + *LEU2*	WT-*ACC1* 1*2*	This study
JMY7590	Y195+pTEF-*DGA1-Leu2* ex	*DGA1*	This study
JMY7593	Y195+pTEF-*DGA1-Leu2* ex+*URA3*	*DGA1*	This study
JMY7596	Y7497 + pTEF-*DGA1-Leu2* ex	*DGA1-ACC1*	This study
JMY7599	Y7500 + pTEF-*DGA1-Leu2* ex	*DGA1-ACC1* 1*	This study
JMY7602	Y7502 + pTEF-*DGA1-Leu2* ex	*DGA1-ACC1* 2*	This study
JMY7605	Y7505 + pTEF-*DGA1-Leu2* ex	*DGA1-ACC1* 1*2*	This study
JMY7608	*pox1-6Δ tgl4Δ* +pTEF-*DGA1-LEU2* ex + *URA3*	Obese	This study
JMY7609	Y3371 + pTEF-*ACC1*cyto-*URA3* ex	Obese-*ACC1*	This study
JMY7612	Y3371 + pTEF-*ACC1*(1*)cyto-*URA3* ex	Obese-*ACC1* 1*	This study
JMY7615	Y3371 + pTEF-*ACC1*(2*)cyto-*URA3* ex	Obese-*ACC1* 2*	This study
JMY7618	Y3371 + pTEF-*ACC1*(1*2*)cyto-*URA3* ex	Obese-*ACC1* 1*2*	This study
JMY6962	JMY195 pTEF-*YlACS2-URA3* ex + *LEU2*		This study
JMY6965	JMY195 pTEF-*CpPCT-URA3* ex + *LEU2*		This study
JMY6969	JMY195 pTEF-*EnPCT-URA3* ex + *LEU2*		This study
JMY6971	JMY195 pTEF-*EcPCT-LEU2* ex + *URA3*		This study
JMY6974	JMY195 pTEF-*RePCT-URA3* ex + *LEU2*		This study
JMY6979	JMY195 pTEF-*EcPrpE-LEU2* ex + *URA3*		This study
JMY6981	JMY195 pTEF-*SePrpE-URA3* ex + *LEU2*		This study
JMY7228	*phd1Δ mfe1Δ tgl4Δ* +pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3* ex	Obese-L	Park and Nicaud, 2019
JMY7775	JMY7228 + *LEU2* ex	Obese-L	This study
JMY7778	JMY7228 + pTEF-*CpPCT-LEU2* ex	Obese-LP	This study
JMY7780	JMY7228 + pTEF-*RePCT-LEU2* ex	Obese-LP	This study
JMY7782	JMY7228 + pTEF-*SePrpE-LEU2* ex	Obese-LP	This study
JMY8438	JMY7228 + pTEF-*RePCT-LEU2* ex + pTEF-*ReBktB-Hygro* ex	Obese-LPB	This study

Chapter 6. *De novo* production of OCFAs from glucose

Strain	Geenotype or description	Abbreviation	Reference
E. coli			
GGE0004	TOPO-P2-TEF		Celińska *et al*. 2017
GGE0009	TOPO-P3-TEF		Celińska *et al*. 2017
GGE0014	TOPO-T1-LIP2		Celińska *et al*. 2017
GGE0015	TOPO-T2-LIP2		Celińska *et al*. 2017
GGE0020	TOPO-T1-3-LIP2		Celińska *et al*. 2017
GGE0021	TOPO-T2-3-LIP2		Celińska *et al*. 2017
GGE0028	pSB1C3		Celińska *et al*. 2017
GGE0029	pSB1A3		Celińska *et al*. 2017
GGE0038	TOPO-ZetaDOWN-NotI		Celińska *et al*. 2017
GGE0067	TOPO-ZetaUP-NotI		Celińska *et al*. 2017
GGE0081	TOPO-T3-LIP2		Celińska *et al*. 2017
GGE0082	TOPO-P1-TEF		Celińska *et al*. 2017
GGE0085	TOPO-M-*URA3* ex		Celińska *et al*. 2017
GGE0376	TOPO-*AAT2*		This study
GGE0377	TOPO-*THR1*		This study
GGE0378	TOPO-*THR4*		This study
GGE0379	pJET-*ILV1*		This study
GGE0380	TOPO-*HOM3*		This study
GGE0381	TOPO-*HOM2*		This study
GGE0382	pJET-*HOM6*		This study
JME0547	pUC-*Cre*		Fickers *et al*. 2003
JME0740	pGEM-T-*PHD1* PUT		Papanikolaou *et al*. 2013
JME1811	pGEM-T-*PHD1* PLT		Papanikolaou *et al*. 2013
JME2563	JMP62-*LEU2* ex-pTEF		Dulermo *et al*. 2017
JME4478	GGV-*URA3* ex-pTEF-*AAT2*	Module A	This study
JME4479	GGV-*URA3* ex-pTEF-*THR1*-pTEF-*THR4*-pTEF-*ILV1*	Module T	This study
JME4632	GGV-*URA3* ex-*HOM3-HOM2-HOM6*	Module H	This study
JME4774	GGV-*URA3* ex-*ylPDA1-ylPDB1*	Module P	This study
JME4775	GGV-*URA3* ex-*ylLPD1-ylLAT1*	Module P	This study
JME4776	JMP62-*LEU2* ex-*ylPDX1*	Module P	This study

Y. lipolytica

JMY195	MATa *ura3-302 leu2-270 xpr2-322*	wt	Barth and Gaillardin. 1996
JMY2900	JMY195 *URA3 LEU2*	wt	Dulermo *et al*. 2014
JMY7201	JMY195 + GGV-*AAT2-URA3* ex	wt-A	This study
JMY7202	JMY195 + GGV-*AAT2*	wt-A	This study
JMY7639	JMY195 + GGV-*AAT2-URA3* ex + *LEU2*	wt-A	This study
JMY7203	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1-URA3* ex	wt-AT	This study
JMY7204	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1*	wt-AT	This study
JMY7353	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex	wt-ATH	This study
JMY7357	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	wt-ATH	This study
JMY7374	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex + *phd1::LEU2* ex	wt-ATH *phd1Δ*	This study
JMY7828	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6*	wt-ATH	This study
JMY7640	JMY195 + GGV-*THR1-THR4-ILV1-URA3* ex	wt-T	This study
JMY7643	JMY195 + GGV-*THR1-THR4-ILV1-URA3* ex + *LEU2*	wt-T	This study
JMY7646	JMY195 + GGV-*HOM3-HOM2-HOM6-URA3* ex	wt-H	This study
JMY7649	JMY195 + GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	wt-H	This study
JMY7822	Y195+*ylPDH*cyto	wt-P	This study
JMY7824	Y195ATH+*ylPDH*cyto	wt-ATHP	This study
JMY3501	*pox1-6Δ tgl4Δ* pTEF-*DGA2-LEU2* ex pTEF-*GPD1-URA3* ex	obese	Lazar *et al*. 2014
JMY3820	*pox1-6Δ tgl4Δ* pTEF-*DGA2* pTEF-*GPD1*	obese	Lazar *et al*. 2014
JMY7206	JMY3820 + GGV-*AAT2-URA3* ex	obese-A	This study
JMY7207	JMY3820 + GGV-*AAT2*	obese-A	This study
JMY7208	JMY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1-URA3* ex	obese-AT	This study
JMY7267	JMY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1*	obese-AT	This study
JMY7412	MY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* + GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	obese-ATH	This study
JMY7413	JMY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* + GGV-*HOM3-HOM2-HOM6*	obese-ATH	This study
JMY7414	JMY7413 + *phd1::LEU2* ex	obese-ATH *phd1Δ*	This study
JMY7417	JMY7413 + *phd1::LEU2* ex + URA3	obese-ATH *phd1Δ*	This study
JMY7826	Y3820ATH+*ylPDH*cyto	obese-ATHP	This study

APPENDIX B. PRIMER LIST

Chapter 3. Development of synthetic biological tools – synthetic inducible promoter

Name	Sequence (5' to 3')	Purpose
P1 TEF FW	GGTCTCTACGGGGGTTGGCGGCG	Amplification for building block construction
P1 TEF RV	GGTCTCTCATTCTTCGGGTGTGAGTTAC	
P1 EYK FW	GGTCTCTACGGCCCATCGATGGAAACCTTAATAGGAGACTACTTCC	
P1 EYK RV	GGTCTCTCATTGGATCCAGTAGATGTGTAAGTG	
P1 EYD FW	GGGGGGTCTCTACGGCCCATCGATGGAAACCTTAATAGGAGACTAC TTCC	
P1 EYD RV	CCCGGTCTCTCATTTGTGTATGTGTGTGTGTGTGTG	
EYD UAS1 MluI Fw	CCTTAATAGGAGACTACTTCCGACGCGTAATTAGG	Addition of the MluI site for EYD1 UAS mutation
EYD UAS1 MluI RV	CCTAATTACGCGTCGGAAGTAGTCTCCTATTAAGG	
EYD UAS2 MluI Fw	GAACTCGATACGCGTGCCGTACTCTGGAAA	
EYD UAS2 MluI RV	TTTCCAGAGTACGGCACGCGTATCGAGTTC	
ZetaUp-internal-FW	TATCTTCTGACGCATTGACCAC	Verification of Golden Gate assembly process
URA3-internal-FW	CATCCAGAGAAGCACACAGG	
URA3-internal-RV	CAACTAACTCGTAACTATTACC	
Redstar-internal-FW	AAGACGGTGGCGTTGTTACT	
RedStar-internal-RV	GACTTGCTTCTTGGCCTTGT	
Tlip2-internal-FW	TGCGTTCCTCTAAGACAAATC	
Tlip2-internal-RV	GATTTGTCTTAGAGGAACGCATA	
ZetaDown-internal-RV	GGTAACGCCGATTCTCTCTG	
LYS5-P1	ATAAGAATGCGGCCGCCGACTAAATTTCGACCCAC	LYS5 disruption
LYS5-P2	CGATTACCCTGTTATCCCTAGCGTAACTCGCTACTAGGCCGCCACC	
LYS5-T1	CGTAGGGATAACAGGGTAATATAGCGTAACTATAACGGTCCTAAGG TAGCGAAGGCGTTGGTGCTCTCTCGGAAGTAG	
LYS5-T2	ATAGTTTAGCGGCCGCAAAAATGTCCGCCATTGAGTGTTG	
LPR-R	GCTAGATAGAGTCGAGAATTACCCTG	
LYS5PR	TCGGTGCGTGTGAAAGACAC	
preTEYK Fw	GTGTTTGACATTTTGTTTTGTGTGAGT	EYK1 verification
postPEYK Rv	TACACACTCACACTCACCAGAACATC	
ClaI-pEYD1-Fw	CCCATCGATGGAAACCTTAATAGGAGACTACTTCC	pEYD1 cloning
no AvrII-pEYD1-Fw	CCTCGTGTCCGGGCTAGGGCAGAAACAGCTC	
no AvrII-pEYD1-Rev	GAGCTGTTTCTGCCCTAGCCCGGACACGAGG	
BamHI-pEYD1-Rev	TGTGTATGTGTGTGTGTGTGTGTGTGTGTTTG	
EYK300-Fw	GCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGG	CalB verification
CalB-prepro-Fw	ATGAAGCTGCTGTCTCTGACC	
CalB-internal-Rev1	CCACCTTAGATCGAATAGAAGGG	
CalB-Rev	TTAAGGGGTGACAATACCAGAAC	
CalB-internal-Fw	TCTCTGCTCCTTCTGTGTGG	
CalB-internal-Rev2	GTCGAACAGAGGTCCACAGA	
ACT-F	TCCAGGCCGTCCTCTCCC	RT-PCR control
ACT-R	GGCCAGCCATATCGAGTCGCA	

Chapter 4. Production of odd-chain fatty acids (OCFAs)

Name	Sequence (5' to 3')	Purpose
P240-F1	CGTGCTGCTAGCGCTATATG	Verification of insert in pINA240
P240-R1	CGAAACAAGCGCTCATGAGC	Verification of insert in pINA240
P240-F2	TGCAATTTCTATGCGCACCC	Verification of insert in pINA240
P240-R2	ATCTTCCCCATCGGTGATG	Verification of insert in pINA240
pTEF-internal-Fw	TCTGGAATCTACGCTTGTTCA	Verification of pTEF1
BamHI-RTS1-Fw	CCGGATCCCACAATGATGCGAGGATTCAAGC	Amplification of *RTS1*
RTS1-noBamHI-Fw	CGGAGTTCCAGCGGATTCAGATCCCCCTCTTC	Deletion of internal *Bam*HI
RTS1-noBamHI-Rev	GAAGAGGGGGATCTGAATCCGCTGGAACTCCG	Deletion of internal *Bam*HI
AvrII-RTS1-Rev	CGCCTAGGCTAGTATCCAAACGGAAAGTCAC	Amplification of *RTS1*
BamHI-E03872g-Fw	CCGGATCCCACAATGGTCTTCAAGTACATC	Amplification of *YALI0E03872g (MFS1)*
AvrII-E03872g-Rev	CGCCTAGGTTAGTTCTGATCTCGATCAAG	Amplification of *YALI0E03872g (MFS1)*

Chapter 5. Engineering precursor pools for increasing lipids production

Name	Sequence (5' to 3')	Purpose
BamHI-ACC1-A-Fw	CGGGATCCCGATGCGACTGCAATTGAGGAC	Cloning
ACC1-A-Rev	GCCGTCGCTGAACCGAAC	
ACC1-B-Fw	GTTCGGTTCAGCGACGGC	
ACC1-B-Rev	CCACCGTCGGCAAGAGGTC	Mutation
ACC1-C-Fw	GACCTCTTGCCGACGGTGG	
ACC1-C-Rev	GTCGGAGACGGCATCAGCTC	
ACC1-D-Fw	GAGCTGATGCCGTCTCCGAC	
ACC1-D-Rev	GTACTCGGGGTCGAGACGAG	Cloning
ACC1-E-Fw	CTCGTCTCGACCCCGAGTAC	
AvrII-ACC1-E-Rev	GGGCCTAGGTCACAACCCCTTGAGCAG	
YlACC1-600-Fw	CCATTGTGGCCCAGCACG	Sequencing
YlACC1-1200-Fw	CCACCGAGATGGTCACCG	
YlAcc1-1800-Fw	GCCCATCGAGCTTCCGAG	
YlAcc1-2400-Fw	GCTTGGACCCCCCACTCTC	
YlAcc1-3000-Fw	GGTGGCCGACCAGGCTG	
YlAcc1-3600-Fw	CGATTGTTGCCGTGCCTCATC	
YlAcc1-4200-Fw	CTGACCGGCTCATGAGCG	
YlAcc1-4800-Fw	GACCCCCGAGTACCCTCG	
YlAcc1-5400-Fw	CAAGCTGCTTGGTCGAGAGG	
YlAcc1-6100-Fw	CACCGGTGAGCTGCGAG	
pTEF-internal-Fw	TCTGGAATCTACGCTTGTTCA	Sequencing Verification of cloning
ACS2-Fw	ATGTCTGAAGACCACCCAGC	
ACS2-intern-Rev	GCTCGTGGGTGTTCCACA	
CpPCT-intern-Rev	GCAACAGAAGCCACGTACTCA	

EnPCT-Fw	ATGACCCACCCCCAGCAG	
EnPCT-internal-Rev	GACCGGCAGATCCAGGTCGG	
EcPCT-Fw	ATGAAACCTGTCAAACCGCC	
EcPCT-internal-Rev	GATTCCTTGCGAAGTGATTCCG	
RePCT-Fw	ATGAAGGTGATTACCGCCAGAG	
RePCT-internal-Rev	CCAATGGGGCCTGCCTC	Sequencing Verification of cloning
EcPrpE-Fw	ATGTCTTTCTCCGAGTTCTACCAG	
EcPrpE-Rev	CTACTCCTCCATAGCCTGTCG	
SePrpE-Fw	ATGTCTTTCTCCGAGTTCTACCAG	
SePrpE-Rev	CTACTCCTCAATGGCCTGTCG	
ScACS1-Fw	ATGACCATTAAGGAGCACAAGG	
ScACS1-Rev	CTACTTCTTCTTCTGAGAGAAGAACTG	
ReBktB-Fw	ATGACCCGAGAGGTGGTGGTGGTC	
ReBktB—internal-Rev	CCTTGAAGTAGCCGGCCTTGATGG	
ReBktB-Rev	GATTCGCTCGAAGATGGCAGCGATG	

Chapter 6. *De novo* production of OCFAs from glucose

Name	Sequence (5’ to 3’)	Purpose
GGP_AAT2_A_F	GGTCTCTAATGCTCCGAACCATCGCC	Golden Gate assembly
GGP_AAT2_A_R	GGTCTCTTAGACTATTTGGTCACCTCGTAGATAGCCTG	Golden Gate assembly
GGP_THR1_A_F	GGTCTCTAATGTCTCGAAAGTTTGAAATTTCTGTGCCCGCGTCCTCGGC	Golden Gate assembly
GGP_THR1_A_R	GGTCTCTTAGATTAGAGCTGCTTCACGTTGGAACC	Golden Gate assembly
THR1_BsaI_del_1_F	ATGTCTCGAAAGTTTGAAATTTCTGTGC	Remove internal BsaI site
THR1_BsaI_del_1_R	GGCCGAGGCGGTGGACACTTCAAAG	Remove internal BsaI site
THR1_BsaI_del_2_F	CTTTGAAGTGTCCACCGCCTCGGCC	Remove internal BsaI site
THR1_BsaI_del_2_R	TTAGAGCTGCTTCACGTTGGAACCG	Remove internal BsaI site
GGP_THR4_B_F	GGTCTCTACAATGACCACCTACTTTTCCACGCG	Golden Gate assembly
GGP_THR4_B_R	GGTCTCtATCCTTACTGCTTCTTCTCCTTGGCCAG	Golden Gate assembly
THR4_BsaI_del_1_F	ATGACCACCTACTTTTCCACGC	Remove internal BsaI site
THR4_BsaI_del_1_R	CATCCTTAATAGTTTCGAGAGTCTGC	Remove internal BsaI site
THR4_BsaI_del_2_F	GCAGACTCTCGAAACTATTAAGGATG	Remove internal BsaI site
THR4_BsaI_del_2_R	GTAGCCCTCCAGGCCCTTGAGG	Remove internal BsaI site
THR4_BsaI_del_3_F	CCTCAAGGGCCTGGAGGGCTAC	Remove internal BsaI site
THR4_BsaI_del_3_R	TTACTGCTTCTTCTCCTTGGCCAGC	Remove internal BsaI site
GGP_ILV1_C_F	GGTCTCTCCACAATGTCCGAACCCGACTATCTGAAG	Golden Gate assembly
GGP_ILV1_C_R	GGTCTCTGTACTTACTTCATAAACTGCTTGTACACCACATTG	Golden Gate assembly
ILV1_BsaI_del_1_F	ATGTCCGAACCCGACTATCTGAAG	Remove internal BsaI site
ILV1_BsaI_del_1_R	GCATCATAAGTTTCCACGGCAATG	Remove internal BsaI site
ILV1_BsaI_del_2_F	GAAACTTATGATGCATGTGCTCTGAAAC	Remove internal BsaI site
ILV1_BsaI_del_2_R	CAGGCCAGCCACCGACAGAGCAC	Remove internal BsaI site
ILV1_BsaI_del_3_F	CGGTGGCTGGCCTGAAGAAGTAC	Remove internal BsaI site
ILV1_BsaI_del_3_R	TTACTTCATAAACTGCTTGTACACCACATTGTCTGTC	Remove internal BsaI site
GGP_HOM3_A_F	GGTCTCTAATGACCTGGATCGTCCAAAAGTTC	Golden Gate assembly
GGP_HOM3_A_R	GGTCTCTTAGACTAGATTTTCCGTCTAGGACACAACAAGC	Golden Gate assembly
GGP_HOM2_B_F	GGTCTCTACAATGGTCAAGACTAAAAAGGCTGGAGTTC	Golden Gate assembly
GGP_HOM2_B_R	GGTCTCTATCCCTAGATGAGATTCTTCTTCAGCAGAGTCTCG	Golden Gate assembly

HOM2_BsaI_del_1_F	ATGGTCAAGACTAAAAAGGCTGGAG	Remove internal BsaI site
HOM2_BsaI_del_1_R	CACCACCAGGCCGGCG	Remove internal BsaI site
HOM2_BsaI_del_2_F	GACGCCGGCCTGGTGGTGATTTC	Remove internal BsaI site
HOM2_BsaI_del_2_R	GGCAGACACCTTGAGCTCCGAGTCGGGAATGTTCTCAAAGGTGGCT CCGTCGGCGCTGACGTGGCCCAGAATCTTCTTAGTTTCCCAC	Remove internal BsaI site
HOM2_BsaI_del_3_F	GTGGGAAACTAAGAAGATTCTGGGCCACGTCAGCGCCGACGGAGCC ACCTTTGAGAACATTCCCGACTCGGAGCTCAAGGTGTCTGCC	Remove internal BsaI site
HOM2_BsaI_del_3_R	CTAGATGAGATTCTTCTTCAGCAGAGTCTCGGCAATCAG	Remove internal BsaI site
GGP_HOM6_C_F	GGTCTCTCCACAATGTCCAAGGCTGTTAACATTGCTATTATC	Golden Gate assembly
GGP_HOM6_C_R	GGTCTCTGTACCTATTTGGCAAGTCGCTGGGC	Golden Gate assembly
HOM6_BsaI_del_1_F	ATGTCCAAGGCTGTTAACATTGCTATTATCGG	Remove internal BsaI site
HOM6_BsaI_del_1_R	CCCTCGACAGTTTCGACCTCGTCACCG	Remove internal BsaI site
HOM6_BsaI_del_2_F	CGGTGACGAGGTCGAAACTGTCGAGGG	Remove internal BsaI site
HOM6_BsaI_del_2_R	CTATTTGGCAAGTCGCTGGGCAGC	Remove internal BsaI site
PDA1-Rev	CGTTAGTTCTTAAAGTAGTAGTCCTCGG	Verification of gene
PDB1-Rev	CTACTCCTCAATGTAGAGGGCGTC	Verification of gene
LAT1-Rev	CTACAACAACATCTCAATGGGGTTTTC	Verification of gene
LPD1-rev	CTTAAAAGTGGATGGCCTTGTCGTAAG	Verification of gene
PDX1-Rev	CTACTCCTCCACAGTCAAATGATAC	Verification of gene
ZetaUp_Intern_Fw	TATCTTCTGACGCATTGACCAC	Verification of assembly
URA3M_Intern_Fw	CATCCAGAGAAGCACACAGG	Verification of assembly
URA3M_Intern_Rv	CAACTAACTCGTAACTATTACC	Verification of assembly
P-TEF_Intern_Fw	TCTGGAATCTACGCTTGTTCA	Verification of assembly
P_TEF_Intern_Rv	CTTAACGATTTCGGGTGTGAGT	Verification of assembly
T_Lip2_Intern_Fw	TGCGTTCCTCTAAGACAAATC	Verification of assembly
T_Lip2_Intern_Rv	GATTTGTCTTAGAGGAACGCATA	Verification of assembly
ZetaDown_Intern_Rv	GGTAACGCCGATTCTCTCTG	Verification of assembly

APPENDIX C. GENE SEQUENCES

Chapter 3. Development of synthetic biological tools – synthetic inducible promoter

Sequences of the promoters.

The *Bsa*I sites are underlined, and their 4-bp overhangs are in bold.

Promoter	Sequence
TEF	GGTCTCT**ACGG**GGGTTGGCGGCGTATTTGTGTCCCAAAAAACAGCCCCAATTGCCCCAATTGACCCCAAATTGACCCAGTAGCG GGCCCAACCCCGGCGAGAGCCCCCTTCACCCCACATATCAAACCTCCCCCGGTTCCCACACTTGCCGTTAAGGGCGTAGGGTAC TGCAGTCTGGAATCTACGCTTGTTCAGACTTTGTACTAGTTTCTTTGTCTGGCCATCCGGGTAACCCATGCCGGACGCAAAATAG ACTACTGAAAATTTTTTTGCTTTGTGGTTGGGACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGAATTACCTTTCCTCTTCTT TTCTCTCTCTCCTTGTCAACTCACACCCGAAGA**ATGA**GAGACC
EYK1	GGTCTCT**ACGG**ATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGGGAAAGCCGCC GCATTAAGCTCCACAGCCTTGCATAATCCGATGACCTGACTAGTGCGGACAAAGACTATTATTTCGAGGCAAGGCCACCACGTA CCGCGGTCCCAAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTCAACGTGGATCAGAAAGAGGGGCAGATCAGCTTCTATAA GAAGCTCCTTTCCCCACAATTGGCCCACACGACACTTCTACACACTTACACATCTACTGGATCC**ATGA**GAGACC
EYK1-2AB	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGG GAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCTTGCAT AATCCGATGACCTGACTAGTGCGGACAAAGACTATTATTTCGAGGCAAGGCCACCACGTACCGCGGTCCCAAACTTTTGCAAAG CTGAAAACAGCGTGGGGGTCAACGTGGATCAGAAAGAGGGGCAGATCAGCTTCTATAAGAAGCTCCTTTCCCCACAATTGGCC CACACGACACTTCTACACACTTACACATCTACTGGATCC**ATGA**GAGACC
EYK1-3AB	GGTCTCT**ACGG**CGATACGCGTAtCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGG GAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGC GGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCTTGCATAATCCGATGACCTGACTAGTGCGGACAAAGACTAT TATTTCGAGGCAAGGCCACCACGTACCGCGGTCCCAAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTCAACGTGGATCAGAA AGAGGGGCAGATCAGCTTCTATAAGAAGCTCCTTTCCCCACAATTGGCCCACACGACACTTCTACACACTTACACATCTACTGGA TCC**ATGA**GAGACC
EYK1-4AB	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGG GAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGC GGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCT CCACAGCCTTGCATAATCCGATGACCTGACTAGTGCGGACAAAGACTATTATTTCGAGGCAAGGCCACCACGTACCGCGGTCCC AAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTCAACGTGGATCAGAAAGAGGGGCAGATCAGCTTCTATAAGAAGCTCCTTT CCCCACAATTGGCCCACACGACACTTCTACACACTTACACATCTACTGGATCC**ATGA**GAGACC
EYK1-5AB	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGG GAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGC GGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCT CCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCTTGCATAATCCGATGACCTGACTAGTG CGGACAAAGACTATTATTTCGAGGCAAGGCCACCACGTACCGCGGTCCCAAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTC AACGTGGATCAGAAAGAGGGGCAGATCAGCTTCTATAAGAAGCTCCTTTCCCCACAATTGGCCCACACGACACTTCTACACACT TACACATCTACTGGATCC**ATGA**GAGACC
EYD1AB	GGTCTCT**ACGG**CCCATCGATGGAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAGACAAAGCG GCTTGGAGTAGGCCTCGTGTCCGGCCTAGGGCAGAAACAGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCTAGA GGAAGTTCCAAGGTCGAGTCTCTTCGATATAAAAGGACGCCATGGAAGCTCTGTAGTTCGATATCAAATACTGACAACAGTTTC CAAACACACAAACACACACACACACACACACACACACATACACAC**ATGA**GAGACC
EYD1A*B	GGTCTCT**ACGG**CCCATCGATGGAAACCTTAATAGGAGACTACTTCCGACGCGTTAATTAGGACTTCCGCGACCCCAGACAAAGC GGCTTGGAGTAGGCCTCGTGTCCGGCCTAGGGCAGAAACAGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCTAG AGGAAGTTCCAAGGTCGAGTCTCTTCGATATAAAAGGACGCCATGGAAGCTCTGTAGTTCGATATCAAATACTGACAACAGTTT CCAAACACACAAACACACACACACACACACACACACATACACAC**ATGA**GAGACC
EYD1AB*	GGTCTCT**ACGG**CCCATCGATGGAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAGACAAAGCG GCTTGGAGTAGGCCTCGTGTCCGGCCTAGGGCAGAAACAGCTCCGGAACTCGATACGCGTGCCGTACTCTGGAAAGTCTAGAG GAAGTTCCAAGGTCGAGTCTCTTCGATATAAAAGGACGCCATGGAAGCTCTGTAGTTCGATATCAAATACTGACAACAGTTTCC AAACACACAAACACACACACACACACACACACACATACACAC**ATGA**GAGACC
EYK UAS1-4AB-TEF	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGGGAAGCGGAATCCCAAAAGG GAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGC GGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCGGGAAGCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCT CCACAGCCTTGCATAATCCGATGACCTGACTAGTGCGGTTGGGACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGAATTAC CTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCCGAAGGATCCCACA**ATGA**GAGACC
EYK UAS1-4AB-R1-TEF	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTCGGAATCCCAAAAGGGAAAGCCG CCGCATTAAGCTCCACAGCCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCGGAATCCCAAAAGGGAAAGCC GCCGCATTAAGCTCCACAGCCGGAATCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCTTGCATAATCCGATGACCTG ACTAGTGCGGTTGGGACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGAATTACCTTTCCTCTTCTTTTCTCTCTCTCCTTGTC AACTCACACCCGAAGGATCCCACA**ATGA**GAGACC

EYK UAS1-4AB-R2-TEF	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTTCCCAAAAGGGAAAGCCGCCGCA TTAAGCTCCACAGCTCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCTCCCAAAAGGGAAAGCCGCCGCATTAAGCTCC ACAGCTCCCAAAAGGGAAAGCCGCCGCATTAAGCTCCACAGCCTTGCATAATCCGATGACCTGACTAGTGCGGTTGGGACTTTA GCCAAGGGTATAAAAGACCACCGTCCCCGAATTACCTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCCGAAGGATCCA CA**ATGA**GAGACC
EYK1/EYD1A-EYK1	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTAAACCTTAATAGGAGACTACTTCC GTTTCCTAATTAGGACTTCCGCGACCCCAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAAACC TTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGG ACTTCCGCGACCCCCTTGCATAATCCGATGACCTGACTAGTGCGGACAAAGACTATTATTTCGAGGCAAGGCCACCACGTACCG CGGTCCCAAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTCAACGTGGATCAGAAAGAGGGGCAGATCAGCTTCTATAAGAA GCTCCTTTCCCCACAATTGGCCCACACGACACTTCTACACACTTACACATCTACTGGATCCACA**ATGA**GAGACC
EYK1/EYD1A-TEF	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTAAACCTTAATAGGAGACTACTTCC GTTTCCTAATTAGGACTTCCGCGACCCCAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAAACC TTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGG ACTTCCGCGACCCCCTTGCATAATCCGATGACCTGACTAGTGGTTGGGACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGA ATTACCTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCCGAAGGATCCACA**ATGA**GAGACC
EYK1/EYD1B-EYK1	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGCTCCGGAACTCGATTGAGAAGC CGTACTCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTAC TCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCCTTGCATAATCCGATGACCTGACTAGTGCGGAC AAAGACTATTATTTCGAGGCAAGGCCACCACGTACCGCGGTCCCAAACTTTTGCAAAGCTGAAAACAGCGTGGGGGTCAACGT GGATCAGAAAGAGGGGCAGATCAGCTTCTATAAGAAGCTCCTTTCCCCACAATTGGCCCACACGACACTTCTACACACTTACAC ATCTACTGGATCCACA**ATGA**GAGACC
EYK1/EYD1B-TEF	GGTCTCT**ACGG**CGATACGCGTATCGATGCATCTACTTTTCTCTATACTGTACGTTTCAATCTGCTCCGGAACTCGATTGAGAAGC CGTACTCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTAC TCTGGAAAGTCGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCCTTGCATAATCCGATGACCTGACTAGTGGTTGG GACTTTAGCCAAGGGTATAAAAGACCACCGTCCCCGAATTACCTTTCCTCTTCTTTTCTCTCTCTCCTTGTCAACTCACACCCGAA GGATCCACA**ATGA**GAGACC

Sequences of the *EYD1* promoters in *Y. lipolytica* and in the *Yarrowia* clade.

The sequences are from *Y. lipolytica* E150 (YALI; *YALI0F01606g*), *Yarrowia galli* (YAGA), *Yarrowia phangngensis* (YAPH), *Yarrowia yakushimensis* (YAYA), and *Yarrowia alimentaria* (YAAL). The ATG of EYD1 is in bold.

Promoter	**Sequence**
YALI-pEYD1	GATTTTTGGAAACCTTAATAGGAGACTACTTCCGTTTCCTAATTAGGACTTCCGCGACCCCAGACAAAGCGGCTTGGAGTAGGCC TCGTGTCCGGCCTAGGGCAGAAACAGCTCCGGAACTCGATTGAGAAGCCGTACTCTGGAAAGTCTAGAGGAAGTTCCAAGGTC GAGTCTCTTCGATATAAAAGGACGCCATGGAAGCTCTGTAGTTCGATATCAAATACTGACAACAGTTTCCAAACACACAAACACA CACACACACACACACACACATACACAA**ATG**GTTTCTTCAGCCGCTACTTCTGCTCTGCCCATCTCGGCACCC
YAGA-pEYD1	TGCGCACCCCAGATTTTACGGAAACCTAAATGGAAGACTACTTCCGTTTCCTAATATGGACATCCGCGACCCCCAGACAAAGCGG CCTGGAGTAGACCTCGTGTCCGGCCTAGGGAAGAAACAGCTCCGGATCTCGATTGAGAAGCCGTTCTATGGAAAGTCTAGAGA CAGAGCTGAGGTGGGTCTCTTTGATATAAAAGGGTCACCACTGTCGTCGTAGTTTCGACATCAATTAACTGACAACACTCTTCTA ACACACACACACACAAACACACACACA**ATG**GTTTCTACAGCCGCTACTTCTGCTCTGCCCATCTCGGCACCT
YAPH-pEYD1	TCCATCTTAAAGTTAGACTATACCCCAGATGACTGAACCTAAAAGACTATAGATTTCTATTTCCCTATTAGGTTGCCGCTCTCCCCC ATATTAACCTAATTCGTGTATGTGTCACTTAGCTCCCTTGTGTGATTACGGATCTTCATTGAGAAAGCCGGAATGTCCAAGAATAG CGGAGTTTCGGAGCTTAGGTATCATCTAGATATAAAAGGGTCAAAGGCTGCTCTCAGGATTCAATACAGTATCTCTACTTACAAC AATAACCCAATCTTCACACACA**ATG**GTTTCCATCACTCCTACTTCTGCCCTGCCCCTCTCTGCTCCT
YAYA-pEYD1	CTTTAAATTAAGACAAGGGCGCCATCGTGCGATTCGAACAAAAAGAAATTACACTTTTAGGCACCCCAGATTTCTAAACCCAGAC TCTAAATGCAATCCAGTTGCGTTTCCTAATGCGGACTTCCGCGACGACACGGCGCCTTACTAGTTGAGAAAACAGTCCCGGATCT CAATTGAGAAGCCGTAGTGAAAAGTGTAGGGAAGCTACAAGACCGTCTGATATATATAGGCTGGGGATTTCTCCAGAGTTGGTA TCAATCAACAGTTATCTCTTCACACA**ATG**GTTTCTACAACTTCCACTTCTGCTCTGCCAATTTCGGCCCCC
YAAL-pEYD1	TTTGTCTCGTGGCTTTTTTTCGGCACCCCGGATTATAACTTTAAAGCTTGCTACTTCCGTTTCCTAATTAGGACATCCGCGACTTCAT ACAAGCGGCTTGTCGGAAGGCCTCGTGTCTCATGAGCGCACTAATAGTCTCGGGTCTGGATTGAGAAGCCGTTCAGCCTTCAAA GTCTAGAATCAAAGTGAAGACGGAGGATTCGATATATAAGGGCACCATGACCACCTCTTGGTAGGCATCAAAACACCCTCACAC ACACACACACACATACAAAACACA**ATG**GTTTCCTCTGCTGCTACCTCCGCACTCCCCATCTCGGCACCT

Nucleic and amino acid sequence of codon optimized CalB.

Bold and underlined sequences correspond to the pre*LIP2*-pro*CalB* targeting sequence of the extracellular lipase Lip2p encoded by the *LIP2* gene.

DNA sequence	**ATGAAGCTGCTGTCTCTGACCGGTGTGGCTGGTGTTCTGGCCACCTGTGTCGCTGCCACCCCTCTGGTGAAGCGACTGCCTTCTGG ATCTGACCCTGCCTTCTCTCAGCCCAAGTCTGTTCTGGACGCTGGTCTGACCTGTCAGGGAGCTTCTCCTTCTTCTGTGTCTAAGCCC ATTCTCCTGGTGCCTGGAACCGGAAC**CACCGGTCCTCAGTCTTTCGACTCGAACTGGATTCCTCTGTCTACCCAGCTGGGATACACCC CCTGTTGGATTTCTCCTCCTCCTTTCATGCTGAACGACACCCAGGTGAACACCGAGTACATGGTGAACGCCATTACCGCTCTGTACGC TGGCTCTGGAAACAACAAGCTGCCCGTTCTGACCTGGTCTCAGGGAGGTCTGGTGGCTCAGTGGGGTCTGACCTTCTTCCCTTCTAT TCGATCTAAGGTGGACCGACTGATGGCCTTCGCTCCCGACTACAAGGGAACCGTTCTGGCTGGTCCTCTGGACGCTCTGGCTGTCTC TGCTCCTTCTGTGTGGCAGCAGACCACCGGCTCTGCTCTGACCACCGCTCTGCGAAACGCTGGAGGTCTGACCCAGATTGTCCCCAC CACCAACCTGTACTCTGCCACCGACGAGATTGTCCAGCCTCAGGTGTCTAACTCTCCTCTGGACTCTTCGTACCTGTTCAACGGAAAG AACATTCAGGCTCAGGCTGTCTGTGGACCTCTGTTCGACATTGACCACGCTGGCTCTCTGACCTCTCAGTTCTCCTACGTGGTTGGAC GATCTGCTCTGCGATCTACCACCGGTCAGGCTCGATCTGCTGACTACGGTATCACCGACTGTAACCCTCTGCCTGCCAACGACCTGAC CCCTGAGCAGAAGGTGGCTGCTGCTGCTCTGCTGGCTCCCGAGGCTGCTGCCATTGTCGCTGGTCCCAAGCAGAACTGCGAGCCCG ACCTGATGCCTTACGCTCGACCCTTCGCTGTTGGAAAGCGAACCTGTTCTGGTATTGTCACCCCT**TAA****
Amino acid sequence	**MKLLSLTGVAGVLATCVAATPLVKR**LPSGSDPAFSQPKSVLDAGLTCQGASPSSVSKPILLVPGTGTTGPQSFDSNWIPLSTQLGYTPCWI SPPPFMLNDTQVNTEYMVNAITALYAGSGNNKLPVLTWSQGGLVAQWGLTFFPSIRSKVDRLMAFAPDYKGTVLAGPLDALAVSAPSV WQQTTGSALTTALRNAGGLTQIVPTTNLYSATDEIVQPQVSNSPLDSSYLFNGKNIQAQAVCGPLFDIDHAGSLTSQFSYVVGRSALRSTT GQARSADYGITDCNPLPANDLTPEQKVAAAALLAPEAAAIVAGPKQNCEPDLMPYARPFAVGKRTCSGIVTP*****

Chapter 4. Production of odd-chain fatty acids

Gene	Sequence
RTS1r (truncated)	ATGATGCGAGGATTCAAGCAGCGAATGCTGTCGCGGTCCAAGTCTCACAGCGAGTCGTCGACCAAAAACAAAAAGAAGGAGGACG GACCCAAGCGCACTTTCTCGCTAGGCGACCGCAAGAAAACCCCTGAGCCCACCCCTGCTGCCGCTACGCCCTCCAATGTCCAGGCCG CCGCTACGCCGGTCTCTTCGTCTTCGAGTGCCAGCAGCATAACCTCTGTCAGCGGGTCCTCTGGTAACAACAACTCCAGCAGCAACA ACAACAGCAACAATAACATCACCAGTGGTGCAACTAACACCACCAATAGCAGCAATGCTAACACCACCAACCCTGCCACATCATCCA ACTCCAACCCTTCGACCGGCAACGTGTCCTCCATGTTGACCCCCCAACAGCCTGCCCAGTCACAGGCAGCGCTCCAGGGCCTGCAGC AGAACCAGCAGCAGCAGGGACAGCAGATGGACACAAACACACCGCCAGCCATTCTCGTTGAGGGACCTACGCAACTGGGCTCGGC CCAGGCGTCGAGTCACGTGACCATGGGCCACGTGACCGGCCACATGAGCACGCACTCGGGCACCCCACATCACCACCCGTCGTCTC CGGGCCAGCTGGAGACCATGCCCACCGATCTGGAGCCTCCCAAGCGCCACTCGTTTGAGCTGCTCAACGCGGACGGCCCTGAGGTC AAAACCCCCCGTCGACACAACTCCAGCCGTCTGGAAATTTCCAAGGAGCGGGAGCTGGAGCGTCTCCCCGGTTTCAACGAGGTGCC TTTCCGAAAGCGCGCGGAGCTGTTTTTGCAGAAGGTCAAGCAGTGCAATACCATTTTCGACTTTGGCGACCCGTCGTCTGACATTCA GGGCAAGGACATTAAGCGGATGGCTCTGCACGAGCTGCTGGACTTTGTCGCTAACACGCGCATCACAATCACGGACGAAATGTACG CCCAGGTGGTGGGCATGTTTGGCAAGAACATCTTCCGGCCCACGCCTCCACTGGTGAACCCGGTAGGAGAGGTTTTTGATCCT	
RTS1	ATGATGCGAGGATTCAAGCAGCGAATGCTGTCGCGGTCCAAGTCTCACAGCGAGTCGTCGACCAAAAACAAAAAGAAGGAGGACG GACCCAAGCGCACTTTCTCGCTAGGCGACCGCAAGAAAACCCCTGAGCCCACCCCTGCTGCCGCTACGCCCTCCAATGTCCAGGCCG CCGCTACGCCGGTCTCTTCGTCTTCGAGTGCCAGCAGCATAACCTCTGTCAGCGGGTCCTCTGGTAACAACAACTCCAGCAGCAACA ACAACAGCAACAATAACATCACCAGTGGTGCAACTAACACCACCAATAGCAGCAATGCTAACACCACCAACCCTGCCACATCATCCA ACTCCAACCCTTCGACCGGCAACGTGTCCTCCATGTTGACCCCCCAACAGCCTGCCCAGTCACAGGCAGCGCTCCAGGGCCTGCAGC AGAACCAGCAGCAGCAGGGACAGCAGATGGACACAAACACACCGCCAGCCATTCTCGTTGAGGGACCTACGCAACTGGGCTCGGC CCAGGCGTCGAGTCACGTGACCATGGGCCACGTGACCGGCCACATGAGCACGCACTCGGGCACCCCACATCACCACCCGTCGTCTC CGGGCCAGCTGGAGACCATGCCCACCGATCTGGAGCCTCCCAAGCGCCACTCGTTTGAGCTGCTCAACGCGGACGGCCCTGAGGTC AAAACCCCCCGTCGACACAACTCCAGCCGTCTGGAAATTTCCAAGGAGCGGGAGCTGGAGCGTCTCCCCGGTTTCAACGAGGTGCC TTTCCGAAAGCGCGCGGAGCTGTTTTTGCAGAAGGTCAAGCAGTGCAATACCATTTTCGACTTTGGCGACCCGTCGTCTGACATTCA GGGCAAGGACATTAAGCGGATGGCTCTGCACGAGCTGCTGGACTTTGTCGCTAACACGCGCATCACAATCACGGACGAAATGTACG CCCAGGTGGTGGGCATGTTTGGCAAGAACATCTTCCGGCCCACGCCTCCACTGGTGAACCCGGTAGGAGAGGTTTTTGATCCTGAC GAGGACGAGCCTGTTTGCGAGGTCGCATGGCCCCACATGCAACTGGTGTACGAGTTTTTCCTCAAGTTCATCGAATCACCGGACTTC AACCACACGTCTGCCAAGAAGTTCATCGACCACCGGTTTGTGCAGAACCTGCTGGAGCTGTTTGACAGCGAAGACATTCGGGAGCG GGACTGCCTCAAAACTACTCTGCACCGAATCTATGGCAAGTTCCTGAATCTGCGAGCGTATATCCGGCGGTCCATCAACAATGTCTTT TTCCAGTTCATTTATGAAACGGAGCGGTTCAATGGCGTGGCCGAGCTGCTGGAGATTTTGGGCTCCATCATCAACGGCTTTGCTCTG CCGCTCAAGGACGAGCACAAGATCTTCTTGTCCAGGGTGCTCATCCCTCTGCATAAGGCCAAGACTCTGAGCCTGTACCACCCACAG CTGGCCTACTGTGTCGTACAGTTCCTGGAAAAGGACCCCTCGCTTACCGAGGAAGTCATTCTGGGCTTGCTGCGGTACTGGCCCAAG GTTAACTCGTCGAAGGAGGTCATGTTCCTGAACGAGATTGAGGACATCTTCGAGGTGATGGAGCCGACGGAGTTCCAGCGGATtCA GATCCCCCTCTTCTCGCAGCTAGCTAAGTGCATCTCGTCGCAGCATTTCCAGGTGGCCGAACGGGCACTGTACTATTGGAACAACGA GTACTTTTGCACGCTCATGTCGGAGAACATCACCGAGATCTTGCCGGTCATATTCCCTGCGCTGTACGAAAACTCCCGCGGCCACTG GAACCGCACCATCCACTCTATGGTGTACAATACTATGAAAATGATCATGGAAGCCAATCCGCAGTTGTTTGACCAGTGCACCATGGT GTACCAAGAGCGACAAGACCTCAAGGAGGAGCACGAGGCGCTGGTGGCGAAGTGGTGGGATATGGTGGTGGAACGTGCGGCCAA	

	TAGTCAGGAGAACGTTGATTCGCCACAATTTGCCAAGCTGCTCAACCAGGCCCAGGAGGAGTTTTCCATGCTCAAGACCGAGGACC GGGATCTGCAGATGAGCGGAATTCCCGAGGAGGGGGAGGAGGATAGCAACAAGCTTGCTAATAACCAGGGTCCCGAGGAGGGCA TGGAACATGACCACCACGAGCAGCACCAACAGCACCAGGGACTTGGACAGCATGAGCAGCAGCAGCAGCAGCAGGGTGTTGA GCAACAACAACAGCCCCACAATATCCAGCCTCTGGGGGTTGTGACTAACCATGAGACTCCTGTCGTTGCTCATAACGATGAGGAGCA TACTGGTTTCCACACCCCTCGCGGTAGCAGTGACTTTCCGTTTGGATACTAG
MFS1r (truncated)	ATGGTCTTCAAGTACATCGGAGACACTTTGGGTCTTACCCCGGTGCTCAAGTCATCCTGGGACGTCCATGCTCTCATTATCATGCGAT TTTTCCGACTCATGGCGTTTGGCCAGATCTCTGTCATTCTCGCCCAGTTTTTTGATGCCCATGGCTTCTCTCAGGACAAGACCGGTCTT TTCATGTCCCTGACTCTCGCCGGAGACGTTGGCCTGTCCCTGTTCCTTACCACATATGGTGACCGACTCGGCCGAAAGAATGTGCTCC TTATTGGAGCCGGCCTCATGACCCTCTCGGGCCTCGTCTTCTTCCACGAGTCCAACTACTATCTCCTTCTTCTGGCCGCCATTTGCGGT GTCATTTCTCCCTCTGGTGCTGAGGTTGGACCCTTCCGAGCAATTGAAGAGTCCACCTTAGCCCAGCTCACAACCCTGGAGGCCCGA GCTGACGTCTTTGCTTGGTACACTCTTTTGGGATCTCTCGGAGCTGCCCTCGGAGCCCTTGTCGGTGGCTGGACCGTCCAGATTGCCC AGGAGAAGTACCACTTCACCAAGCTTGAGGCTTACAAGACCGTTTTCCTGACTTATGCAGCCATTGGCTTCATCAAGTTCATTGCTTC CTGTTTCCTTTCTCGAAAGGTCGAAGCTCCAGCTGAAGAGACCGAGCCTCTTCTTGCCAACGATCAGGAGAACGCCACCGAGGAGC GAGCTGAGCTCCGAAACAACATCAAGAAGCGAGGTGGTCTGCGAAACCTGCTTCCCAACATCTCCCCCGAGTCCCAGAAGATC
MFS1	ATGGTCTTCAAGTACATCGGAGACACTTTGGGTCTTACCCCGGTGCTCAAGTCATCCTGGGACGTCCATGCTCTCATTATCATGCGAT TTTTCCGACTCATGGCGTTTGGCCAGATCTCTGTCATTCTCGCCCAGTTTTTTGATGCCCATGGCTTCTCTCAGGACAAGACCGGTCTT TTCATGTCCCTGACTCTCGCCGGAGACGTTGGCCTGTCCCTGTTCCTTACCACATATGGTGACCGACTCGGCCGAAAGAATGTGCTCC TTATTGGAGCCGGCCTCATGACCCTCTCGGGCCTCGTCTTCTTCCACGAGTCCAACTACTATCTCCTTCTTCTGGCCGCCATTTGCGGT GTCATTTCTCCCTCTGGTGCTGAGGTTGGACCCTTCCGAGCAATTGAAGAGTCCACCTTAGCCCAGCTCACAACCCTGGAGGCCCGA GCTGACGTCTTTGCTTGGTACACTCTTTTGGGATCTCTCGGAGCTGCCCTCGGAGCCCTTGTCGGTGGCTGGACCGTCCAGATTGCCC AGGAGAAGTACCACTTCACCAAGCTTGAGGCTTACAAGACCGTTTTCCTGACTTATGCAGCCATTGGCTTCATCAAGTTCATTGCTTC CTGTTTCCTTTCTCGAAAGGTCGAAGCTCCAGCTGAAGAGACCGAGCCTCTTCTTGCCAACGATCAGGAGAACGCCACCGAGGAGC GAGCTGAGCTCCGAAACAACATCAAGAAGCGAGGTGGTCTGCGAAACCTGCTTCCCAACATCTCCCCCGAGTCCCAGAAGATCATTA CTACTCTGTGTCTTCTGTTTGCCATCGACTCAATGGGTTCTTCCCTTGCGAACTTCTCCTGGATCTCCTACTACATTCAACGAAAGTTTG ACATTTCTACCGGATACCTTGGTTCCGTCTTCTTCGTCACAGGTATCGTGGGTGCTCTGGCCACCCTTGTTGGATCTGGTATCTCTCGA CGTCTCGGTCCTCTTCTGACCATGGTCGTCACCCACCTTCCTTCCTCTGCTTTGATCATTCTTCTGCCCGTTCCTTCTACCGTGCAAGGT GTCATCACCATTCTCGCTATCCGAGCCTGCACTGCCCAGATGGATGTTGCTCCTCGACAAGCCTTCCTGTCCGCCGTGGTACTCAAGA ACGAGCGAACTCTGGTCATGGGATGGGTTGGAACTATCAAGACTCTTGCCCAGCTGCCTGGACCTTACATCACCGGTTACCTTGCTC GACACAACAAGCAGTGGATTGCTTTTGTTGCTGCCGGTTCTCTCAAGGTCATCTACGATTTGTCTATCCTCTCTTACTTCACCCGGGTG AAGCTTGATCGAGATCAGAACTAA
MFS2	ATGTCTCGAGATTCTGAAACGAACGAAATGTCCAGGTTTGACCGGTTCATTCGGGAAATCGGATGGCACTCGCTCGTGAACTCTTCA TGTGACATCAAGATCCTCATTGCACAAAAAGTGCTGCGGATGATCGCCTACGGTCAGAGTACTTTGATTCTGGTGCAGTTTTTCCGTG AAATACACGTGTCCGACGTTGGTCTCGGGTACTTTATGACCCTGACTCTGCTTGGAGATGTAATCATTAGCTACTTTTTGACATTGTA CGCCGACCAGCTTGGACGGCGCCTTGTGCTCCTCCTTGGTTCGTTTCTCATGATGGTCAGCGGCATAGTTTTTGTATTTTCCGACCACT TTGTAGTGCTGTTGATAGCAGCCATCGTCGGTGTAATTAGCCCTTCTGGAGACGAGACGGGTCCCTTCAAGTCCATTGAGGAGTCTG TAGTAGCCCATCTCACTCCAGCCAAAGAGCTCTCCGACATCTACGCCTGGTATGGGCTGTTTGGCACGGTCGGAAGTGCACTTGGAA GCGTTTCTGCGGGCATCATGATTGATGGACTTGTTGCAAACTTCGGATGGACGCAGTTGAAGGTGTATCGGTTCATGTTTGCCCAGT ACGCTCTGCTGGCATTTTTGAAACTGATGCTCAACTGGTTTCTCACAGATAAGTGTGAGATCGACCCAGATAACAATGACAATGAAG AACACGAAGCTGGTCTTCTTTTCGATACCCACGATCATTATCACCAACTCGAACACGAAGCTGATCAACTCCATGGTTCCAAATTCTC CATCTGGGGAACACCTCTCAGTCCCAGGAGCACATCAGTTGTGTGGAAACTATGCATTCTTTTTGCTCTGGATTCAATGGGCTACGG CTTCATGCCCATCTCGTGGGTGGTGACCTATTTTTTCGACCGATGGAAGGCAAGCGAAGTCACTGTCGGCACCCTCTTCTTCTTCACC AGCGTGCTCAACGCTCTTTCCTCTCTGGCATCTTCGTCCATGTACAAACGTCTGGGACCGATCTTTGCCATTGTGGCTACCCATTTCCC CTCTGCAATGGCCATGTCCTTCATCCCCCTGGCCCCTACCATGGGAATTGCGATGGGCCTTCTTCTCTTCAGAGCATCCACTGCTGTCA TGGACGTAGTTCCCAGACAAGCGTTTCTGTCCCATGTGGTCTCGTCCTCTGAGCGAACCAAGGTTATGGGCATTGTCAACGTGGTCA AGACTCTGGCCCGATCTGTGGGTCCCATCTTCACTGGAATGTTTGCCCAGCGAGATATTCTCGGCTTTGCGTTCTTGATCACGGGCCT CTTGGAAGCTGCCCATGACCTGGGAATGCTCGGACTCTTCTGGAAGTATAACCGGATCATTGAGCATTAA
MFS3	ATGTCTCGAGGTCCTGAATTGCCGGAGCCCGCGTTCAAGCGGTTCACGAAGGAGATTGGGTGGAGTTCGCTGGTGCATTCGTCACG TGACATCAAGATTCTGATTGCGCAAAAGGTGTTGCGAATGATTGCCTATGGTCAGAGCACTTTGATTCTGGTGCAGTTTTTCCGGGC GATTCACGTGTCGGATGTTAGTCTGGGGTACTTTATGACGCTGACGTTGCTCGGGGACGTAATTATTAGCTACTTTTTGACGCTTTAC GCCGACCAGATTGGGCGCCGACATGTGCTGTTGCTGGGATCTTTCCTGATGATGGTCAGTGGGATTGTTTTTGTCTTTTCCGATCACT TTGTTGTACTGTTGATAGCCGCAATTGTTGGCGTGATTAGTCCGTCGGGCGACGAGACCGGGCCCTTCAAGTCCATTGAGGAGTCTG TAGTTGCTCATTTGACTCCTACCAAGGAGTTGTCGGACATTTACGCGTGGTACGGGCTGTTTGGCACTGTTGGCAGTGCGGTGGGAA GTGTGTCTGCGGGACTCATGATCGATGGGCTGGTTGACCATTATGGCTGGACCAAGCTCAAGGTCTACCGGTTCATGTTTGCCCAAT ATGCCTTTTTTGCGTTCCTCAAGCTGCTGCTCAATTGGGGTCTAAGCGACAAGTGCGAGCTGCGAAAACGATCGGAGGAGGAGGAG CGACGGGACGAGGCCATTGCCACTGGTGACGAGTCTGATCCTTTGTTGCGTCCCACCGACCACCACGACCCCACTCCGTCCCAGTCC CCCAAGTTCTCAGTCTGGGGCACTCCTCTCAGCCGTAGAAGTCGACAGGTGGTGTGGAAGCTGTGCATTCTGTTTGCTCTGGACTCC ATGGGCTATGGCTTCATGCCAATCTCCTGGGTCGTCACCTACTTTCTGGACCGATGGAAGGCCAGTGAAAGCACCGTGGGCACTCTG TTCTTCTTCACCAACGTGCTCAACGCCCTGTCGTCGCTGGCCTCTTCGTCCATGTACAAGCGTCTGGGCCCCATCATTGCCATTGTCGC TGCACACTTCCCCTCAGCCATGGCCATGTCCTTCATTCCACTGGCTCCAAACATGCCCATTGCCATGGCTCTGCTGCTGTTCCGAGCGT CCACAGCCGTCATGGACGTGGTACCGCGACAGGCCTTCTTGTCGCACGTGGTGTCTGCCGAGGAGCGAACCAAGGTCATGGGCATC GTCAACGTGGTCAAGACGCTGGCTCGATCTGTGGGTCCCATTTTCACCGGCATGTTTGCCGAAAAGAACATGCTGGGCTTTGCTTTC CTCATCACCGGTCTTCTCGAGGCCGCCCACGATATGGGCATGTTGACTCTCTTCTGGAAGTACAACCGGATCATCAAGCACTGA

Chapter 5. Engineering precursor pools for increasing lipids production

Gene	Sequence
YlLDP1	ATGCCTTCTGTCGCCGACAACACTTCCAACGGTCCCATTGAGGGCAACGTTCTGCCCCAGTCCAAGTTCATCCAGCACCTGAGCGAG TATCCAGCTGTTGCTGCCGTGACTGGCTTCGCTGCCTCTTTCCCCGTTGTCAAGATTTTTGCATCCAACGCCGTTCCTCTCATCCAGGC CATCCAGAACCGAGGTGCCCCCGTCGCCGAGCCCGTCGTCAAGCGAGCCGCCCCCTACATCTCTCAGATTGACAACGCTGCCGACGA GGCCCTCAACCGACTCGACAAGGCCGTGCCCTCTCTCAAGAACACCAAGCCCGACGAGGTCTACAGCCGAATCGTGACCCAGCCCTT GGAGAACGTGCGAGGTACTGTCGACAAGTACGCCGACGAGACCAAGAACACTGTCTCCCGAGTGGTTGTGCAGCCCATCCGAGAC GTTGCCTCCAGAGTCCAGTCCCAGGTTGTCACCTACTACGATGCCCATGGAAAGCCCATTGTCCACGCCCGACTCGACCCCATCTTCC ACCCTCTCAACGACCGTCTCGAAGCCCTGATCAACGCCTACCTGCCCAAGGGCCAGGAGATTGTGACTGATGCCGAGAACGAGCTG GCCCGAGCATGGCGCCTCACCGTGGTTGCTTTTGACCGAGCCCGACCTCTGATTGAGCAGCAGACTTCCCAGATTCAGGAAATCAAC CAGCACACCCGAGAACACATCCAGAAGGTTTACGACGGCAAGCGATCGGAAATCGACGATAAGAAGACTGTCTCTGGTCCAGTATA CGCCACCGTCGCCACTGTCCGAGATCTTTCACAGGAGGGCCTCCAGTACGCCCAGTCCATTCTGAATGCCAAGAAGCCCGAGGAGA AGGCCGACTCTAACTCTGGTGTTGCTCCCGTGAGCCAGCCTCACACCACCTCCGCTGTTGACAACTCCCTCGCTGCTCCCACCGCCGT TCACGAGGTTACTGCTTCTGCTTAA
Repct	ATGAAGGTGATTACCGCCAGAGAAGCAGCGGCTCTTGTGCAGGACGGTTGGACTGTTGCATCGGCTGGATTCGTTGGCGCAGGCC ATGCTGAGGCAGTCACCGAAGCCCTTGAGCAGCGATTTCTGCAATCGGGTCTGCCACGAGATCTGACCCTCGTCTACTCTGCTGGAC AGGGCGATCGTGGTGCCCGAGGTGTGAACCACTTCGGCAATGCCGGCATGACCGCCAGCATCGTCGGCGGCCATTGGAGATCCGC AACCAGACTCGCCACCCTGGCCATGGCTGAGCAGTGTGAGGGCTACAACCTGCCTCAAGGCGTCCTTACGCACCTGTACCGAGCCAT TGCTGGCGGTAAACCTGGTGTCATGACCAAGATCGGCCTCCATACGTTCGTCGACCCACGAACCGCCCAAGATGCCCGATACCATGG CGGCGCGGTTAACGAGCGAGCACGGCAGGCCATTGCCGAGGGAAAGGCTTGCTGGGTTGACGCCGTGGACTTTCGAGGCGATGA GTACCTGTTCTACCCCTCGTTTCCCATCCACTGTGCGCTCATTCGGTGCACTGCCGCTGACGCCCGAGGAAACCTCTCCACTCACAGA GAGGCCTTTCACCACGAACTTTTGGCAATGGCCCAAGCTGCTCACAACTCCGGAGGCATCGTCATCGCGCAGGTGGAGTCCCTCGTG GACCACCACGAGATTCTGCAGGCCATCCACGTTCCAGGCATTCTGGTGGACTACGTCGTGGTTTGCGACAACCCCGCTAATCACCAG ATGACCTTCGCCGAGTCCTACAACCCTGCGTACGTCACGCCTTGGCAGGGAGAAGCTGCCGTGGCCGAAGCCGAGGCCGCTCCCGT CGCTGCTGGACCCCTTGACGCGCGGACCATCGTGCAGCGTCGAGCCGTTATGGAGCTGGCCCGACGAGCCCCGCGAGTTGTGAACC TCGGTGTCGGAATGCCTGCTGCCGTTGGTATGCTCGCCCATCAGGCTGGACTCGACGGCTTCACCCTGACTGTGGAGGCAGGCCCC ATTGGTGGTACTCCCGCTGACGGACTGTCCTTTGGTGCCTCTGCTTATCCGGAGGCTGTCGTCGACCAGCCTGCCCAGTTCGACTTCT ACGAAGGCGGTGGCATTGACCTTGCCATCCTCGGCTTGGCTGAGCTCGATGGTCACGGCAACGTCAACGTGTCCAAGTTCGGTGAG GGAGAAGGAGCCTCCATTGCTGGTGTTGGCGGTTTCATCAACATCACCCAGTCTGCTCGAGCCGTCGTGTTCATGGGAACACTGACA GCAGGTGGACTTGAGGTTCGAGCTGGTGATGGAGGACTCCAGATCGTCCGAGAGGGCCGAGTCAAGAAGATCGTCCCTGAGGTGT CTCACCTGTCCTTTAACGGTCCCTATGTGGCTTCTCTCGGAATCCCTGTCCTGTACATCACTGAGCGAGCTGTTTTCGAGATGCGAGC TGGAGCTGATGGCGAAGCCCGATTGACTCTGGTGGAGATTGCGCCCGGTGTCGACCTTCAGCGGGACGTTTTGGACCAGTGTAGCA CACCCATTGCTGTCGCCCAGGATCTGCGTGAGATGGATGCCCGTCTGTTTCAGGCCGGTCCCCTGCATCTGTAA
Cppct	ATGCGAAAGGTTCCCATCATCACTGCTGACGAGGCTGCCAAGCTCATCAAGGACGGAGATACCGTTACTACTTCGGGTTTTGTCGGA AACGCTATCCCTGAGGCTCTGGACCGAGCTGTCGAGAAGCGATTCCTCGAGACCGGCGAGCCTAAGAACATTACTTACGTTTACTGT GGATCTCAGGGTAACCGAGACGGACGAGGTGCTGAGCACTTTGCCCATGAGGGCCTGCTCAAGCGATACATTGCTGGACACTGGG CCACCGTTCCCGCTCTGGGAAAGATGGCCATGGAGAACAAGATGGAGGCTTACAACGTGTCCCAGGGAGCCCTGTGCCACCTCTTC CGAGACATCGCCTCGCATAAGCCCGGTGTTTTCACCAAGGTCGGCATCGGAACTTTTATTGACCCTCGAAACGGCGGCGGCAAGGT CAACGACATCACCAAGGAAGACATTGTTGAGCTGGTGGAGATTAAGGGCCAGGAGTACCTCTTCTACCCCGCCTTTCCTATCCACGT GGCTCTGATTCGAGGAACCTACGCCGACGAGTCCGGTAACATCACTTTTGAGAAGGAAGCCGCTCCCCTCGAGGGAACCTCTGTCT GTCAGGCTGTTAAGAACTCCGGCGGAATTGTGGTCGTTCAGGTCGAGCGAGTGGTCAAGGCCGGAACTCTGGACCCCCGACATGTC AAGGTTCCTGGTATCTACGTGGATTACGTTGTGGTCGCTGACCCCGAGGATCACCAGCAGTCGCTGGACTGCGAGTACGATCCCGCC CTCTCTGGCGAGCATCGACGACCTGAGGTTGTGGGAGAGCCCCTGCCTCTCTCGGCTAAGAAGGTCATCGGCCGACGAGGAGCCAT TGAGCTGGAGAAGGACGTGGCTGTCAACCTCGGTGTGGGAGCTCCTGAGTACGTGGCTTCTGTTGCTGACGAGGAAGGCATCGTC GATTTCATGACCCTGACTGCCGAGTCTGGAGCTATTGGTGGCGTGCCTGCTGGAGGTGTCCGATTCGGTGCCTCCTACAACGCCGAC GCTCTGATTGATCAGGGCTACCAGTTTGACTACTACGATGGCGGAGGTCTGGACCTCTGTTACCTGGGTCTCGCTGAGTGCGATGAG AAGGGCAACATCAACGTGTCCCGATTCGGTCCCCGAATTGCCGGCTGTGGCGGCTTCATCAACATTACCCAGAACACTCCTAAGGTT TTCTTTTGCGGCACCTTCACTGCTGGTGGCCTGAAGGTGAAGATCGAGGACGGCAAGGTCATCATTGTCCAGGAAGGCAAGCAGAA GAAGTTCCTGAAGGCCGTCGAGCAGATCACCTTTAACGGAGACGTTGCCCTCGCTAACAAGCAGCAGGTGACCTACATTACTGAGC GATGTGTCTTCCTGCTCAAGGAAGACGGTCTGCACCTCTCTGAGATTGCTCCTGGCATTGATCTGCAGACCCAGATCCTCGACGTGAT GGATTTTGCTCCTATCATTGACCGAGATGCCAACGGCCAGATTAAGCTGATGGATGCTGCTCTGTTTGCTGAGGGTCTGATGGGCCT GAAGGAGATGAAGTCTTAG
Ecpct	ATGAAACCTGTCAAACCGCCTCGAATCAACGGCCGAGTTCCAGTTCTCTCTGCCCAGGAAGCCGTTAACTACATTCCCGATGAGGCT ACCCTCTGTGTCCTTGGCGCTGGAGGAGGCATTCTTGAGGCCACCACGCTGATTACAGCCCTGGCTGACAAGTACAAGCAGACGCA GACTCCCCGAAATCTGTCCATTATCTCTCCCACAGGACTTGGTGATCGAGCTGATCGAGGCATTTCCCCTCTGGCACAAGAGGGACT GGTGAAGTGGGCGCTGTGCGGTCATTGGGGCCAGTCTCCACGAATTAGCGATCTGGCCGAACAGAACAAGATTATTGCCTACAACT ACCCTCAGGGTGTGCTTACCCAGACCCTCCGAGCCGCAGCTGCCCATCAACCCGGCATTATCTCCGACATCGGCATTGGAACCTTTGT CGATCCCCGACAGCAGGGCGGCAAGCTGAACGAGGTGACCAAAGAGGACCTCATCAAGTTGGTTGAGTTCGACAACAAGGAGTAC CTTTACTACAAGGCCATTGCTCCCGATATTGCCTTCATTCGTGCAACCACCTGCGATTCCGAAGGCTACGCCACTTTTGAGGACGAGG TGATGTATCTCGACGCCCTGGTTATTGCGCAAGCTGTCCACAACAACGGTGGAATCGTGATGATGCAGGTCCAGAAGATGGTTAAG AAGGCCACGCTTCACCCCAAGTCCGTGCGTATCCCCGGTTACCTCGTCGACATCGTGGTCGTTGACCCGGATCAGTCTCAGTTGTATG GTGGCGCCCCAGTCAACCGATTCATCTCTGGCGACTTCACCCTCGACGACTCCACCAAGCTGTCGCTTCCCCTCAATCAGCGGAAGCT TGTCGCTAGACGAGCACTGTTTGAGATGCGGAAAGGAGCGGTCGGAAACGTGGGTGTCGGCATTGCCGATGGTATCGGACTCGTT GCCCGAGAAGAAGGTTGTGCTGACGACTTCATTTTGACCGTCGAGACTGGCCCTATCGGCGGAATCACTTCGCAAGGAATCGCCTTT GGCGCCAATGTCAACACCCGAGCCATCCTTGACATGACGTCCCAGTTTGACTTCTACCACGGAGGAGGTCTGGACGTGTGCTACCTG TCGTTTGCAGAAGTCGACCAGCATGGCAACGTTGGTGTCCACAAGTTCAACGGCAAGATCATGGGAACCGGAGGCTTCATCGACAT CTCCGCTACTTCCAAGAAGATCATCTTCTGTGGCACACTCACCGCTGGTTCTCTCAAGACTGAGATTGCTGACGGTAAGCTGAACATT

	GTGCAGGAGGGCCGAGTCAAGAAGTTCATCCGAGAACTGCCTGAGATCACCTTCAGCGGCAAGATCGCCCTGGAGAGAGGTCTGG ATGTGCGGTACATCACAGAGAGAGCTGTGTTTACTCTGAAAGAGGATGGTCTGCACTTGATCGAGATTGCTCCTGGTGTTGACCTGC AGAAGGACATCCTCGACAAGATGGATTTCACTCCCGTGATCTCCCCTGAGCTGAAGCTGATGGACGAGCGACTCTTCATTGACGCTG CCATGGGTTTTGTCCTCCCCGAGGCTGCGCACTAA
Enpct	ATGACCCACCCCCAGCAGGCCGTTCACGCCGCTTCGCTCCAGAACCCCGAGGCTTTTTGGTCCCATCACGCCCAGCAGCTCCATTGGC ACAAGAAGCCCTCGCGAGCCATTGGCCGATCTACCAAGACTCTGGCTTCTGGAGCCTCCCACGAGTCCTGGTCGTGGTTCCCTGACG GAGAGATCTCCACCACTTACAACTGTGTGGATCGACATGTCCTGAACGGCAACGGAGACAACGTGGCCATCATTTGGGATTCTGCTG TCACCGGCAAGAAGGAGAAGTACACTTACCGACAGCTGCTCGACGAGGTCGAGGTTCTGGCTGGTGTCCTCCGAGAGGAGGGCGT TAAGAAGGGAGACGTGGTCATCATCTACATGCCCATGATCCCTGCCGCTCTGATTGGAGCTCTCGCTGTCGCTCGACTGGGTGCTAT TCACGCCGCTGTTTTCGGCGGATTTGCCGCTAAGTCCCTGGCTCAGCGAATTGAGGCTGCTCGACCCCGAGCTATCCTCACCGCTTCT TGCGGTATTGAGGGCGCCAAGGGACCCATCGCTTACCGACCTCTGGTGGAGGGCGCTATTGAGGCCTCTTCCTTCAAGCCCGAGAA GGTCCTGATCTGGCAGCGAGACCAGCTCCGATGGAACAACCCTGATAAGCTGGGTGGCCAGCGAAACTGGAACCGACTCGTGAAG TCCGCCCGAATGCGAGGCATTCGAGCTGAGCCCGTGCCTGTCCGATCTACCGACGGACTGTACATCATCTACACTTCCGGTACCACT GGCCTCCCCAAGGGAGTTGTGCGAGAGGCCGGAGGTCACGCTGTGGGTCTGTCTCTCTCCATCAAGTACCTGTTCGACATTCATGGT CCCGGCGATACCATGTTTTGTGCCTCCGACATTGGTTGGGTCGTTGGCCACTCGTACATCCTGTACGCCCCTCTGCTCGTCGGAGCTA CCACTGTTCTCTTCGAGGGAAAGCCTGTGGGTACCCCTGACGCTGGTACTTTTTGGCGAGTGGTCGCCGAGCATAAGGCTAACGTCC TGTTCACCGCTCCCACTGCCCTCCGAGCTATTCGAAAGGAGGACCCTGATAACAAGCACTTTGAGAAGGTGGCCGGTGACAACAAC CTGCGACATCTCCGAGCCCTGTTCCTCGCTGGCGAGCGATCGGAGCCCTCTATCGTCCGAGCCTACCAGGACCTGCTCACCAAGCAC GCCGCTCGAGGAGCTCTGGTTGTGGATAACTGGTGGTCGTCTGAGTCGGGCTCTCCTATTTCCGGACTGGCTCTCCGATCGGCTGTC GGTCGAGTTCCTCCTCGATCGGACGAGTACGATGTGGCCCCCCTGGCTATCCGACCTGGATCTGCCGGTCTCCCCATGCCTGGTTTC GACGTCCGAGTCGTTGACGATGAGGGCAACGAGGTTGCCCAGGGCACCATGGGAAACATTGTGATGGCTACTCCCCTGGCCCCTAC CGCTTTCACTCGACTCTTTAACGACGATGAGCGATTCTACAAGGGATACCTGAAGCGATTTGGCGGACGATGGCTCGACACCGGCG ACGCTGGTATGATCGACCAGGATGGCTACATTCACGTGATGTCCCGATCGGACGATATCATTAACGTCGCCGCTCACCGATTCTCTA CTGGACAGGGTTCCATCGAGCAGGCCATTCTGTCGCACCCCGCCATTGGAGAGGCTTCTGTGGTCGGCATCCCCGACGCCCTGAAG GGACATCTCCCTTTCGCTTTTATCACCCTGAAGCAGTCCGGTGGTAACTCGCCTGCTCGACCTTCTGCTGAGCTGTTCAACTCCGTTAA CCGACTCGTGCGAGAGCAGATCGGAGCTATTGCCTCCCTGGGAGGAATGATCCAGGGCCAGGGAATGATTCCCAAGACCCGATCTG GCAAGACTCTCCGACGAGTGCTGCGAGAGCTCGTCGAGAACGGAGCCCGAGGTGAGTTCGAGAAGGAGGTTGCTGTGCCTCCTAC CGTGGAGGACCGAGGCGTTGTGGAGGTTGCCCGAGAGAAGGTGCGAGAGTACTTCGAGTCTCAGTCCGGATCGCCCAAGGCTAAG CTGTAG
EcprpE	ATGTCTTTCTCCGAGTTCTACCAGCGATCTATCAACGAGCCTGAGCAGTTCTGGGCTGAGCAGGCTCGACGAATTGACTGGCAGACC CCCTTCACCCAGACCCTGGACCACTCCAACCCTCCCTTCGCCCGATGGTTCTGTGAGGGCCGAACCAACCTGTGCCACAACGCTATCG ACCGATGGCTGGAGAAGCAGCCTGAGGCTCTGGCTCTGATTGCCGTCTCTTCCGAGACTGAGGAAGAGCGAACCTTCACCTTCCGA CAGCTGCACGACGAGGTGAACGCCGTCGCTTCTATGCTGCGATCCCTGGGAGTGCAGCGAGGTGACCGAGTGCTGGTCTACATGCC CATGATCGCCGAGGCTCACATTACCCTGCTGGCCTGTGCTCGAATCGGTGCCATTCACTCTGTGGTCTTCGGCGGATTCGCTTCTCAC TCCGTGGCCGCTCGAATCGACGACGCCAAGCCCGTGCTGATTGTGTCCGCTGACGCTGGAGCTCGAGGTGGCAAGATCATTCCCTA CAAGAAGCTGCTGGACGACGCTATCTCTCAGGCTCAGCACCAGCCCCGACACGTGCTGCTGGTGGACCGAGGCCTGGCTAAGATGG CTCGAGTGTCTGGACGAGACGTCGACTTCGCTTCTCTGCGACACCAGCACATTGGTGCTCGAGTGCCTGTGGCTTGGCTGGAGTCTA ACGAGACTTCCTGCATTCTGTACACCTCTGGTACCACCGGCAAGCCCAAGGGAGTGCAGCGAGACGTCGGAGGTTACGCTGTCGCC CTGGCTACCTCCATGGACACCATTTTCGGCGGAAAGGCCGGATCTGTGTTCTTCTGTGCTTCCGACATCGGCTGGGTGGTCGGACAC TCCTACATTGTCTACGCTCCCCTGCTGGCCGGCATGGCTACCATCGTGTACGAGGGACTGCCCACCTGGCCTGACTGTGGTGTGTGG TGGACCATTGTCGAGAAGTACCAGGTGTCTCGAATGTTCTCCGCCCCCACCGCTATCCGAGTGCTGAAGAAGTTCCCCACCGCCGAG ATTCGAAAGCACGACCTGTCTTCCCTGGAGGTCCTGTACCTGGCTGGAGAGCCTCTGGACGAGCCTACCGCTTCTTGGGTGTCCAAC ACCCTGGACGTGCCCGTCATCGACAACTACTGGCAGACCGAGTCTGGTTGGCCCATCATGGCCATTGCTCGAGGCCTGGACGACCG ACCTACCCGACTGGGCTCTCCCGGTGTCCCCATGTACGGATACAACGTCCAGCTGCTGAACGAGGTGACCGGAGAGCCCTGTGGCG TGAACGAGAAGGGTATGCTGGTGGTGGAGGGTCCTCTGCCTCCCGGTTGCATCCAGACCATTTGGGGCGACGACGGACGATTCGTC AAGACCTACTGGTCCCTGTTCTCTCGACCCGTGTACGCCACCTTCGACTGGGGAATCCGAGACGCTGACGGTTACCACTTCATCCTGG GCCGAACCGACGACGTGATTAACGTCGCCGGACACCGACTGGGTACCCGAGAGATCGAGGAGTCTATTTCTTCTCACCCTGGAGTG GCTGAGGTGGCTGTGGTCGGAGTCAAGGACGCTCTGAAGGGTCAGGTGGCCGTCGCTTTCGTGATTCCCAAGGAGTCTGACTCCCT GGAGGACCGAGACGTCGCCCACTCTCAGGAGAAGGCCATCATGGCTCTGGTGGACTCCCAGATTGGTAACTTCGGCCGACCCGCTC ACGTGTGGTTCGTCTCTCAGCTGCCCAAGACCCGATCCGGAAAGATGCTGCGACGAACCATCCAGGCCATTTGTGAGGGTCGAGAT CCCGGCGACCTGACCACCATCGACGACCCTGCTTCCCTGGACCAGATTCGACAGGCTATGGAGGAGTAG
SeprpE	ATGTCTTTCTCCGAGTTCTACCAGCGATCTATCAACGAGCCTGAGGCTTTCTGGGCTGAGCAGGCTCGACGAATTGACTGGCGACAG CCCTTCACCCAGACCCTGGACCACTCTCGACCTCCCTTCGCTCGATGGTTCTGTGGCGGAACCACCAACCTGTGCCACAACGCCGTCG ACCGATGGCGAGACAAGCAGCCTGAGGCTCTGGCTCTGATTGCCGTGTCTTCCGAGACTGACGAGGAGCGAACCTTCACCTTCTCTC AGCTGCACGACGAGGTGAACATTGTCGCCGCTATGCTGCTGTCCCTGGGCGTGCAGCGAGGCGACCGAGTGCTGGTCTACATGCCC ATGATCGCCGAGGCTCAGATTACCCTGCTGGCCTGTGCTCGAATCGGCGCTATTCACTCTGTGGTCTTCGGTGGCTTCGCTTCTCACT CTGTGGCTGCTCGAATCGACGACGCTCGACCCGCCCTGATTGTGTCTGCTGACGCTGGAGCTCGAGGCGGCAAGATCCTGCCCTACA AGAAGCTGCTGGACGACGCTATTGCCCAGGCTCAGCACCAGCCCAAGCACGTGCTGCTGGTCGACCGAGGTCTGGCTAAGATGGCC TGGGTGGACGGCCGAGATCTGGACTTCGCTACCCTGCGACAGCAGCACCTGGGTGCTTCTGTGCCCGTCGCCTGGCTGGAGTCTAA CGAGACTTCCTGCATCCTGTACACCTCCGGCACCACCGGAAAGCCTAAGGGAGTGCAGCGAGACGTCGGCGGATACGCTGTGGCTC TGGCTACCTCTATGGACACCATTTTCGGTGGCAAGGCTGGAGGTGTGTTCTTCTGTGCCTCTGACATCGGTTGGGTGGTCGGCCACT CCTACATTGTCTACGCTCCCCTGCTGGCTGGTATGGCCACCATCGTGTACGAGGGCCTGCCTACCTACCCTGACTGCGGAGTGTGGT GGAAGATTGTCGAGAAGTACCAGGTGAACCGAATGTTCTCCGCTCCCACTGCCATCCGAGTCCTGAAGAAGTTCCCCACCGCCCAGA TTCGAAACCACGACCTGTCTTCTCTGGAGGCTCTGTACCTGGCTGGAGAGCCTCTGGACGAGCCTACCGCCTCTTGGGTGACCGAGA CTCTGGGCGTGCCCGTCATCGACAACTACTGGCAGACCGAGTCCGGTTGGCCCATTATGGCTCTGGCTCGAGCTCTGGACGACCGAC CTTCCCGACTGGGTTCCCCCGGAGTCCCTATGTACGGATACAACGTCCAGCTGCTGAACGAGGTGACCGGAGAGCCCTGTGGTATC AACGAGAAGGGCATGCTGGTCATCGAGGGACCTCTGCCTCCCGGTTGCATCCAGACCATTTGGGGAGACGACGCCCGATTCGTCAA GACCTACTGGTCCCTGTTCAACCGACAGGTGTACGCTACCTTCGACTGGGGCATCCGAGACGCCGAGGGATACTACTTCATTCTGGG TCGAACCGACGACGTGATCAACATTGCCGGCCACCGACTGGGAACCCGAGAGATCGAGGAGTCTATTTCTTCCTACCCTAACGTGGC TGAGGTGGCTGTGGTCGGCATCAAGGACGCTCTGAAGGGACAGGTGGCCGTCGCTTTCGTCATTCCCAAGCAGTCTGACACCCTGG

	CTGACCGAGAGGCTGCTCGAGACGAGGAGAACGCTATCATGGCCCTGGTGGACAACCAGATTGGACACTTCGGTCGACCCGCTCAC GTGTGGTTCGTCTCTCAGCTGCCCAAGACCCGATCCGGCAAGATGCTGCGACGAACCATCCAGGCCATTTGTGAGGGTCGAGATCC CGGCGACCTGACCACCATCGACGACCCTGCTTCCCTGCAGCAGATCCGACAGGCCATTGAGGAGTAG
YlACS2	ATGTCTGAAGACCACCCAGCCATCCACCCACCCTCCGAGTTCAAGGACAACCACCCCCACTTCGGAGGCCCCCACCTCGACTGTCTGC AGGACTACCACCAGCTGCACAAGGAGTCCATTGAGGACCCCAAGGCCTTCTGGAAGAAGATGGCCAACGAGCTCATCTCCTGGTCA ACCCCCTTTGAAACTGTGCGATCTGGCGGCTTCGAGCACGGCGACGTGGCCTGGTTCCCCGAGGGCCAGCTCAACGCCTCCTACAAC TGTGTGGATCGACACGCCTTTGCCAACCCCGACAAGCCCGCCATCATTTTTGAGGCCGATGAGCCGGGCCAGGGCCGAATCGTCAC CTACGGCGAACTGCTGCGACAGGTGTCTCAGGTCGCAGCCACCCTGCGATCCTTCGGCGTCCAGAAGGGCGATACTGTGGCCGTCT ACCTGCCCATGATCCCCGAGGCCATTGTCACTCTGCTGGCCATCACCCGAATTGGCGCTGTCCACTCGGTCATCTTCGCCGGCTTCTC CTCCGGTTCTCTGCGAGACCGAATCAACGACGCCAAGTCCAAGGTTGTCGTCACCACCGACGCCTCCATGCGAGGAGGCAAGACCA TCGACACCAAGAAGATTGTCGATGAAGCCTTGCGAGACTGCCCCTCTGTTACCCACACCCTGGTCTTCCGACGAGCAGGTGTCGAGA ACCTGGCCTGGACTGAGGGCCGGGACTTCTGGTGGCACGAGGAGGTCGTCAAGCACCGACCCTACCTTGCCCCCGTCCCCGTTGCC TCCGAGGACCCCATCTTCCTGCTTTACACCTCTGGATCCACCGGCACCCCCAAGGGTCTGGCCCACGCTACCGGTGGCTACCTGCTTG GTGCTGCCCTGACCGCCAAGTACGTGTTTGACATCCACGGAGACGACAAGCTGTTCACCGCTGGAGACGTTGGCTGGATCACCGGC CACACCTACGTGCTCTACGGTCCTCTGATGCTCGGAGCCACCACTGTTGTGTTCGAGGGAACCCCTGCCTACCCCTCCTTCTCGCGAT ACTGGGACATTGTCGACGACCACAAGATCACCCACTTCTACGTGGCTCCCACCGCCCTGCGTCTCCTGAAGCGGGCCGGCACCCATC ACATTAAGCACGACCTGTCGTCTCTGCGAACCCTCGGCTCTGTGGGTGAGCCCATTGCCCCCGACGTGTGGCAGTGGTACAACGACA ACATTGGCCGAGGCAAGGCCCACATCTGTGACACCTACTGGCAGACCGAGACTGGCTCGCATATCATTGCCCCCATGGCCGGCGTG ACCCCCACCAAGCCCGGTTCTGCTTCCCTGCCTGTCTTTGGAATTGATCCCGTTATCATTGATCCCGTGTCTGGCGAGGAGCTCAAGG GTAACAACGTTGAGGGTGTTCTTGCCCTGCGATCTCCCTGGCCCTCCATGGCCCGAACCGTGTGGAACACCCACGAGCGATACATGG AGACCTACCTGCGGCCCTACCCCGGCTACTACTTCACCGGTGATGGTGCTGCCCGAGACAATGACGGCTTTTACTGGATCCGAGGCC GAGTCGACGACGTTGTCAACGTTTCTGGCCACCGTCTTTCCACCGCCGAGATTGAGGCTGCTCTCATTGAGCACGCTCAGGTGTCTG AGTCTGCCGTTGTTGGTGTCCATGACGATCTGACTGGCCAGGCCGTCAACGCCTTTGTGGCTCTCAAGAACCCCGTCGAGGATGTGG ACGCTCTGCGAAAGGAGCTTGTTGTGCAGGTGCGAAAGACCATTGGACCCTTTGCTGCTCCCAAGAATGTCATCATCGTGGACGATC TGCCCAAGACTCGGTCTGGCAAGATCATGCGACGAATTCTGCGAAAGGTGCTTGCTGGCGAGGAGGACCAGCTCGGAGACATTTCC ACTCTTGCTAACCCCGACGTTGTCCAGACCATCATTGAGGTTGTTCACTCGTTGAAAAAGTAA
RebktB	ATGACCCGAGAGGTGGTGGTGGTCAGCGGTGTTCGAACCGCCATCGGCACCTTCGGCGGCTCCCTGAAGGACGTCGCCCCCGCTGA GCTGGGCGCTCTGGTTGTCCGAGAGGCCCTGGCCCGAGCTCAGGTGTCTGGTGACGACGTGGGCCACGTCGTCTTCGGCAACGTCA TTCAGACCGAGCCCCGAGACATGTACCTGGGACGAGTCGCTGCCGTCAACGGCGGCGTCACCATTAACGCCCCCGCCCTCACCGTG AACCGACTGTGCGGTTCCGGCCTGCAGGCCATCGTCTCCGCCGCTCAGACCATCCTGCTCGGCGACACCGACGTTGCCATCGGCGGC GGTGCCGAGTCCATGTCCCGAGCTCCCTACCTGGCCCCCGCCGCTCGATGGGGTGCTCGAATGGGTGACGCCGGTCTGGTTGATAT GATGCTGGGAGCCCTGCACGACCCTTTCCACCGAATTCACATGGGCGTTACCGCTGAGAACGTCGCCAAGGAGTACGACATCTCCC GAGCCCAGCAGGACGAGGCTGCCCTGGAGTCCCACCGACGAGCCTCCGCTGCCATCAAGGCCGGCTACTTCAAGGACCAGATCGTC CCCGTCGTTTCCAAGGGCCGAAAGGGCGACGTCACCTTCGACACCGACGAGCACGTCCGACACGACGCCACCATCGACGACATGAC CAAGCTGCGACCCGTCTTCGTCAAGGAGAACGGCACCGTGACCGCCGGAAACGCCTCCGGACTGAACGACGCCGCCGCCGCTGTCG TGATGATGGAGCGAGCCGAGGCCGAGCGACGAGGACTGAAGCCCCTGGCCCGACTGGTCAGCTATGGTCACGCCGGTGTGGACCC CAAGGCTATGGGAATTGGTCCCGTCCCCGCCACCAAGATCGCCCTGGAGCGAGCCGGACTGCAGGTGTCCGACCTGGACGTTATCG AGGCCAACGAGGCTTTCGCTGCTCAGGCCTGCGCCGTTACCAAGGCCCTGGGCCTGGACCCCGCCAAGGTCAACCCCAACGGATCT GGCATCTCCCTGGGCCACCCCATCGGCGCTACCGGTGCTCTGATCACCGTCAAGGCCCTGCACGAGCTCAACCGAGTCCAGGGTCG ATACGCCCTGGTCACCATGTGCATCGGAGGCGGCCAGGGCATCGCTGCCATCTTCGAGCGAATCTAA

Chapter 6. *De novo* production of OCFAs from glucose

Gene	Sequence
AAT2 *(YALI0B02178g)*	GGTCTCtAATGCTCCGAACCATCGCCCGAACCCACGCTGTGGCCGCCACCAAGGTCGTGCGAGCCTCCACTTTTTCTGCCACCCCC GCCGCCTCTTTTGTGCGATTCCAGTCCGTCTGGGCCAAGGTCCCCCAGGGTCCCCCCGACGCCATTCTCGGAATCACCGAGGCGT TCAAGAAGGACGCCTTTGAGCAGAAGATCAACCTCGGTGTTGGCGCCTACCGAGATGACGGCGGAAAGCCCTTCGTTCTTCCCT CCGTCCGAGAGGCCGAGAAGGAGGTGGTGAACAAGGCCCTCGACAAGGAGTACGCCCCCATCACCGGAGTCCCCGCCTTCACC AAGGCTGCTGCCGAGCTCGCTTACGGCGCCGACTCCCCCGCCGTCCTCGAGGACCGAATTGCCATCACCCAGACCATCTCCGGTA CCGGTGCTCTGCGAATCGGAGCCGAGTTCCTCAACAAGTTCTACTCCTCCAAGAAGATTCTGCTCCCCCAGCCTTCTTGGGCTAAC CACAAGGCCGTTTTCACCGCCGCCGGCCTCGAGCCCGCCACCTACCGGTACTACGACCCCAAGAACATTGCCCTCGACTTTGAGG GTCTGCTCGCCGACCTGGAGGCTGCCCCCAACGGAACCGCCGTCCTTCTGCACGCCTGTGCCCACAACCCCACCGGTGTTGACCC CACTCCCGAGCAGTGGCGAAAGATTGAGGAGGTCGTCAAGGCCAAGGGCCACTTCCCCTTCTTCGACATGGCCTACCAGGGCTT TGCCACCGGTGACGTCAACCGAGACGCCTACCCCATCCGATACTTTGTCGAGCAGGGCCACGAGGTTGCTCTGTGCCAGTCCTTC GCCAAGAACATGGGTCTTTACGGTGAGCGAGTCGGTGCTTTCTCTCTTGTCTGCCAGGACACCGCCGAGAAGAACCGAGTTGAC TCTCAGCTGAAGATTATCATCCGACCCTTCTACTCCAACCCCCCCGTCCACGGTGCCCGAATCGCCGCCACCATCCTCAACAACCC CGAGCTCAAGAAGCAGTGGCTCGGTGAGGTCAAGCAGATGGCCGACCGAATGATCAAGATGCGAGCTCTGCTCAAGGAGAACC TCGAGAGACTTGGCTCCCAGCAGAACTGGGACCACGTCACCTCTCAGATTGGTATGTTCTGCTACACCGGTCTGTCTCCTGAGCA GGTTGAGCGACTTGCCAAGGAGTTCTCCGTCTACGGAACCAAGGACGGCCGAATCTCCATTGCCGGAATCACCTCTCAGAACGT CGGCCGACTGGCCCAGGCTATCTACGAGGTGACCAAATAG
HOM3 *(YALI0D11704g)*	ATGACCTGGATCGTCCAAAAGTTCGGAGGAACATCGGTCGGCAAGTTCCCCAAAAACATCTGCAACGACATTGTGCAGCAGTAC TCGCAGTCTTACGACGTGGCTGTGGTCTGCTCAGCCCGATCTACCGGAACCAAGGCCGAGGGAACCACCACCCGTCTTATTGCT GCTGCTGACTCTGCTCTTCTCGGATCAGACGCCTACAACCCCATCATTGAGTCTATCCGAGAAGACCATCTTGCAGCTGCTAAGC GTGACGTCAAGAACCCCGAACTGCTCGAAAACCTCAATGCCGACATCAACGGCGAGTGCGACCAGCTGTTGCGAATCCTGGCTG CTGCTGAAATCATTTCTGAGATCTCCCCCCGAACACTCGACTCCATCATGGCCATTGGGGAGAAGCTCAGTTGCATGTACATGAC TGCAGTTATGCGGGACGCTGGTGTTAACGCACGGTACTTTGACCTGTCTCACGCAGTTACCACCACCAATGCCGAAGATCCCAAG TTCTATTCGGACCTCGGACGTGTTCTGGGCGAGATCATCACTCTTCCCGGCTCTCCTAACACGGACTCTGCTCCTGTCAAGGGAA ACATGGTGCCCGTGTTGACCGGTTTCTTTGGCCCCGTCAAGGGAGGCCTCCTAAGCCAGATCGGACGAGGATACACCGATCTGT GTGCTGCTCTGGTGGCTGTGGGTCTGGACGCCAAGGAGCTTCAGATCTGGAAAGAAGTCGATGGTGTCTTCACTGCCGATCCCA GAAAGGTGTCCACTGCCCGTCTGCTGCCCATCATTACCCCCGAGGAGGCCGCTGAGTTGACCTACTACGGATCTGAAGTCATCCA CCCCTTCACCATGGAGCAGGTCATTAAGGCCCATATTCCTATCCGAATCAAAAACGTGGAAAACCCCCTGGGAGGAGGAACTAT CATCTACCCATCTGCTGACGGCTCGGGCAACCTGAAAACGAAAAACAATGTCCAGGATCTGGCTTCTTCCACCGACTCGCTAACT TCTCTGTCCTCTGGCTCATTCACTCCCCAGGTGTCTGCTCCTGTAGATGACGAGAAGAAGCCTACAGCTGTCACTACCAAATCGAA TATCACCGTTCTCAACGTTCATTCGAATAAGCGAACCAAGAGTCATGGCTTCTTGAACAAGATCTTTGCTACTCTCGACCAGCAGA AACTTGTCGTCGATCTCATCTCCACTTCTGAGGTCCATGTGTCGATGGCCTTCCATGCTCCCGACTCCAACCTCAAGCAGGCTGTG GAGGAGCTGCGAAAGTACGGCACAGTAGATGTGAAGCGAGGCATGACCATTGTGTCGCTGATCGGAACGAGAATGAAGGCCA TGGTCGGATGTGCAGGCATGTTCTTCTCTACTCTGGCCCAGGCAGGTATTAACATTGAGATGATTTCTCAGGGAGCCAACGAAAT TAACATCTCGTGTGTTATTGAGGAAAAAGACGCGCTGAAGGCGTTGAACGTCATCCACCAGGGCTTGTTGTGTCCTAGACGGAA AATCTAG
HOM2 *(YALI0D13596g)*	ATGGTCAAGACTAAAAAGGCTGGAGTTCTCGGTGCCACCGGATCTGTGGGCCAGCGATTCATTCTGCTGCTCTCGGAGCACCCC GAGTTCGAGCTGTCTGTTCTGGGAGCTTCTTCTCGATCTGCCGGCAAGAAGTACGTTGATGCTTGTGACTGGAAGCAGACGGAT GTTCTCCCCGAGGCTGCTGGCCAGACCGTGGTCAAGGAGTGCACTCCCGAGAACTTTGCCGAGTGCGACGTGGTCTTTTCCGGC CTGGACGCCGACTACGCTGGTGACATTGAGAAGGCTTTTGTTGACGCCGGCCTGGTGGTGATTTCCAACGCCAAGAACTACCGA CGAGAGCCTACCGTGCCCCTGGTTGTCCCCACCGCCAACTCCGAGCACCTGGACTGCTTTTCCGAGCGAGTGGTTGCTGCCCGAA AGGCTGGCAAGAAGCAGGGCTACCAGATCTGCAACTCCAACTGCTCTACCGCCGGCCTTGTGATCCCTCTCAAGGCTCTGGTCG ACGCCTTTGGCCCCATTGACAAGGTCATTGTCACAACCATGCAGGCCGTTTCCGGTGCTGGCTTCTCTCCCGGTGTCCCCTCCATG GATGTTCTCGATAACCTCATCCCTTACATTTCCGGCGAGGAGGACAAGATGGAGTGGGAAACTAAGAAGATTCTGGGCCACGTC AGCGCCGACGGAGCCACCTTTGAGAACATTCCCGACTCGGAGCTCAAGGTGTCTGCCACCTGCAACCGAGTGGCTGTCATTGAC GGCCACACCGAATGTGTCGCCTTCTCCTTCAAGTCCGACAAGAAGCCCTCCGTCGACGAGGTCAAGAAGGTGCTCAACGACTAC GTGTCCGAGCCACAAAAGCTGGGCTGTCCCTCCGCCCCCAAGAAGGCCATCCACCTGCTTGAGCAGCCCGACAGACCCCAGCCC CGTCTGGACCGAAACCGAGACAACGGCTACGCCGCATCTGTCGGCCGAGTCCGAGAGGACGCCGTGCTCGACTTCAAGTTCACC GTTCTATCCCACAACACTGTCATTGGTGCTGCCGGATCCGGTATTCTGATTGCCGAGACTCTGCTGAAGAAGAATCTCATCTAG
HOM6 *(YALI0D01089g)*	ATGTCCAAGGCTGTTAACATTGCTATTATCGGAACCGGCCTTGTCGGAAAGGCCTTCATCAACCAGCTTGCCGCCGTCAAATCCT CCATTGCCTACAATGTCGTTCTCATCGCTCGATCTTCTAAGACCCTCATCTCCAAGGACTTCAAGCCCCTCTCCCTGACCAACTGGG AGTCCGAGCTCAACTCCTCCCCCGTCGGAGCCATGTCTTTCACCGAGATCAACGACTTCCTCAAGAAGTCTCCTCTGCCCGTCATT CTGGTTGACAACACCTCCAACGAGGCCCTGGCCAACGAGTACCCTACTTTTGTCAACTCTGGTATCTCCATTGCCACCCCCAACAA GAAGGCCTTCTCCTCCGATCTTAAGACCTGGGAGGCCATCTTCGGCGGCGCTGAGAAGTCCGGCGCTCTTGTCTACCATGAGGC TACTGTCGGTGCCGGTCTGCCCGTCATCTCTACCCTCAATGACCTCATTGCCACCGGTGACGAGGTCGAaACtGTCGAGGGTATT GTTTCCGGAACTCTGTCTTACATCTTCAACGAGTTCTCCACCCTCTCTGGCTCTGACGTTAAGTTCTCCGACGTTGTCACCAAGGCC AAGCAGCTCGGATACACCGAGCCCGATCCCCGAGAGGATCTCAATGGTCTGGATGTCGCCCGAAAGGTCACCATTCTGGCTCGA CTCTCTGGCTTCGACGTGGAGTCTCCCACTGCCTTCCCTGTTCAGTCCCTGATTCCCAAGCCCCTCGAGACTGCCTCTTCCGCCGAT GAGTTCCTCCAGAAGCTCCCCGAGTATGACGCCGACCTTGCTAAGCTTCGAGACGAGGCTTTCGCCGAGAAGAAGGTTCTCCGA TTCGTCGGTTCCATCAACAAGGGCACCGGCAAGGTCGAGGTTGGTATTCAGAAGTACGATGCCTCTCATCCCTTTGCCTCCCTCA AGGGCTCCGATAACATTATCGCCTTCAAGACCAAGCGATACCCCAACCCTCTGGTCATCCAGGGAGCTGGTGCTGGAGACGAGG TCACTGCTGCTGGTGTTCTTGCCGACGTTTTCAAGGCTGCCCAGCGACTTGCCAAATAG
THR1 *(YALI0F13453g)*	ATGTCTCGAAAGTTTGAAATTTCTGTGCCCGCGTCCTCGGCCAACATTGGTCCCGGTTTCGACGTGCTCGGCCTCGCTCTGGCCA AGTTCCTCGTGATCAACGTCGAAATCGACTCGTCCAAAACCTCGGACCGAAAGGACCCCAACAACTGCATTATCACCTACGAGG GCGAGGGAGCTGACGGCGTTCCTCTCGACAGTGACCACAACCTTGTCACCCGAGTGGCTCTCTACGTTCTGCGATGCAACGGCA TTAGATCTTTCCCCTCGGGCACCTTTGTGCACGTCAACAACCCCATTCCTCTGGGTCGAGGTCTGGGCTCGTCTGGAGCGGCTGT GGTGGCTGGTGTCATGCTCGGAAACGAGGTCGGAAAGCTGGGCTTCTCCAAGCAGCGAATGCTCGACTATTGTCTCATGATCGA

	GCGACATCCCGACAACATCACCGCAGCCATGATGGGCGGCTTTGTGGGTTCTTACCTGCGGGAGCTGTCTGCTGCTGATCTCGA GCGAGTGGAGATCCCCCTTGCTGAGGTTCTCCCCGAGCCTGCCGGAGGCCGAGATACCGGCCTTGTACCCCCCGAGCCTCCTCTC AACATCGGACACCACATCAAGTACGACTGGTGCCGAGAAATCAAGGCCATTGTGGTGATTCCCAACTTTGAAGTGTCCACCGCCT CGGCCCGAGGAGTGCTCCCCACTGCCTACACTGCTTCAGATATGATCTTCAACCTGCAGCGACTGGCCGTTCTCACCACCGCCCT CACCCGATCGCCTCCCCAGGCCGACCTCATCTATCAGGCCATGCAGGACAAGGTCCACCAGCCCTACCGAAAGACCCTGATCCCC GGTCTGCCTGAGATTCTGGCCTCCGTTACCCCCAAGACTCACGAGGGTCTGCTGGGCATCTGTCTGTCTGGCGCTGGGCCCACCA TTCTGGCTCTGGCCACCGACAACTTCGAGACAATCGCCAAGGAGATTGTGTCGCGATTCAACAAGGAGGGCATCGACTGCCGAT GGGAGGTCCAGGAGTTGGCCTACGACGGTTCCAACGTGAAGCAGCTCTAA
THR4 *(YALI0F23221g)*	ATGACCACCTACTTTTCCACGCGATCATCCAATGAGCCCATTTCCTTCGAGGCTGCCGTCATGAAGGGTCTGGCCCCCGATGGAG GTCTTTACATTCCCACCTCCATCCCCAAGCTGCCCTCCGACTTCCTCACCAAGTGGGCCGATCTGAGCTTTGCCGAGTTGGCCTTC GAGATTCTCAGTCTGTACATTCCCGAGTCTGAGATCTCGCGAGCCGACCTGAAGGAGCTTGTCACCCGGTCCTACTCTACTTTCC GATCCGACGAGGTCACCCCCGTGGTTGAGCTCAGCAAGGAGAAGCAGCTTTATCTGCTGGAGCTATTCCACGGCCCCACCTACG CCTTCAAGGACGTGGCTCTGCAGTTTGTTGGCAACCTGTTTGAGTACTTCCTGACCCGAAAGAACGTTGGCAAGGAGGGCACCG ACCGAGATACTCTTACCGTCGTGGGAGCCACCTCTGGTGACACCGGCTCCGCCGCCATCTACGGTCTGCGAGGCAAGAAGGACG TTTCTGTCTTCATTCTGTACCCCACCGGCCGAGTCTCCCCCATCCAGGAGGACCAGATGACGACTGTCTCTGACGCCAACGTGCAC ACTATCTCCGTTGCTGGTACCTTTGACGACTGCCAGGACATTGTTAAACAGGTCTTTGGTGATGCCGAGTTTAACGCCAAGCACCC ACGTCGGTGCCGTCAACTCTATCAACTGGGCCCGAATCCTCGCCCAGATCACCTACTACTTCCACTCCTTCTTCCAGGTCCAGAAG AAGTTCGGCACCTCCTCCGCCATCAAGTACTCTGTCCCCACTGGTAACTTTGGAGACATTCTCGCTGGCTTTTACGCCCGACGAAT GGGTCTGCCCATCCAGGAGCTCACCATCGCCACTAACTCCAACGACATTCTCGACCGATTCCTCAAGACTGGCTCCTACTCGAAAT CCGACGGAGCTTCTGCCGAGGTCCATGCCACCCTTTCCCCCGCCATGGACATCCTCGTCTCCTCCAACTTTGAGCGATTCCTTTGG TACGTTGCCCGAGAGAACGTTGCTTCTTCGGACGCTGAGGCTGGAGCCACCCTCAACAAGTGGATGCAGTCTCTCAAGACCGAC GGTGTCATCACTGTCGACGCCAAGGTCCTTGGAGCCGCAAAGTCCGAGTTTTCCTCCGAGCGAGTCTCCGACGAGCAGACTCTC GAAACTATTAAGGATGTGTTTACCAACATTTCCAAGGGCTATATTTTGGACCCCCACTCCTCTGTTGGTGTCACTGCCGCTCTGCG AAAGCTTGAGGGAACCGACTCCGTGTACATTGCTCTGTCAACCGCTCACCCCGCCAAGTTCTCTGACGCCGTCGACCAGGCCCTC AAGGGCCTGGAGGGCTACAACTTTGAGCGAGATGTTCTCCCTCAGGAGTTCAAGGACTTTGCTAACAAGGACAAGAAGAAGCT GTTCAGCAAGGCCGACGTCAAGGAGGTCGAAAGCATCATTGAGGAGGAGCTGGCCAAGGAGAAGAAGCAGTAA
ILV1 *(YALI0D02497g)*	ATGTCCGAACCCGACTATCTGAAGCTCATCTTGAAGAGCCGCGTCTACGACGTGTGCAAGGAAACACCTGTGACATCTGCTCATG GTCTGAGCGAGAAGCTGGGCTGCAAAGTGCTGCTCAAGCGGGAAGATCTTCAGCCGGTTTTCTCGTTCAAGCTGCGAGGAGCCT ACAACATGATTTCGCAGCTGAGTGACGAGGAAAAGTGGAAGGGAGTGATTGCGTGTAGCGCCGGTAACCATGCCCAAGGAGTC GCCTTTTCAGCCAACTATCTCAACATTCCAGCGACTATTGTCATGCCGTTGGCCACTCCTTCCATCAAGCACAGTAATGTTTCTAGA CTAGGTGGCAAGGTGGTTTTGCACGGAGACGATTTTGATTCGGCCAAGGCCCACTGCAAGCAGCTGTGTGAGAAATATGGACTC ACAGATATCCCTCCCTTTGATCACCCCCACGTGATTGCAGGCCAGGGAACTATTGGTATGGAGATTCTTCGTCAGGCGTCGGACA ACCTGAAGGCCGTGTTTATCTGTGTTGGAGGCGGCGGTCTGATTGCCGGAGTAGGCGCTTACATCAAGCGGATCCAGCCCGATG TCAAAATCATTGCCGTGGAAACTTATGATGCATGTGCTCTGAAACAGAGTCTCATCAAGGGCGAACGGGTGACTCTGCCTGAAG TCGGTCTGTTTGCCGATGGAGCTGCTGTCAAGCTGTGTGGCGAGGAGACTTTCCGACTCTGTCGCAAGTACGTTGATGGAGTTG TGCTTGTGAACACGGACGAGATCTGCGCCGCTATCAAAGATGTATTTGAGGCCACTAGATCGGTGGTGGAGCCTGCTGGTGCTC TGTCGGTGGCTGGCCTGAAGAAGTACTGCTCCGACCCCTCGGCCATTTGGTGGTCACCTGAGTCCGATTCCGCAAAGGCCAATG GTATCCCCACTAACGTTGCCATCTCAGAAACCGACGAGTATCTGTCAATTCTCTCTGGAGCCAACATGAACTTTGACCGGCTTCGA TTCGTGGCCGAACGAGCTATGCTTGGAGAAGGAACCGAAGTCTTCATGGTCGTCACCATCCCCGATATTCCCGGAGCGTTTGAA AAGCTGCACGAGATCATTCTCCCCAGAGCTGTCACCGAGTTCTCCTACAGAAAGAAGTCCACTGCTGAGAACGAAGACGCTAAC ATTTTTGTGTCTTTTTCAGTCAAAAACCGACAAGAGGAAATTGCAGACGTGCTGGAAAAGCTGCAAGCTGCCGGTATGAGCGGA GTCGACGTTTCAGACAACGAACTGGCAAAGACCCACGCTAGATATCTCGTGGGAGGCCAGCCAGACGTGCCTAATGAGAGACT GTTCCGGTTCGAGTTCCCTGAACGACCCAACGCGCTCAAAAACTTCCTCGGAGGTGTCCAGACAAAGTGGAATATCACCCTGTTC CACTACAGAAACAACGGCAGTGATATTGGAAAGATTCTGACAGCCTTGGACGTGCCGGAAAGCGACAATGAGGCGCTCAAGGA GTTTCTTGAGAAGCTCAAGTACCCCTTTGTGGAGGAGACAGACAATGTGGTGTACAAGCAGTTTATGAAGTAA
PDA1 *(YALI0F20702g)*	ATGTCCATCGCTGACGATGCCGACAAGAAATGCACAATCACGCTCAAGGAGGATTCTTACACCACCTACATGCTTGATTCTCCCC CTCCTCTCGAGTTCGAGATGACCAAGGGTGAGCTTCTGCAAATGTACAAGGACATGGTGACCGTCCGACGACTCGAGATGGCTG CTGATGCCCTCTACAAGGCCAAGAAGATCCGAGGTTTCTGCCATCTGTCTACTGGTCAGGAGGCTGTTGCCGTCGGTATCGAGA AGGCCATCGACCACGACGATTCTGTCATCACCGCCTACCGATGCCACGGTTTCGCCTACATGCGAGGTGCCTCTGTCCGAGCAAT CATCGCCGAGCTGCTCGGAAAGCGAACCGGTGTCTCCTACGGTAAGGGTGGcTCCATGCACATGTTCACCGAGGGTTTCTACGG AGGAAACGGTATTGTCGGAGCCCAGGTCCCCGTCGGAGCTGGcCTCGCCTTCGCCCACAAGTACCTCGAGCAGACCGGAAAGG CCACCTTTGCCCTGTACGGTGACGGTGCTTCCAACCAGGGTCAGATCTTCGAGGCCTACAACATGGCCAAGCTCTGGGACCTCCC CTGCATCTTTGCATGCGAGAACAACAAGTACGGAATGGGTACCGCTGCTGCTCGATCCTCTGCCCTGACGCAGTACTACAAGCG AGGTCAGTACATTCCCGGcCTCAAGGTTAACGGAATGGACATTCTGTCCGTCTACCAGGGAGCCAAGTTCGCCAAGGAGTGGAC CACACACGGCAAGGGTCCCCTCGTCATGGAGTTCGAGACtTACCGATACGGTGGTCACTCCATGTCCGATCCCGGAACCACCTAC CGAACCCGAGAGGAGATCCAGTACATGCGATCCCACAACGATCCTATTTCTGGcCTCAAGGCCCACATCCTGGAGCTTAATTTCG CCACTGAGGACGAGCTTAAGTCTGTGGACAAGGCTGCTCGAGCTATGGTTGACAAGGAGGTTGCCCTTGCTGAGTCCGACCCTG CTCCTGAGGCTACTGCCAAGGTTCTGTTTGAGGATATCTACGTTCCCGGCACCGAGCCTCCTGTGATCCGAGGCCGAATCCCTTC CGAGGACTACTACTTTAAGAACTAA

PDB1 *(YALI0E27005g)*	ATGGCCTCAACCGAAGGCGGCGCCACTAACATGACTGTCAGAGACGCCCTCAACACCGCACTGCGAGAGGAGATGGACCGAAA CGATAATGTTTTCATCATGGGTGAGGAGGTCGGCCAGTACAACGGTGCCTACAAGGTCACCAAGGGCCTTCTCGACAAGTTCGG CGAGAAGCGAGTGGTTGACACCCCTATCACCGAGATGGGTTTCGCCGGTGTTTGTGTCGGTGCCGCCCTGGCCGGcCTCACCCC CGTCTGCGAGTTCATGACCTGGAACTTCGCCATGCAGGCCATTGATCAGATCATCAATTCCGGTGCCAAGACCTACTACATGTCC GGAGGTACCCAGCAGTGCAATGTCACCTTCCGAGGTCCTAACGGTGCCGCCGCTGGTGTTGCTGCCCAACACTCTCAGGATTTCA CCGGGTGGTACGGCCAGATTCCCGGcCTCAAGGTCGTCTCTCCCTACAGCTCTGAGGATGCCAAGGGTCTGCTCAAGGCCGCCA TCCGAGAtCCCAACGTGACTGTTTTCCTCGAGAACGAGATCATGTACGGAGAGTCTTTCCCCATGTCTGAGGAGGCCATGTCCCC CGACTTCGTTCTGCCCCTTGGAAAGGCCAAGATTGAGCGAGAGGGTAAGGATATCACTCTTGTCGGTCACTCCCGAAACGTCGA GACtGCCCTCAAGGCCGCCGACCTCCTCAAGAAGCACCACAACGTCGATGCCGAGGTCATTAACCTGCGAACTGTCAAGCCTCTC GACACTGAGACtATTTTCAACTCCATCAAGAAGACTAACCGACTTGTCTCTGTCGAGGCTGGCTTCCCCGCCTTTGGCATGGGCTC CGAGCTCTGTGGTGTCGTCAACGACTCCTGGGCCTGGGATTACCTTGATGCCCCCATCCAGCGAGTTACCGGAGCTGAGGTTCCC ACTCCTTACGCCATTGAGCTTGAGAACTTCGCCTTCCCCACACCCGAGATTGTTGTCAAGGCTGCCAAGGACGCCCTCTACATTGA GGAGTAG
LAT1 *(YALI0D23683g)*	ATGGCCGCCAAGTCCTGGCCTAGCCACACAGTCATCGACATGCCCGCCCTGTCCCCTACCATGACCCAGGGTAACATTGGCGCCT GGCAAAAGTCTGTCGGCGACGCTCTTGCTCCCGGCGAGGTTCTCGTCGAGATTGAGACTGACAAGGCCCAGATGGACTTTGAGT TCCAGGATGATGGCTACCTGGCCAAGATTCTGCTCGACGCCGGAGCCAAGGACATTGCCGTTGGCACCCCCATTGGTGTCTACG TCGAGGACGAGGCCGACGTGGCTGCCTTCAAGGACTTCACCATTGACGACGCCGGAGGAGTCCCCAAGCCTCCCAAGACCGAG GAGCAGAAGGAAGAGGAGGAATACGAGGCCGAGAAGGCCGAGAAGGCCGAGAAGGAGGCCGAGGCTTCCAAGGAGACTGC TTCTCCCGCCCCCTCTTCTCAGTCCTCTGCCCCTGCTGCCCCCACTCCCCCCTCTTCTCGAATCTTTGCTTCTCCCATGGCCAAGACC ATTGCTCTGGAGAAGGGCATCAAGCTTAGCGAGATCAAGGGCTCCGGTCCCGGTGGTCGAATCATCAAGCGAGACGTCGAGAA CTGGACCCCTCCCGCCGCTCCCGCCGCCAAGGCTGCCCCTGCCAAGGGCGCTGCCCCTGCTGCTGCCGCTGCTGCTGGATCTGCT TACACCGACATTCCTCTCACCAACATGCGAAAGACAATTGCTTCTCGACTGACCCAGTCCAAGAACACATCTCCCGACTACATTGT GTCTTCCACCGTGTCTGTGTCCAAGCTGCTCAAGCTGCGAGCTGCTCTCAACGCCTCCTCCGATGGTACCTACAAGCTGTCCATCA ACGATCTGCTTGTCAAGGCCCTGGCCGTGGCCAACACCAAGGTCCCCCAGGTCAACTCCCAGTGGCTCGAGTCTGAGGGTGTTA TTCGACAGTTCACCAACGTGGACGTTTCTGTCGCCGTTGCCACCCCCACTGGTCTGATCACCCCTGTGGTCAAGAACGCCAACCTC AAGGGTCTGGCGGAAATCTCCAAGGAGATTAAGGCTCTGGGCAAGAAGGCCAAGGACGGCAAGCTGGCTCCCGAAGAGTACC AGGGCGGTACCGTGACCATTTCCAACCTGGGCATGAACCACGCCGTGTCCTTCTTCACTGCCATCATCAACCCTCCCCAGGCCGC CATTCTCGCTGTCGGCACCACCGAGCGAAAGGCCATTGAGGACGTTGACTCCGAGGCCGGCTTTGTCTTTGACGACGTTGTTACT CTGACCACCTCTTTCGACCACCGAGTCGTCGATGGAGCTGTTGGAGGCGAGTGGGTCAAGGCTCTCAAGCAGGTGGTTGAAAAC CCCATTGAGATGTTGTTGTAG
LPD1 *(YALI0D20768g)*	ATGAGCTCCGCAAACGAGGAGCTTGATGTCCTTGTTATCGGAGGTGGCCCCGGTGGATACGTCGCCGCCATCAAGGCCGCCCAG GCCGGCCTCAAGACCGGCTGTATCGAGAAGCGAGGCTCCCTCGGAGGAACCTGTCTCAACGTCGGATGTATCCCCTCTAAGTCT CTGCTCAACAACTCCCAGATGTACCACGCCATCAAAACCGACTCCGCCAACCGAGGAATCGAGGTgTCTGACGTCAAGATGAACA TCGCCAAGCTGCAGGAGGCCAAGGAGACtTCCGTCAAGGGcCTCACCGGCGGTATCGAGATGCTGTTCAAGAAGAACAAGGTC AACTACTACAAGGGCGCCGGTTCTTTTGTGTCCGACTCCGAGGTCAAGGTCGACCCCATTGATGGCGGCGAGGCCGTCACCCTC AAGGCCAAGAACATCATCATTGCCACCGGCTCTGAGCCCACCCCCTTCCCCGGCATCACCATTGACGAGAAGAAGATTGTTTCCT CCACTGGTGCCCTTGCCCTCGAGGCCGTCCCCAAGAAGATGGTCATCATCGGAGGAGGTATCATTGGcCTCGAGATGGGCTCCG TCTGGTCCCGACTCGGCTCCGAGGTGACTGTTGTCGAGTTCCAGAACGCCATTGGCGCTGGTATGGACGACGAGATCGCCAAGG CCGCCCAGAAGATGCTCACCAAGCAGGGTATCAAGTTCAAGCTTGGCACCAAGGTGCTTTCCGGTGCCATTGAGGGCGACGGC GTCAAGGTCGAGGTCGAGAACGTCAAGAAGGGCGACAAGGAGACtCTTGATGCCGACGTTCTGCTCGTTGCCATTGGCCGACG ACCCTACTCCGAGGGCTTGAACCTCGAGGCTGCCGGTGTCGAGAAGGACGACAAGGGCCGAATCATCATCGACCAGGAGTACC GAACCAACAAGTCCAACATCCGATGCATTGGTGACGTCACTTTCGGCCCCATGCTGGCCCACAAGGCCGAGGAGGAGGGAGTT GCTACCGCTGAGTACATTGCCACCGGTCACGGCCACGTTAACTACGCCGCCATCCCCTCTGTCATGTACACCCACCCTGAGGTTG CTTGGGTTGGACAGACTGAGCAGCAGGTCAAGGAGGCCGGCATCAAGTACAACGTCGGCAAGTTCCCCTTTGCCGCCAACTCTC GAGCCAAGACCAACCTCGACACCGAGGGTACCGTCAAGTTCATTGCTGACAAGGAGACTGACCGAATTCTCGGTATCCACATCA TTGGCCCCAACGCCGGTGAGATGATCGCCGAGGGTGTTCTTGCCCTTGAGTACGGTGCTTCTTGCGAGGACATTGCTCGAACCT GCCACGCCCACCCCACTCTCTCCGAGGCTTTCAAGGAGGCCGCCATGGCCACTTACGACAAGGCCATCCACTTTTAA
PDX1 *(YALI0B09845g)*	ATGACCACCCCTCGCCTCTATCAGGCCAGCAACTTTGCCATGCCAGCCATGAGTCCCACCATGACCGAGGGAGGCATTGTGTCGT GGAAGGTCAAGGAGGGCGACGAGTTTTCAGCTGGTGATGTCATCCTTGAGATCGAAACCGACAAGGCCCAGATCGACGTCGAG GCTGCTGACGATGGAGTCATGGCCAAGATCTACAAGAAGGACGGTGACAAGGACATTCAGGTCGGAGACACCATTGCTGTGAT TGCTGAGCCCGGAGATGACATCAAAACCATTGATATTCCTGCTCCTGTCGAGTCGGACGGCAAGCCTGCTCCCAAGGAGGAGGC CAAGGAGGAGGTCAAGGAGGCCCCTAAGGAGGAGGCTAAGGCCCCTGCCCCTAAGGCTCCTTCTACCCCCAAGGAGGCTCCCA AGACCGAATCGTCTTCTGCTCCCTCATCATCCGGCTACTCTGCCCCCGCTAACCCCGCACAGACCCTTCTCCCTTCGGTGTCCTCGC TTCTTGTGGCCAATGGCATCTCCAAGGAGGACGCCTTTGCCAAGATCAAGGCCACCGGTCCCCACGGCCGGCTTCTCAAGGGCG ACATTCTCGCCTACCTCGGAAAGGTCCCTGAGGGCTCTCCTGGCGCTGTTGCCGACGAGATTAACAAGCGATCCCATCTCGACTT GTCCAACATCAAGCCCGCCAAGAAGAGCGATGCTGCTGCTCCCACTGGGGCTGCCAAGGCTGGAGATGCTGCTGCTCCTGCTAC CAAGGCTGCTCCCGAGCCTGTCATCTTCACCTCGTTCCTCGATGTGGCCCACTTTGAGCCCGAGGACCAGCTCGAGCTGCAGCGA ATCGTCAAGCAGGCCATCAAGCTGGCCAAATACGACGCCGTGGAGCTCGCAAAGCCCCGCCGATCCGCCAACGTGGACCCCGA CTTTGAGGCTATCATCGGCCCCGCCAAGGGCACCAAGTTCTACGACGTGAACGTCATCTACCCCAAGTCCACAAAGCGGGCTCTC TCCAATGGGGCCGACCTGTATGACATTCTGTCGGACCGAAAGCCCCGAAAGGCTGCTGTCCAGGTGCCTTCCAACACTGTGCAG GTCGATGTAACAGTCAACGACAAGGTCCCCGGTGCCGAGAAGCGAGCCAAGCTTTTCTTGAACAGACTGGAGTATCATTTGACT GTGGAGGAGTAG

APPENDIX D. RESUME EN FRANÇAIS

Les préoccupations environnementales et énergétiques deviennent une demande sociétale de plus en plus forte. En effet, la production de biomolécules comme alternative aux produits issus des combustibles fossiles est de plus en plus demandées de nos jours. En outre, de nombreux aliments, produits pharmaceutiques et cosmétiques sont obtenus à partir de sources naturelles (plantes, fruits, poissons, *etc*.) dont l'offre est limitée par exemple par le pays, les saisons et leur concentration peut nécessiter des méthodes d'extraction complexes et couteuses. La production microbienne de biomolécules peut bénéficier des plus récents outils d'ingénierie métabolique et de biologie synthétique pour pallier à ces limitations, permettant ainsi de produire de nouveaux bioproduits avancés qui ont des propriétés supérieures aux produits existants.

Yarrowia lipolytica est une levure GRAS (Generally Recognized As Safe, généralement considérés comme sûrs). Selon les conditions de culture ou la souche utilisée, *Y. lipolytica* peut croitre sous forme de levure ou sous forme de filament. C'est une levure oléagineuse, capable d'accumuler une grande quantité des lipides sous forme de triacylglycérols (TAG), qui peuvent représenter plus de 40% du poids sec dans des conditions de limitation de l'azote (Figure 1). Son génome a été séquencé et de nombreux outils d'ingénierie génétique sont disponibles. Récemment, de nombreux efforts ont été déployés pour concevoir des souches présentant une capacité à utiliser un spectre de substrats plus large et produisant différentes biomolécules d'intérêt biotechnologie (Figure 2).

Récemment, de nombreux groupes se sont impliqués dans l'ingénierie de la levure *Y. lipolytica* afin d'améliorer la production de lipides habituels, triglycérides (TAG) à haute teneur en acide oléique (HO-TAG) et en acide linoléique (AL-TAG) et des dérivés d'acides gras (FAME, FAEE, des acides gras polyinsaturés et des alcools gras). Les travaux ont aussi porté sur la production d'acides gras inhabituels, considérés comme des produits à plus haute valeur biotechnologique, en raison de leurs applications dans l'industries pharmaceutique, alimentaire et en oléochimie, mais qui sont produit naturellement en faible quantité. Parmi ces acides gras inhabituels, on peut citer les triglycérides riches en acides gras impairs (agi-TAG), les triglycérides acétylés (ac-TAG), et les acides gras inhabituels dérivés comme par exemple les acides gras impaires (AGI), les acides linoléiques conjugués (CLA), les acides cyclopropaniques (CFA) et l'acide ricinoléique (RA). La production microbienne de ces lipides inhabituels pourrait représenter une production plus économiquement viable.

L'objectif de cette thèse est de développer *Y. lipolytica* comme souche plateforme prometteuse, par l'ingénierie métabolique, pour la production de lipides inhabituels, en particulier d'acides gras à chaînes impaires (AGI).

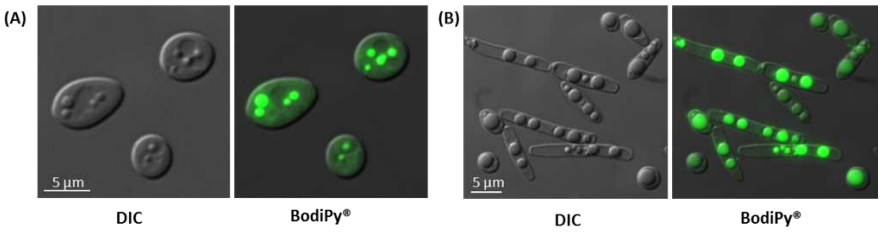

Figure 1. Morphologie cellulaire et accumulation de lipides par *Y. lipolytica*.

(A) La souche de type sauvage (JMY2900) pousse sous forme de levure et accumule moins de lipides. (B) La souche modifiée génétiquement (JMY3501) accumule une plus grande quantité de lipides et pousse principalement sous forme d'hyphes. L'accumulation de lipides dans les corps lipidiques est visualisée en vert après marquage au Bodipy®.

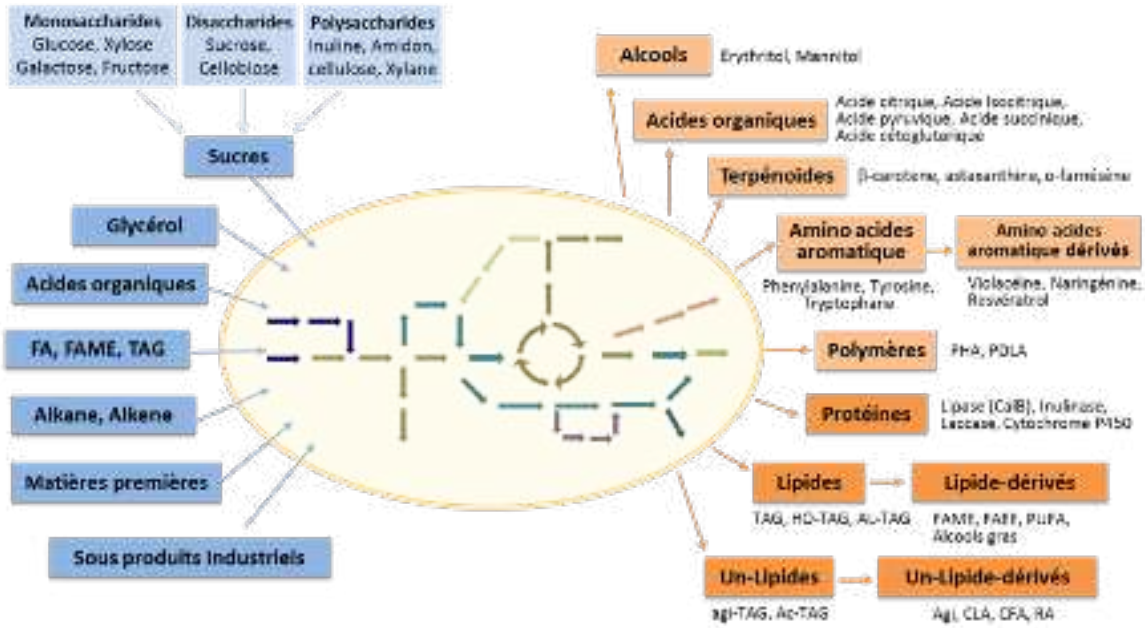

Figure 2. *Y. lipolytica* comme châssis pour des applications biotechnologiques.

Les substrats utilisables naturellement ou par des souches génétiquement modifiées de *Y. lipolytica* sont indiqués dans les encadrés bleus. Les différents types de biomolécules produites par des souches sauvages ou génétiquement modifiées de *Y. lipolytica* sont indiqués dans des encadrés oranges. Les axes étudiés au cours de cette thèse sont encadrés en orange foncé.

Abréviations : PHA, polyhydroxyalcanoate; PDLA, poly-D-lactate ; TAG, triacylglycérol; HO-TAG, triacylglycérol à haute teneur en acide oléique; AL-TAG, triacylglycérol riche en acide linoléique; FAME, ester méthylique d'acides gras; FAEE, ester éthylique d'acides gras; PUFA, acide gras polyinsaturé; agi-TAG, triacylglycérol à haute teneur en acide gras impaire ; Ac-TAG, triacylglycérol acéthylé ; Agi, acides gras à chaine impaire ; CLA, acide linoléique conjugué; CFA, acide cyclopropanique; RA, acide ricinoléique.

Développement de promoteurs hybrides synthétiques inductibles par l'érythritol

Afin de faire de *Y. lipolytica* le châssis le plus prometteur pour des applications biotechnologiques, divers outils génétiques efficaces sont nécessaires. Dans cette thèse, j'ai participé à l'identification et la caractérisation des motifs de régulation des gènes *EYK1* et *EYD1* impliqués dans le catabolisme de l'érythritol. L'objectif était de développer un ensemble de promoteurs inductibles par l'érythritol et/ou l'érythrulose pour des applications biotechnologiques (Figure 3). Ce travail a permis, dans un premier temps, d'identifier des régions conservées dans les promoteurs de ces gènes (CRM : *cis*-regulatory module) (Figure 3A). L'évaluation des promoteurs sauvages et mutés, en utilisant la protéine fluorescente RedStarII comme protéine reportrice, a permis d'identifier les régions activatrices (UAS) pour la construction de promoteurs inductibles par l'érythritol. De nombreux promoteurs sauvages, mutés et hybrides ont été évalués, par exemple les promoteurs EYK1 et les promoteurs hybrides EYK1-2AB, EYK1-2AB, EYK1-3AB, EYK1-4AB et EYK1-4AB-coreTEF (Figure 3B). Ces promoteurs présentent différents niveaux d'expression, d'inductibilité, et de niveau d'expression en présence ou absence d'érythritol ou d'érythrulose. Ils dépendent du génotype de la souche, si elle est sauvage pour le gène *EYK1* (l'érythritol est utilisée comme source de carbone pour la croissance et comme inducteur) ou une souche délétée pour le gène *EYK1* (*eyk1Δ*) (le glucose est utilisé comme source de carbone pour la croissance et l'érythritol comme inducteur). La série de promoteurs hybrides composée de répétitions multiples d'UAS (upstream activation sequence), d'UAS mutées, et hybrides avec différents core promoteurs (Figure 3B) a mis en évidence la diversité de l'inductibilité à l'érythritol avec un niveau d'induction allant de 2,2 à 32,3 fois dans la souche WT et de 2,9 à 896,1 fois dans une souche *eyk1Δ* (Figure 3C). Ce travail a fait l'objet d'une publication dans FEMS Yeast Research (Park *et al.*, FEMS Yeast Research (2019) 19:foy105).

Comme preuve de concept, un ensemble de vecteurs d'expression inductibles ont été construits et validés par l'expression d'une protéine d'intérêt biotechnologique, la lipase CalB de Candida antarctica. En comparant l'expression du gène et la production de lipase sous le contrôle de chaque promoteur, il a été démontré que pEYK1-3AB est le promoteur le plus approprié pour la production de cette lipase. Dans un bioréacteur discontinu, environ 45000 U/mL de lipase CalB ont été obtenus en 24 h, ce qui représente à ce jour le processus le plus efficace identifié pour la production de cette enzyme dans une levure. Ce travail a fait l'objet d'une publication dans Microbial Cell Factories (Park et al., Microbial Cell Factories (2019) 18:167). Ce travail a aussi permis de construire un ensemble de biobriques pour des assemblages modulaires Golden Gate (GGA) et des promoteurs hybrides inductibles par l'érythritol pour la biologie synthétique et l'ingénierie métabolique (Figure 3D).

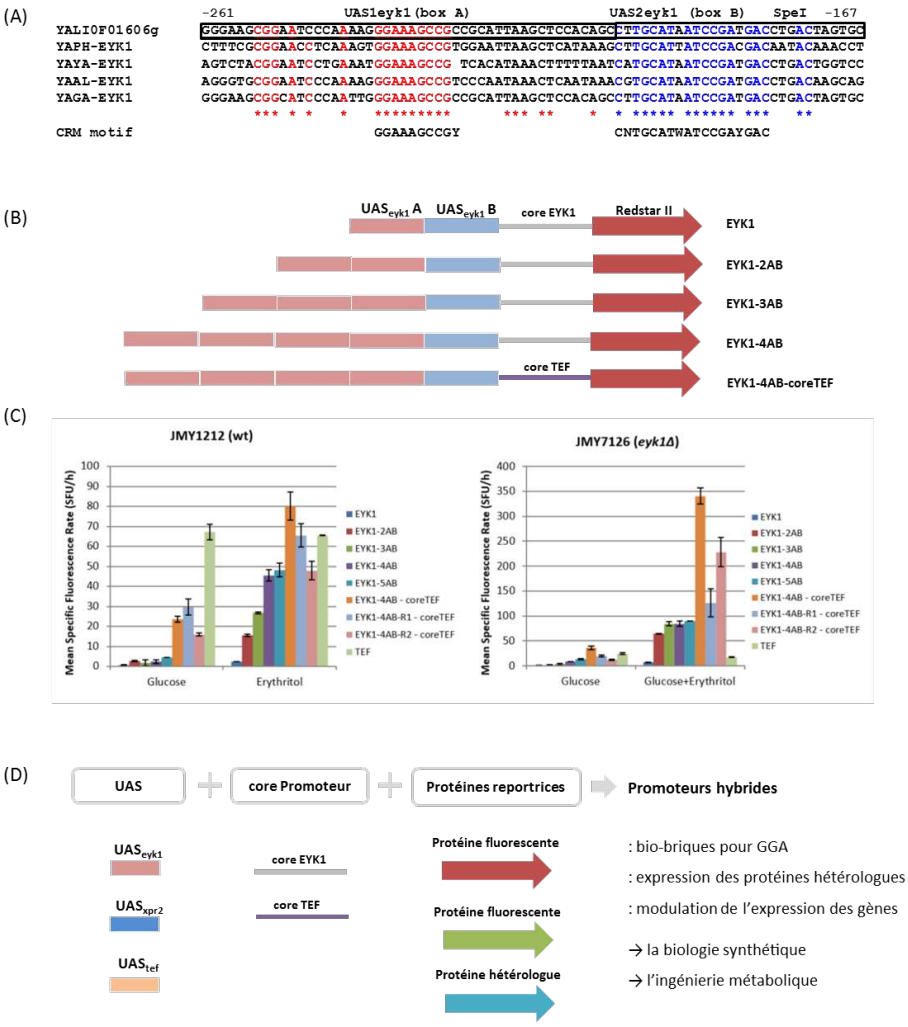

Figure 3. Promoteurs hybrides synthétiques inductible par l'érythritol.

(A) Alignement multiple partiel des régions du promoteur des gènes *EYK1* chez *Y. lipolytica* (YALI ; *YALI0F01606g*) et des souches du clade *Yarrowia phangngensis* (YAPH), *Yarrowia yakushimensis* (YAYA), *Yarrowia alimentaria* (YAAL) et *Yarrowia galli* (YAGA). Il met en évidence des régions conservées (CRM) permettant de mettre en évidence les UAS (upstrean activating sequence), impliquées la régulation de l'expression par l'érythritol et l'érythrulose.

(B) Représentation schématique du promoteur EYK1 et des promoteurs hybrides EYK1-2AB, EYK1-2AB, EYK1-3AB, EYK1-4AB et EYK1-4AB-coreTEF pour contrôler l'expression de la RedStarII.

(C) Expression et force de promoteurs sauvages et hybrides *EYK1* en fonction du milieu et du génotype de la souche. La force du promoteur a été déterminée en quantifiant l'expression de la protéine fluorescente RedStarII et en comparant le taux moyen de fluorescence spécifique (SFU/h) obtenu dans la souche sauvage (*EYK1*, JMY1212) et la souche délétée pour le gène *EYK1* (*eyk1Δ*, JMY7126) cultivé en milieu glucose et érythritol ou en milieu glucose et glucose + érythritol pour la souche délétée.

(D) Stratégie générale de construction de promoteurs hybrides combinant des UAS avec un core promoteur pour l'expression de protéines reportrices (protéine fluorescente ou protéine hétérologue) afin de déterminer leurs forces, leur niveau d'expression, le facteur d'induction. Ces promoteurs hybrides sont utilisés pour des assemblages modulaires Golden Gate (GGA), pour la construction de vecteurs d'expression et pour la biologie synthétique et l'ingénierie métabolique.

Production d'acide gras inhabituels, les acides gras impairs.

La production d'acides gras impaires (AGI) chez *Y. lipolytica* a été étudiée dans cette thèse. Les AGI sont largement utilisés dans l'industries pour des applications thérapeutiques, nutritionnelles et chimiques, y compris les biocarburants (Figure 4). Par exemple, l'acide *cis*-9-heptadécénoïque peut aider à traiter le psoriasis, les allergies et les maladies auto-immunes grâce à des effets anti-inflammatoires. Les AGI sont aussi utilisés comme biomarqueurs de la consommation alimentaire, du risque de maladie coronarienne, et du risque de diabète (T2D). Cependant, les AGI ont jusqu'à présent été produits à partir de plantes ou par synthèse chimique, et leur niveau de production dans les microorganismes sauvages est faible. Nous avons décidé d'utiliser *Y. lipolytica* comme souche châssis pour une production efficace d'AGI.

Production d'acides gras impairs : toxicité du propionate

L'utilisation du propionate est très importante pour la synthèse des AGI dans la mesure où il fournit un précurseur clé des AGI, le propionyl-CoA. Nous avons montré que la levure *Y. lipolytica* est sensible à la concentration de propionate. Sa croissance est fortement affectée en fonction de la concentration en propionate dans le milieu de culture. Afin d'identifier des gènes permettant une meilleure résistance au propionate, un crible pour sélectionner des souches plus résistantes a été développé et une banque de vecteurs contenant des fragments d'ADN génomique dans un vecteur réplicatif a été criblée (Figure 5).

Deux souches présentant une meilleure résistance au propionate ont été isolées, JMY7588 et JMY7589. Le séquençage des régions génomiques ont permis d'identifier deux gènes, *RTS1* et *MFS1* (Figure 5B), conférant une meilleure tolérance au propionate.

Les souches exprimant ces deux gènes dans des vecteurs réplicatifs ou dans des cassettes d'expression intégrées dans le génome, présentent des tolérances améliorées dépendant de la concentration en propionate (e.g. Figure 5C). Ces phénotypes pourraient être dûs à des mécanismes différents de tolérance. Toutefois, des études plus détaillées sur l'analyse de l'expression et des réseaux de régulation dans des conditions de stress induit par le propionate sont nécessaires pour comprendre pleinement le rôle de ces deux gènes dans la tolérance au propionate.

Ce travail a fait l'objet d'une publication dans Yeast (Park and Nicaud*,* Yeast (2020) 337:131-140).

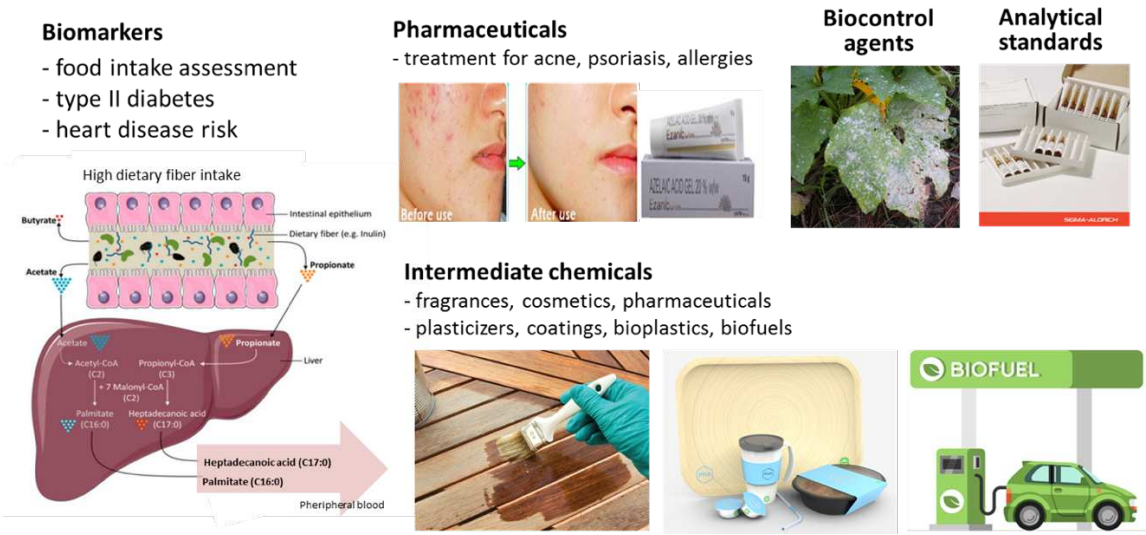

Figure 4. Applications des AGI dans les industries pharmaceutique, alimentaire, cosmétique, chimique et des carburants.

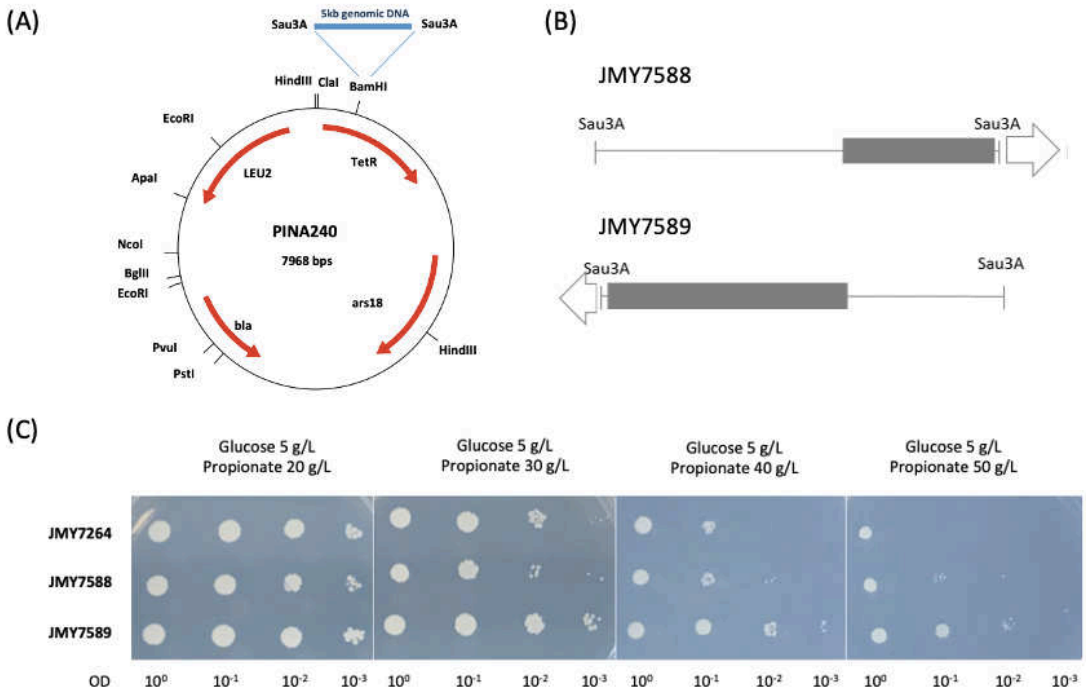

Figure 5. Identification de gènes conférant une meilleure tolérance au propionate par le criblage d'une banque génomique.

(A) Carte schématique du plasmide réplicatif pINA240 utilisé pour la construction de la banque d'ADN génomique. Les fragments génomiques partiels ont été clonés au site BamH1. (B) Fragments d'ADN génomique de JMY7588 et JMY7589 dans les souches résistantes au propionate. Les barres grises indiquent les ORFs, et les flèches blanches montrent la partie des gènes absente dans le fragment génomique. (C) Test de tolérance au propionate de JMY7588 (gène *RTS1*) et JMY7589 (gène *MFS1*) par analyse en test en goutte sur milieux minimum contenant différentes concentrations de propionate ont été utilisés. Les photos ont été prises après 3 jours de croissance à 28 °C.

Production d'acides gras impairs : optimisation de la production

Les stratégies d'ingénierie métabolique utilisées pour augmenter la production des AGI à partir du propionate sont présentées dans la Figure 6. Dans un premier temps, l'inhibition de la consommation du propionyl-CoA par délétion du gène *PHD1* de la voie du methyl citrate a permis d'augmenter de la production des AGI de 28,3% à 46,8% (% d'AGI dans les lipides totaux) dans la souche sauvage WT (Figure 7A et 7B). Cette délétion a été introduite dans une souche « obèse » capable d'accumuler davantage de lipides. En effet, cette souche est améliorée pour l'accumulation de lipides, par délétion des voies de remobilisation des lipides (délétion du gène *TGL4* codant pour la triglycéride lipase Tgl4) et de dégradation des acides gras (délétion du gène *MFE1* codant pour l'enzyme multifonctionnelle Mfe1 impliquée dans la voie de la β-oxydation). La production d'AGI a atteint 0,57 g/L.

La première partie de ce travail a fait l'objet d'une publication dans Biotechnology for Biofuels (Park *et al.*, Biotechnol Biofuels 2018, 7 :11 :158. Optimization of odd chain fatty acid production by *Yarrowia lipolytica*).

Dans un deuxième temps, mon travail a porté sur l'ingénierie des pools de précurseurs. En particulier, l'augmentation du pool de propionyl-CoA pour la synthèse des AGI, a été explorée par l'introduction d'enzymes activant le propionate en propionyl-CoA. La souche la plus performante (surexprimant *Repct*) est capable d'accumuler des AGI jusqu'à 53,2% des lipides totaux, soit 3,8 fois plus que la souche WT, en condition de supplémentation en acétate. Il a été révélé que l'équilibre de deux précurseurs, le propionyl-CoA et l'acétyl-CoA, est un critère crucial pour améliorer la production d'AGI. Une étape supplémentaire d'ingénierie a été réalisée pour stimuler la production du C5-CoA, précurseur à cinq carbones, β-ketovaleryl-CoA, en surexprimant *RebktB*, permettant la production d'AGI jusqu'à 1,87 g/L et représentant 62% des lipides totaux dans cette nouvelle souche modifiée (Figure 7B). C'est le titre AGI le plus élevé produit dans les bactéries et les levures recombinantes publié ce jour.

La deuxième partie de ce travail fait l'objet d'un article soumis à publication (Park *et al*., Engineering precursor pools for increasing production of odd-chain fatty acids in *Yarrowia lipolytica*).

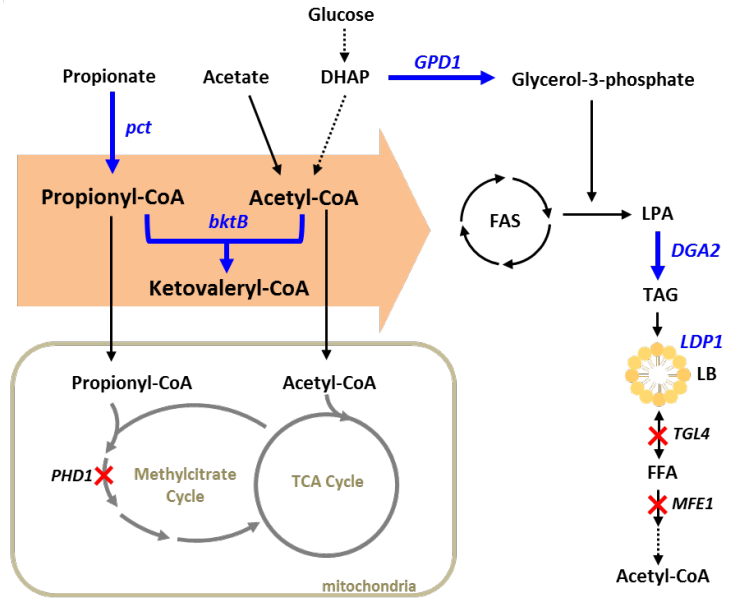

Figure 6. L'ingénierie métabolique de *Y. lipolytica* pour la production d'AGI.

Les principales étapes de biosynthèse modifiées par surexpression ou inactivation de gènes sont indiquées respectivement par les flèches bleues et les croix rouges. Les précurseurs des AGI sont regroupés dans la flèche orange. Les étapes multiples sont indiquées par des flèches en pointillés. pct, propionyl-CoA transférase; bktB, β-ketothiolase; GPD1, glycérol-3-phosphate déshydrogénase; DGA2, acyl-CoA, diacylglycérol acyltransférase; LDP1, protéine de gouttelettes lipidiques; PHD1, 2-méthylcitrate déshydratase; TGL4, triglycéride lipase; MFE1, enzyme multifonctionnelle; LB, corps lipidique.

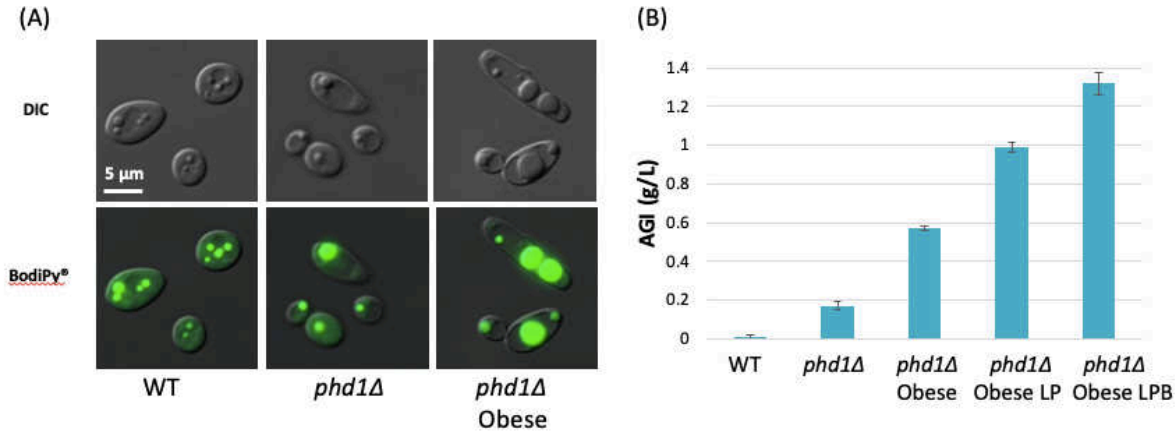

Figure 7. L'accumulation d'AGI par les souches de *Y. lipolytica* génétiquement modifiées.

(A) Images prises au microscope des cellules (DIC) et visualisation des corps lipidiques par marquage au Bodipy dans les souches WT, WT *phd1Δ* et obèse *phd1Δ*. (B) Augmentation de l'accumulation des AGI par ingénierie métabolique dans les souches WT, WT *phd1Δ*, obèse *phd1Δ*, obèse LP *phd1Δ*, et obèse LPB *phd1Δ*.

Production d'acides gras impairs : production *de novo* sans supplémentation de propionate.

La première partie de mon travail a porté sur les conditions d'apport du propionate, l'augmentation de tolérance au propionate, de l'activation du propionate en propionyl-CoA et sur l'invalidation de la voie du catabolisme du propionyl-CoA.

Dans une deuxième partie, mon travail a porté sur la production *de novo* d'AGI. C'est-à-dire que le propionyl-CoA produit dans la cellule ne provient pas d'une source exogène de propionate, mais est directement produit *de novo* pas la cellule.

Cette production endogène de propionate-CoA peut être obtenue par surexpression de la voie de synthèse de la thréonine (Figure 8). La voie aspartate/α-ketobutyrate est composée de 7 étapes que nous avons décomposé en 3 modules ; le module A correspondant au gène *AAT2*, le module H, correspondant aux gènes *HOM3*, *HOM2* et *HOM6* et le module T aux gènes *THR1*, *THR4* et *ILV1* (Figure 8A). Les différentes cassettes d'expression, construites par assemblage modulaire (Figure 8B) ont été introduites individuellement et successivement dans la souche «sauvage» et la souche «obèse».

L'introduction d'une seule cassette n'a pas d'effet significatif sur l'accumulation d'AGI. En revanche, l'introduction simultanée a permis d'augmenter fortement la production d'AGI dans la souche sauvage et dans la souche « obèse » (Figure 8C). La production d'AGI a été augmentée, passant de 0,84% à 3,86% des lipides totaux. La production d'AGI par litre a été multipliée par 12, passant de 0,03 g/L dans la souche sauvage a 0,36 g/L pour la souche « obèse ». Ces résultats sont très prometteurs, car cela permettrait de pouvoir produire des AGI à partir de glucose sans supplémentation en propionate. Toutefois le titre d'AGI final (0,36 g/L) et très inférieur au titre obtenue (1,87 g/L) avec supplémentation de propionate et d'acétate.

La production *de novo* de propionyl-CoA et la production d'AGI sur glucose sans supplémentation en propionate a fait l'objet d'un article publié dans Frontiers in Bioengineering and Biotechnology (Park *et al.*, Frontiers in Bioengineering and Biotechnology (2020) 7:484).

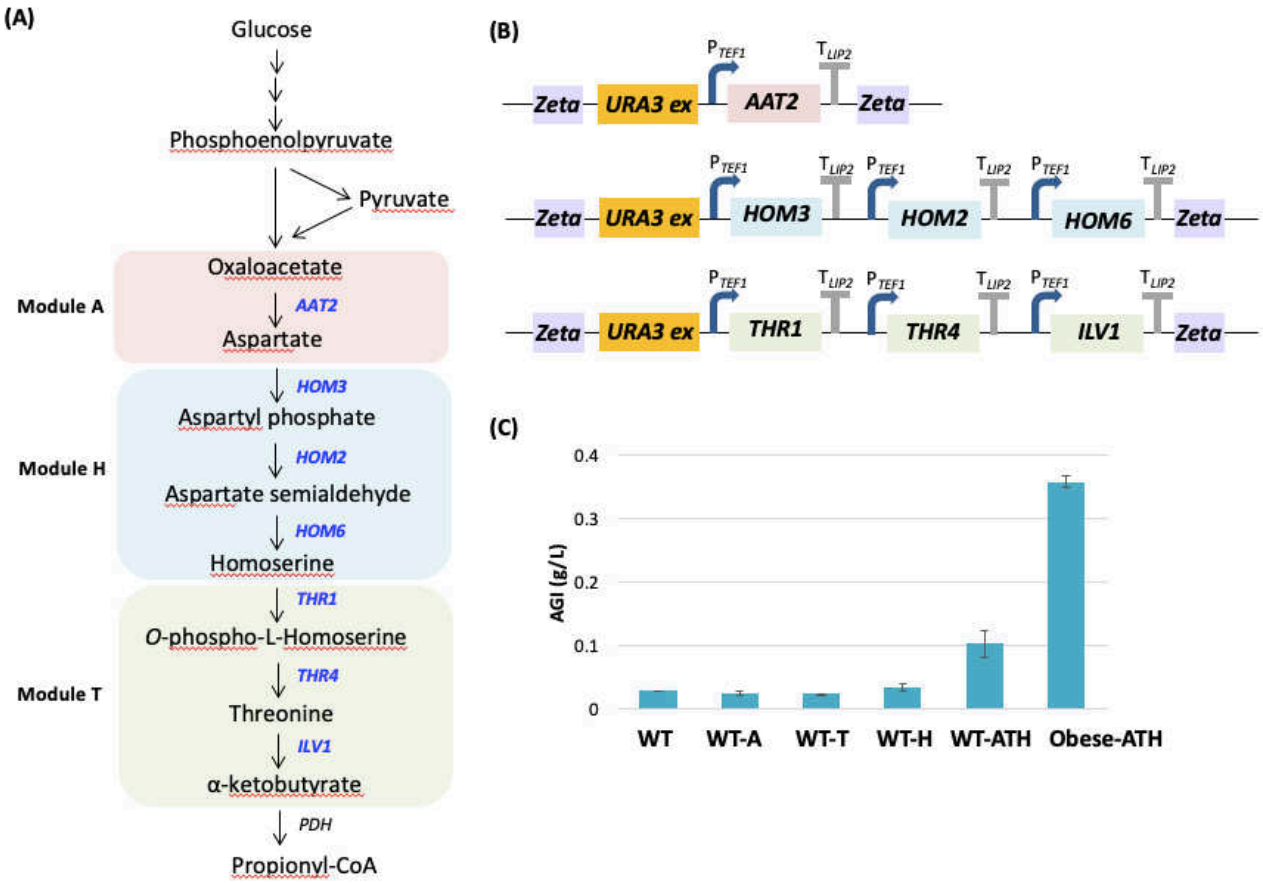

Figure 8. Biosynthèse d'acides gras impaires d*e novo*.

(A) Voie métabolique de synthèse du propionyl-CoA à partir du glucose. Les 7 gènes surexprimés dans cette étude sont indiqués en bleu.

(B) Structure des modules multigéniques des gènes de la voie aspartate/α-ketobutyrate qui ont été construits pour surexprimer les 7 gènes de la voie. Les modules A, H et T permettent la surexpression des gènes *AAT2* (module A), des gènes *HOM3*, *HOM2* et *HOM6* (module H), et des gènes *THR1*, *THR4* et *ILV1* (module T), chaque cassette d'expression a été construite par assemblage modulaire, chaque gène est exprimé sous le contrôle du promoteur *TEF1* (P_{TEF}) et du terminateur du gène *LIP2* (T_{LIP2}).

(C) Accumulation d'AGI en fonction du niveau d'ingénierie de la voie et du génotype « sauvage » et « obèse ». Souche sauvage contrôle, WT ; souche sauvage avec le module A (WT-A), souche sauvage avec le module T (WT-T), souche sauvage avec le module H (WT-H), souche sauvage avec les trois modules A, T, H (WT-ATH) et souche « obèse » avec les trois modules A, T, H (Obèse-ATH).

Conclusions et perspectives

Au cours de cette thèse, plusieurs stratégies d'ingénierie métabolique ont été étudiées pour produire des acides gras impairs (AGI) chez *Y. lipolytica* (Figure 9). Le criblage d'une banque d'ADN génomique a permis de mettre en évidence l'implication des gènes *RTS1* et *MFS1* dans la résistance au propionate. La surexpression de ces gènes dans une souche optimisée pour la production d'AGI devrait permettre d'augmenter la tolérance au propionate. Cela pourrait permettre amélioration supplémentaire pour la production d'AGI.

Pour la synthèse d'acides gras pairs (AGP) et d'acides gras impairs (AGI), le précurseur principal est l'acétyl-CoA, ce précurseur peut être synthétisé par plusieurs voies métaboliques. Dans cette étude, le gène *ACS2* codant pour l'acétyl-CoA synthétase a été surexprimée, mais aucune amélioration significative de la production d'AGI a été démontrée.

De même, la combinaison de *ACS2* avec *ACC1* codant pour l'acétyl-CoA carboxylase n'a pas amélioré la production d'AGI. Toutefois, l'effet de la surexpression d'*ACC1* dépend fortement du génotype de la souche, des conditions de culture telles que la concentration du substrat et le rapport entre le carbone et l'azote (C/N). Une forte augmentation de la production d'AGI a été obtenue par délétion de *PHD1* impliqué dans la voie du catabolisme du propionyl-CoA et dans l'étape clef de l'activation du propionate en propionyl-CoA. L'introduction d'une voie synthétique des 7 gènes de la voie aspartate/a-ketobutyrate a permis d'augmenter la production d'AGI.

En résumé, malgré l'intérêt biotechnologique des AGI, les recherche se sont uniquement concentrées sur les procédés de fermentation de microorganismes non modifiés pour la production d'AGI. Au cours de cette thèse, par des approches d'ingénierie métabolique et de biologie synthétique, nous avons développé des souches châssis de *Yarrowia lipolytica* capable d'accumuler de grande quantité de lipides riche en AGI. Cette étude ouvre la voie à une production microbienne efficace d'AGI et de ses dérivés ayant un potentiel élevé pour des applications biotechnologiques.

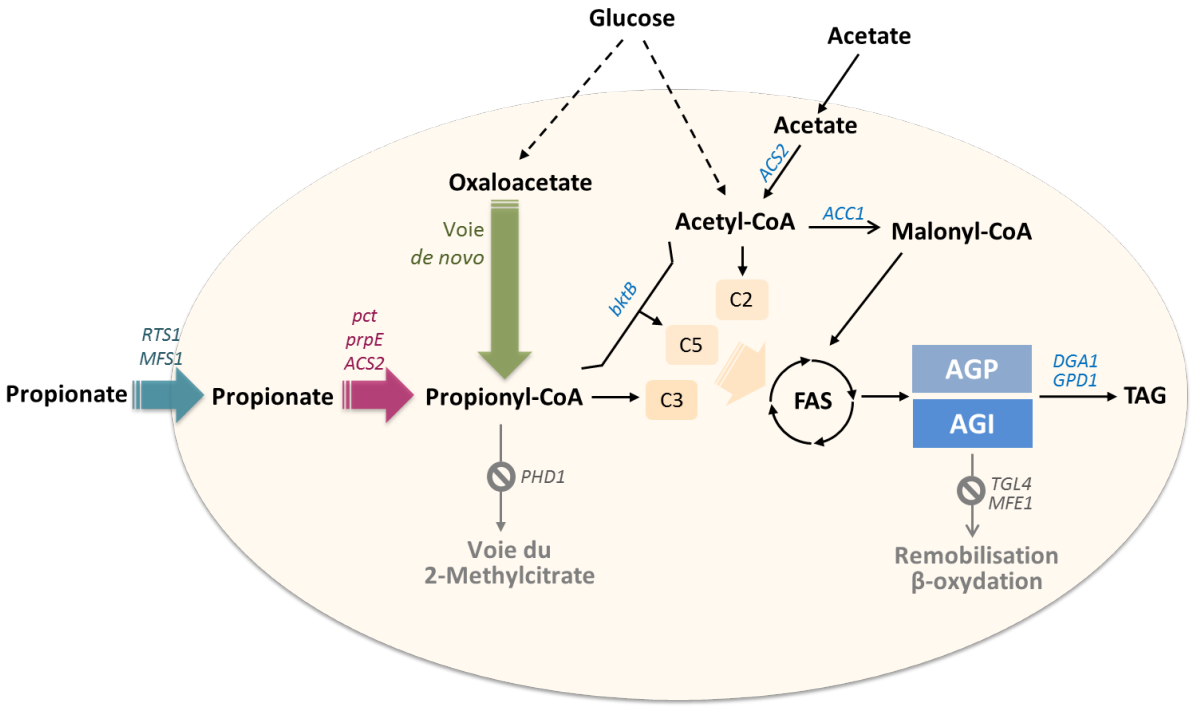

Figure 9. Résumé des stratégies d'ingénierie métabolique étudiées dans cette thèse.

Le travail de cette thèse a porté sur : **1)** Implication des gènes *RTS1* et *MFS1* dans la résistance au propionate (flèche bleue turquoise), **2)** l'effet de la surexpression d'enzymes capable d'activer le propionate en propionyl-CoA (*pct*, *prpE*, *ACS2*) pour augmenter le pool de propionyl-CoA (C3 précurseur) (flèche bordeaux), **3)** effet de la surexpression d'enzymes capable d'activer l'acétate en acétyl-CoA (*ACS2*), la synthèse du malonyl-CoA (*ACC1*) et la condensation de l'acétyl-CoA avec un propionyl-CoA pour la production d'un précurseur C5 (*bktB*) pour alimenter la FAS pour la synthèse d'acides gras pairs (AGP) et d'acides gras impairs (AGI). **4)** Rôle de la délétion de *PHD1* impliqué dans le catabolisme du propionyl-CoA et effet de ces modifications dans une souche sauvage et une souche obèse, invalidé pour la remobilisation et la β-oxydation (*TGL4* et *MFE1* délétés, respectivement) et augmenté pour la synthèse de TAG (*DGA1* et *GPD1* surexprimés) et **5)** effet de l'introduction d'une voie synthétique de production de propionyl-CoA pour la synthèse *de novo* d'AGI (flèche verte).

APPENDIX E. LIST OF CONTRIBUTIONS AND COMMUNICATIONS

Journal articles relating the thesis

YK Park*, F Bordes, F Letisse, JM Nicaud, Engineering precursor pools for increasing production of odd-chain fatty acids in *Yarrowia lipolytica* (Accepted)

YK Park*, R Ledesma-Amaro*, JM Nicaud, *De novo* biosynthesis of odd-chain fatty acids in *Yarrowia lipolytica* enabled by modular pathway engineering, Frontiers in Bioengineering and Biotechnology (2020) 7:484

YK Park*, JM Nicaud, Screening a genomic library for genes involved in propionate tolerance in *Yarrowia lipolytica*, Yeast (2020) 337:131-140

YK Park[1], M Vandermies[1], P Soudier, S Telek, S Thomas, JM Nicaud, P Fickers, Efficient expression vectors and host strain for the production of recombinant proteins by *Yarrowia lipolytica* in process conditions, Microbial Cell Factories (2019) 18:167

YK Park, P Korpys, M Kubiak, E Celińska, P Soudier, P Trebulle, M Larroude, T Rossignol, JM Nicaud, Engineering the architecture of erythritol-inducible promoters for regulated and enhanced gene expression in *Yarrowia lipolytica*, FEMS Yeast Research (2019) 19:foy105

YK Park, T Dulermo, R Ledesma-Amaro, JM Nicaud, Optimization of odd chain fatty acid production by *Yarrowia lipolytica*, Biotechnology for Biofuels (2018) 11:158

Presentations (Awarded presentation is indicated with ※.)

Korean Society for Microbiology and Biotechnology International symposium — *2019, Jeju, South Korea*

- Metabolic engineering of *Yarrowia lipolytica* for production of odd chain fatty acids (Poster※)
- Engineering architecture of inducible promoters for regulated and enhanced gene expression in *Yarrowia lipolytica* (poster)

Yeast Lipid Conference — *2019, Ljubljana, Slovenia*

- Push and pull of odd chain fatty acids production by *Yarrowia lipolytica* (poster)

4th Applied Synthetic Biology in Europe — *2018, Toulouse, France*

- Metabolic engineering of *Yarrowia lipolytica* for production of odd chain fatty acids (Poster※)

BioSynSys 2018 — *2018, Toulouse, France*

- Metabolic engineering of *Yarrowia lipolytica* for production of odd chain fatty acids (Poster)

Non-conventional Yeasts 2018 — *2018, Rzeszow, Poland*

- Metabolic engineering of *Yarrowia lipolytica* for production of odd chain fatty acids (Poster※)
- Engineering architecture of inducible promoters for regulated and enhanced gene expression in *Yarrowia lipolytica* (poster)

Doc'Micalis 2019 (Internal symposium) — *2019, Jouy-en-Josas, France*

- Metabolic engineering of *Yarrowia lipolytica* for production of odd chain fatty acids

Supplementary journal articles

PJ Trotter, K Juco, HT Le, K Nelson, L Tamayo, JM Nicaud, **YK Park**, Glutamate dehydrogenases in the oleaginous yeast *Yarrowia lipolytica*, Yeast 37 (2020) 103-115

M Larroude, **YK Park**, P Soudier, M Kubiak, JM Nicaud, T Rossignol, A goldengate toolkit for *Yarrowia lipolytica* synthetic biology, Microbial Biotechnology (2019) 12(6), 1249– 1259

H Gamboa-Melendez, M Larroude, **YK Park**, P Trebulle, JM Nicaud, R Ledesma-Amaro, Synthetic biology to improve the production of lipases and esterases (Review), Lipases and Phospholipases, Methods in Molecular Biology 1835 (2018) 229-242

YK Park, JM Nicaud, R Ledesma-Amaro, The engineering potential of *Rhodosporidium toruloides* as a workhorse for biotechnological applications, Trends in Biotechnology 36 (2018) 304-317 (Cover page)

FEMS Yeast Research, 19, 2019, foy105

doi: 10.1093/femsyr/foy105
Advance Access Publication Date: 26 September 2018
Research Article

RESEARCH ARTICLE

Engineering the architecture of erythritol-inducible promoters for regulated and enhanced gene expression in *Yarrowia lipolytica*

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One sentence summary: This study identified cis-regulatory modules (CRMs) for the *EYK1* and *EYD1* promoters in *Yarrowia lipolytica*, which allowed the development of erythritol-inducible hybrid promoters with practical applications in metabolic engineering and synthetic biology.
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ABSTRACT

The non-conventional model yeast *Yarrowia lipolytica* is of increasing interest as a cell factory for producing recombinant proteins or biomolecules with biotechnological or pharmaceutical applications. To further develop the yeast's efficiency and construct inducible promoters, it is crucial to better understand and engineer promoter architecture. Four conserved cis-regulatory modules (CRMs) were identified via phylogenetic footprinting within the promoter regions of *EYD1* and *EYK1*, two genes that have recently been shown to be involved in erythritol catabolism. Using CRM mutagenesis and hybrid promoter construction, we identified four upstream activation sequences (UASs) that are involved in promoter induction by erythritol. Using RedStarII fluorescence as a reporter, the strength of the promoters and the degree of erythritol-based inducibility were determined in two genetic backgrounds: the *EYK1* wild type and the *eyk1*Δ mutant. We successfully developed inducible promoters with variable strengths, which ranged from 0.1 SFU/h to 457.5 SFU/h. Erythritol-based induction increased 2.2 to 32.3 fold in the *EYK1* + wild type and 2.9 to 896.1 fold in the *eyk1*Δ mutant. This set of erythritol-inducible hybrid promoters could allow the modulation and fine-tuning of gene expression levels. These promoters have direct applications in protein production, metabolic engineering and synthetic biology.

Keywords: *Yarrowia lipolytica*; promoter; inducible; erythritol; Golden Gate; gene expression; synthetic biology

INTRODUCTION

Yarrowia lipolytica is an oleaginous yeast species that serves as a non-conventional model organism in research on lipid turnover and bio-oil production (Beopoulos *et al.* 2008, 2009), dimorphic transition and fungal differentiation (Martinez-Vazquez *et al.* 2013), and secretory protein synthesis (Matoba *et al.* 1988; Matoba and Ogrydziak 1989; Boisramé *et al.* 1998; Pignède *et al.* 2000; Nicaud *et al.* 2002). *Y. lipolytica* is also the focus of increasing

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interest because it can serve as an industrial workhorse in a number of processes (Bankar, Kumar and Zinjarde 2009; Coelho, Amaral and Belo 2010; Groenewald *et al.* 2014). Indeed, *Y. lipolytica* has been used as a biocatalyst in the high-level production of citric acid (Rywińska, Rymowicz and Marcinkiewicz 2010; Holz *et al.* 2011; Rywińska *et al.* 2011), erythritol (Rymowicz, Rywińska and Marcinkiewicz 2009; Carly *et al.* 2017a), aroma compounds (Pagot *et al.* 1998; Gomes, Teixeira and Belo 2010; Celińska, Olkowicz and Grajek 2015), and a number of proteins of diverse origins (Nicaud *et al.* 2002; Madzak 2015, Dulermo et al. 2017).

Given the growing number of research areas in which *Y. lipolytica* has been found to be a model organism of choice, the need for efficient molecular tools dedicated to this species has concomitantly grown. The systematic examination of a specific metabolic phenomenon requires the construction and testing of several genetic variants to obtain useful, well-supported conclusions. Thus, high-throughput techniques that allow broad-scale genetic manipulation and the testing of extensive clone libraries are continuously being developed and adopted. Recently, genetic engineering tools used to manipulate the *Y. lipolytica* genome have greatly grown in number thanks to CRISPR-Cas9 technology (Schwartz *et al.* 2016, 2017; Wong *et al.* 2017) and modular cloning techniques (Leplat, Nicaud and Rossignol 2015; Celińska *et al.* 2017; Larroude *et al.* 2017). Simultaneously, high-throughput screening techniques for evaluating traits of interest have been developed; they include droplet-based microfluidic screening and micro bioreactor culturing (Bordes *et al.* 2007; Leplat, Nicaud and Rossignol 2015; Weizhu *et al.* 2015; Back *et al.* 2016; Beneyton *et al.* 2017).

When carrying out the heterologous overexpression of a given protein or metabolically engineering a pathway of interest, it is crucial to carefully examine and select the regulatory elements driving the expression of the genes to be manipulated. Promoter sequences play a major role: transcription is initiated by harnessing the appropriate transcription factors and polymerase. Thus, not surprisingly, the selection and optimization of promoter sequences is one of the most frequently adopted strategies in the fine-tuning of gene expression. In *Y. lipolytica*, the promoter that natively regulates expression of the *XPR2* gene, which encodes an alkaline extracellular protease, was the first to be examined and remains the most extensively studied (Blanchin-Roland, Cordero Otero and Gaillardin 1994; Madzak *et al.* 1999). This regulatory sequence has been subject to great scrutiny, and its characteristics appear to render it unfit for applications related to industrial protein production or basic research, as it requires very specific conditions for full induction. Nevertheless, the knowledge gained during past studies has allowed researchers to design and develop a strong, hybrid, synthetic promoter that is semi-constitutive (Blanchin-Roland, Cordero Otero and Gaillardin 1994; Madzak, Treton and Blanchin-Roland 2000). It is composed of upstream activation sequences (UASs) and involves a minimal promoter of the *LEU2* gene. It has been incorporated in commercially available YLEX vectors (Yeastern Biotech Co.; Taiwan) and has successfully been used in a large number of applications. In addition to the XPR2-based promoter and its derivatives, several other promoter sequences have been analyzed and described, most notably in a comprehensive study by Müller *et al.* (1998). The functional dissection of pXPR2 allowed the identification of one of its UASs ($UAS1B_{XPR2}$). The hybrid hp4d promoter contains four direct repeats of the 109-bp $UAS1B_{XPR2}$ sequence, which is found upstream from the minimal LEU2 promoter (mLEU2) (Madzak, Treton and Blanchin-Roland 2000). Shabbir Hussain *et al.* (2016) investigated promoter strength by shuffling the constitutive

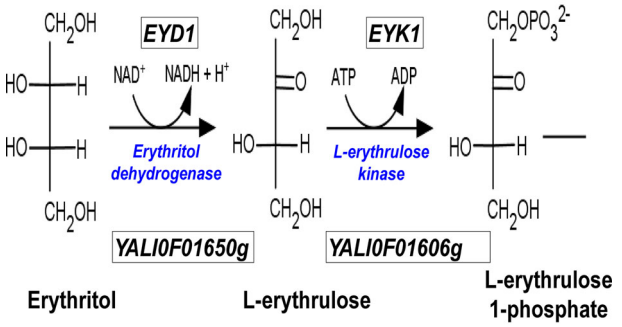

Figure 1. Pathways of erythritol catabolism in *Y. lipolytica*. Erythritol is converted into erythrulose by the erythritol dehydrogenase encoded by *EYD1* (YALI0F01650g). The erythrulose then becomes erythrulose-phosphate via a phosphorylation reaction catalyzed by the erythrulose kinase encoded by *EYK1* (YALI0F1606g) (Carly *et al.* 2017b, 2018).

elements (UAS, proximal promoter, TATA box and core promoter) of various fungal gene promoters (TEF, POX2, LEU2 and PAT1) in *Y. lipolytica*.

In synthetic biology, gene expression must be fine-tuned to ensure optimal flows in related pathways or to avoid metabolic burdens. Cis-regulatory modules (CRMs) are non-coding DNA elements that help regulate gene expression via the binding of transcription factors to motifs in CRM sequences, thus facilitating cell adaptation to internal conditions and the exterior environment. Predicting CRMs is thus a key part of understanding the complex processes underlying cell regulation; it is also necessary if researchers wish to design efficient cellular factories, notably by engineering new promoters with context-specific expression. As indicated in a review by Aerts (Aerts 2012), many computational strategies have been developed throughout the years to identify CRMs. One such strategy—phylogenetic footprinting—exploits the fact that regulatory modules have been evolutionarily conserved among related species. Motifs identified in the promoters of orthologous genes can be tested for functionality, and the corresponding UASs can then be used to construct hybrid promoters.

Recently, the catabolic pathway of erythritol was identified (Fig. 1). It involves the conversion of erythritol into erythrulose, catalyzed by the erythritol dehydrogenase encoded by *EYD1* (YALI0F01650g) (Carly *et al.* 2018), and then the phosphorylation of erythrulose into erythrulose-phosphate, catalyzed by the erythrulose kinase encoded by *EYK1* (YALI0F01606g) (Carly *et al.* 2017b).

Expression of both genes has been shown to be induced by erythritol; the *EYD1* gene displayed 46-fold higher expression on erythritol medium than on glucose medium, a pattern that is similar to the 41-fold increase observed for *EYK1* (Carly *et al.* 2017b, 2018; Carly and Fickers 2018). Consequently, both genes might contain CRMs that respond to erythritol or erythrulose. Two CRMs were identified within the *EYK1* promoter region using sequence conservation among members of the *Yarrowia* clade, which led to the identification of a UAS1-eyk1 motif that responds to erythritol, thus allowing the development of the first erythritol-induced hybrid promoters (Trassaert *et al.* 2017).

However, to engineer complex pathways, a large set of promoters with different strengths and expression profiles is needed. Differential expression in the exponential phase (such as that seen with the constitutive pTEF1) or in the late exponential phase (such as that seen with the promoter hp4d

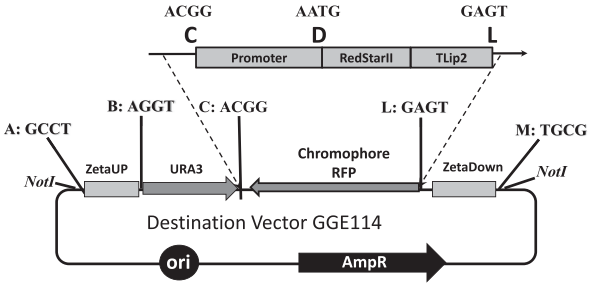

Figure 2. Schematic representation of the Golden Gate assembly technique used to study the promoters. The GG biobricks containing the promoter (overhangs **C** and **D**) were assembled alongside the fragment carrying RedStarII and the Lip2 terminator (overhangs **D** and **L**) and incorporated into the destination vector GGE114. The assembled vector contained the zeta region for expression cassette integration, the *URA3* marker for *Y. lipolytica* selection, and RedStarII as a reporter gene. The chromophore red fluorescent protein RFP was eliminated upon successful cloning of the biobricks. The expression cassette was released via *Not*I digestion.

that contains UAS1B-xpr2) as well as inducible expression could be used to switch on expression at a defined time or to switch off expression upon inducer removal or depletion.

In this study, we identified UASs for *EYK1* and *EYD1* and constructed a set of inducible promoter biobricks useful in Golden Gate assembly (GGAS) in *Y. lipolytica*; gene expression can be regulated by adapting or creating promoters with different behaviors (e.g. with different strengths, expression profiles and degrees of inducibility) with a view to fine-tuning gene expression in *Y. lipolytica*. Here, we constructed expression cassettes using Golden Gate assembly that carried various promoters upstream of a reporter fluorescent protein (RedStarII), which was used to characterize the new promoters.

MATERIALS AND METHODS

Plasmid construction by Golden Gate assembly

Most of the promoter amplicons were cloned using donor vectors (pCR Blunt II TOPO vectors; Thermo Fisher Scientific, Villebon sur Yvette, France), a process that was verified via *Bsa*I digestion and sequencing. Some of the promoters were synthesized and cloned in a donor vector (pUC57) from GeneScript Biotech (New Jersey, US) (see Table 1 and Table S1, Supporting Information). All the primers used to amplify the promoters were designed to have the upstream overhang **'ACGG'** and the downstream overhang **'AATG'** (see Table 2, Fig. 2), which were utilized as part of the Golden Gate assembly process. Other Golden Gate assembly building blocks (destination vector, RedStarII, and Lip2 terminator) were prepared by purifying plasmids from our own GGE collection (Golden Gate *E. coli* collection). The destination vector GGE114, pSB1A3-ZetaUP-URA3-RFP-ZetaDOWN (Table 1) contains the following components: zeta UP, *URA3*ex, RFP (red fluorescent protein, which can be used to generate a red *E. coli* colony) and zeta DOWN, as described in Fig. 2. The promoter names, primer pairs and templates used in PCR are described in Table S1 (Supporting Information). The Golden Gate reaction conditions have been described elsewhere (Celińska *et al.* 2017). The reaction mixture contained a predetermined equimolar amount of each Golden Gate biobrick and of the destination vector (50 pmoles of ends); 1 μL of T4 DNA ligase buffer (NEB); 5 U of *Bsa*I, 200 U of T4; and up to 10 μL of ddH_2O. The following thermal profile was applied: 37°C for 5 min, 16°C for 5 min for 60 cycles, 55°C for 5 min, 80°C for 5 min and 15°C ∞. The reaction mixture was then used for *E. coli DH5α* transformation (Sambrook and Russell 2001). White colonies were screened for the presence of the complete assembly. Afterwards, PCR and restriction enzyme digestion of the plasmids were conducted for verification purposes. All the biobricks were verified by sequencing before the Golden Gate assembly reaction.

Strains, growth media and culture conditions

The *E. coli* and *Y. lipolytica* strains used in the study are described in Table 1. The *EYK1* wild-type (WT) strain, JMY1212 (MatA *ura3-302 xpr2-322*, *LEU2*, zeta platform, derived from Po1d, wild-type for *EYK1*), was used as the basis for characterizing promoters in this study. The *eyk1*Δ strain, JMY7126, which displays a deletion of *EYK1*, was used to examine the inducible expression of promoters in a strain that cannot use erythritol as a carbon source. In this genetic background, erythritol is used as an inducer rather than as a carbon source. Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described below. The YPD medium contained 10 g/L of yeast extract (Difco, Paris, France), 10 g/L of Peptone (Difco, Paris, France) and 10 g/L of glucose (Sigma Aldrich, Saint-Quentin Fallavier, France). The YNB medium contained 1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate (YNBww; Difco, Paris, France), 10 g/L of glucose (Sigma), 5.0 g/L of NH_4Cl and 50 mM phosphate buffer (pH 6.8). To meet auxotrophic requirements, uracil (0.1 g/L), lysine (0.8 g/L) and leucine (0.1 g/L) were added to the culture medium when necessary. Solid media were created by adding 1.5% agar.

Construction of Y. lipolytica strains

The *eyk1*Δ strain JMY7126 was derived from the *EYK1* WT strain JMY1212, via successive gene deletion (*LYS5* and *EYK1*) and marker rescue. The PUT plasmids (Promoter-*URA3*ex marker-Terminator) were constructed for gene disruption as described in Fickers *et al.* (2003) and Vandermies *et al.* (2017) for *LYS5* and *EYK1*, respectively. The disruption cassettes were prepared by digesting PUT plasmids and used for the transformation of the *Y. lipolytica* strains. Transformants were selected on YNB-leucine or YNB-leucine-lysine medium, depending on genotype. The replicative plasmids (JME547, JME4265) harboring the Cre recombinase gene were used for excising the *URA3*ex marker. Strains from previous promoter studies are described in Table S1 (Supporting Information). The plasmids used in promoter analysis (assembled as described above) were digested by *Not*I, which allowed the expression cassette to be released prior to JMY1212 and JMY7126 transformation. Transformation employed 100 ng of DNA and the lithium acetate method (Le Dall, Nicaud and Gaillardin 1994); transformants were then selected using YNB or YNB-lysine medium, depending on genotype. Florescence tests were carried out for 12 transformants from each construct category, and a representative clone was selected (Table 1).

Microplate growth and florescence analysis

Yarrowia lipolytica pre-cultures were grown overnight in YNBD. They were then centrifuged, washed with an equal volume of YNB medium without a carbon source, and resuspended in 1 mL of the same medium. Microplates (96 well) containing 200 μL of the appropriate medium (final volume) were inoculated with washed cells at an OD_{600nm} of 0.1. YNB medium supplemented with glucose (10 g/L) or erythritol (10 g/L) was used

Table 1. List of strains and plasmids.

Strain	Genotype or description	Reference
E. coli		
DH5α	Φ80*lacZ*Δm15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Promega
pUC57	GeneScript Biotech donor vector	GeneScript Biotech
GGE114	pSB1A3-ZetaUP-URA3-RFP-ZetaDOWN	(Celińska *et al.* 2017)
GGE077	pCR4Blunt-TOPO-G1-RedStarII	(Celińska *et al.* 2017)
GGE020	pCR4Blunt-TOPO-T1-3Lip2	(Celińska *et al.* 2017)
GGE085	pCR4Blunt-TOPO-pTEF1	(Celińska *et al.* 2017)
JME547	php4d-Cre_Hyg	(Fickers *et al.* 2003)
JME3267	PUT of LYS5	This study
JME4056	PUT of EYK1 (RIE124)	(Vandermies *et al.* 2017)
JME4265	pTEF-EYK1_hp4d-Cre (RIE132)	(Vandermies *et al.* 2017)
GGE238	pCR4Blunt-TOPO-pEYK1	This study
GGE0130	pCR4Blunt-TOPO-pEYK1-2AB	This study
GGE0104	pCR4Blunt-TOPO-pEYK1-3AB	This study
GGE0132	pCR4Blunt-TOPO-pEYK1-4AB	This study
GGE250	pCR4Blunt-TOPO-pEYK1-5AB	This study
GGE140	pCR4Blunt-TOPO-pEYD1AB	This study
GGE172	pCR4Blunt-TOPO-pEYD1A*B	This study
GGE174	pCR4Blunt-TOPO-pEYD1AB*	This study
JME4417	pUC57-EYK1-4AB-coreTEF	This study
JME4418	pUC57-EYK1-4AB-R1-coreTEF	This study
JME4419	pUC57-EYK1-4AB-R2-coreTEF	This study
JME4420	pUC57-EYK1/EYD1A-coreEYK1	This study
JME4421	pUC57-EYK1/EYD1A-coreTEF	This study
JME4422	pUC57-EYK1/EYD1B-coreEYK1	This study
JME4423	pUC57-EYK1/EYD1B-coreTEF	This study
Y. lipolytica		
JMY195 (Po1d)	*MATA ura3-302 leu2-270 xpr2-322*	(Barth and Gaillardin 1996)
JMY2900	Po1d, Ura^+ Leu^+	(Barth and Gaillardin 1996)
JMY1212	Po1d *lip2*Δ *lip7*Δ *lip8*Δ *LEU2*-ZETA	(Emond *et al.* 2010)
JMY5207	JMY1212 *lys5::URA3 ex*	(Soudier *et al.* unpublished)
JMY7121	JMY1212 *lys5*Δ	(Soudier *et al.* unpublished)
JMY7123	JMY1212 *lys5*Δ *eyk1::URA3 ex*	(Soudier *et al.* unpublished)
JMY7126	JMY1212 *lys5*Δ *eyk1*Δ	(Soudier *et al.* unpublished)

for the growth and florescence analysis. The *eyk1*Δ strain was grown in YNB-lysine medium containing glucose (2.5 g/L) as the carbon source and erythritol (2.5 g/L) as the inducer, as described previously (Trassaert *et al.* 2017). The strains were maintained at 28°C and 110 rpm in a Synergy microplate reader (Biotek, Colmar, France) in accordance with the manufacturer's instructions. OD_{600nm} and red fluorescence were measured every 30 min for 120 h. Red fluorescence was analyzed at the following wavelength settings: excitation at 558 nm and emission at 586 nm. Fluorescence was expressed as mean specific fluorescence value per hour (SFU/h, mean value of SFU per hour). RedStarII fluorescence was expressed in specific fluorescence units per hour. For the RedStarII measurements, no intrinsic fluorescence was detected. Cultures were performed at least in duplicate.

Sequence analysis

The genome sequences of *Yarrowia* species were assembled and annotated by Cécile Neuvéglise, Hugo Devillers and their colleagues (to be published). Homologs of *EYD1* in *Yarrowia* species were identified using BLAST at the private GRYC website (Genome Resources for Yeast Chromosomes; http://gryc.inra.fr) was searched using the *EYD1* gene as a template, as described previously (Carly *et al.* 2018). Promoter regions were retrieved using the download functionality developed by H. Devillers. Multiple alignment of the nucleotide sequences of the *EYK1* and *EYD1* gene promoters among the *Yarrowia* clade (*Y. lipolytica* [YALI], *Y. phangngensis* [YAPH], *Y. yakushimensis* [YAYA], *Y. alimentaria* [YAAL] and *Y. galli* [YAGA]) was then performed using the program Clustal Omega (Larkin *et al.* 2007), which is available at http://www.ebi.ac.uk/Tools/msa/clustalo/. The alignment results highlighted the CRM motifs that have been conserved through evolution and that are thus more likely to have a regulatory function. The conserved motifs were named Box A and Box B. To test their ability to function as UASs, the region containing these motifs plus the 5 to 17 bases on either side of the motifs were selected.

RESULTS

Identification of CRMs within EYK1 and EYD1 promoters

The catabolic pathway of erythritol involves *EYD1* and *EYK1* (Fig. 1), which has been shown to be inducible by erythritol (Carly *et al.* 2017b,2018). We previously reported that the

Table 2. List of primers.

Primer	Sequence	Use
P1 TEF FW	**GGTCTC**T**ACGG**GGGTTGGCGGCG	Amplification for building block construction
P1 TEF RV	**GGTCTC**T**CATT**CTTCGGGTGTGAGTTAC	
P1 EYK FW	**GGTCTC**T**ACGG**CCCATCGATGGAAACCTTAATAGGAGACTACTTCC	
P1 EYK RV	**GGTCTC**T**CATT**GGATCCAGTAGATGTGTAAGTG	
P1 EYD FW	GGGG**GGTCTC**T**ACGG**CCCATCGATGGAAACCTTAATAGGAGACTACTTCC	
P1 EYD RV	CCC**GGTCTC**T**CATT**TGTGTATGTGTGTGTGTGTGTGTG	
EYD UAS1 MluI Fw	CCTTAATAGGAGACTACTTCCGACGCGTAATTAGG	Addition of the MluI site for EYD1 UAS mutation
EYD UAS1 MluI RV	CCTAATTACGCGTCGGAAGTAGTCTCCTATTAAGG	
EYD UAS2 MluI Fw	GAACTCGATACGCGTGCCGTACTCTGGAAA	
EYD UAS2 MluI RV	TTTCCAGAGTACGGCACGCGTATCGAGTTC	
ZetaUp-internal-FW	TATCTTCTGACGCATTGACCAC	Verification of Golden Gate assembly process
URA3-internal-FW	CATCCAGAGAAGCACACAGG	
URA3-internal-RV	CAACTAACTCGTAACTATTACC	
Redstar-internal-FW	AAGACGGTGGCGTTGTTACT	
RedStar-internal-RV	GACTTGCTTCTTGGCCTTGT	
Tlip2-internal-FW	TGCGTTCCTCTAAGACAAATC	
Tlip2-internal-RV	GATTTGTCTTAGAGGAACGCATA	
ZetaDown-internal-RV	GGTAACGCCGATTCTCTCTG	

The bold underlined bases correspond to the *Bsa*1 site; the overhang is in italics.

300-bp EYK1 promoter is not induced on glucose and glycerol media but is induced by erythritol (Trassaert *et al.* 2017). When sequence conservation within the *Yarrowia* clade was examined, two CRMs were identified within the EYK1 promoter region. They were named UAS1-eyk1 (Box A), which had the consensus sequence [**CGGNANCNNNANNNGGAAAGCCG**], and UAS2-eyk1 (Box B), which had the minimal consensus sequence [**CNTGCATNATCCGANGAC**]; both are located upstream from the *Spe*I restriction site (Fig. 3A). In a previous study, thanks to the mutagenesis of the two CRMs (i.e. performed via the introduction of a *Mlu*I restriction site) and the construction of hybrid promoters, researchers identified a UAS1-eyk1 motif that responded to erythritol, thus allowing the development of the first erythritol-inducible hybrid promoters (Trassaert *et al.* 2017). In the latter study, YFP was used a reporter; however, we have observed that *Y. lipolytica* displays a high degree of auto fluorescence, which depends on growth phase and media composition (Trassaert *et al.* 2017 and unpublished results). Therefore, we now use RedStarII as a reporter.

To identify the regulatory element (i.e. UAS) within the EYD1 promoter region, we analyzed the intergenic region between YALI0F01650g (*EYD1*) and the upstream gene YALI0F01672g, using a similar CRM search. Since this intergenic region was longer than 5500 bp (i.e. 5591 bp; Fig. 4), we analyzed the upstream region using the 800-bp nucleic acid sequence found upstream from *EYD1*. BLAST analysis of the EYD1 promoter did not yield evidence of any conserved motif within the *Y. lipolytica* genome (data not shown). Therefore, we examined how the promoter region of the *EYD1* gene in *Y. lipolytica* compared with that of other species in the *Yarrowia* clade (Fig. 4). This alignment process highlighted the existence of three putative conserved elements within the region 300 bp upstream; these elements were a putative TATA box (Box TATA; GATATAWA) and two CRMs. The first box, which had the main signature (ANTTNNNTTTCCNNATNNGG), was named CRM1-eyd1 (Box A). The second box, which had the main signature (CGGNNCTNNATTGAGAANNC), was named CRM2-eyd1 (Box B) and had a variable number of CA repeats just before the ATG. Like the EYK1 promoter, the EYD1 promoter also had two CRMs, which may also represent motifs required for erythritol and/or erythrulose regulation.

Promoter biobrick construction

Each promoter biobrick was designed and constructed to be compatible with *Y. lipolytica* GGAS, previously described by Celińska *et al.* (2017). First, the presence of internal *Bsa*I sites within the promoter sequence was analyzed. Depending on the number of *Bsa*I sites, either the sites were eliminated by PCR mutagenesis or promoters were purchased from GeneScript Biotech in the form of synthetic DNA fragments or plasmids. Second, we added *Bsa*I sites at both ends of the promoter using PCR and specific overhangs, namely the upstream overhang **C** (ACGG) and the downstream overhang **D** (AATG). Third, we purified the PCR products by gel extraction and cloned them into a TOPO vector (Table 1).

Construction of expression cassettes by Golden Gate assembly for promoter analysis

The assemblies we designed contained different promoter variants; the ORF encoding fluorescent protein RedStarII; and the Lip2 terminator, which were all incorporated using the *Bsa*I sites C and D as well as the L overhang (Fig. 2). The three corresponding fragments were assembled with the destination vector GGE114 by adding equimolar concentrations of each fragment type and carrying out a digestion/ligation PCR, as described above. *Escherichia coli* was transformed using the GGAS reaction, and white colonies were selected on LB ampicillin plates. Four positive transformants were screened by colony PCR using the primer pair URA3-internal-FW/RedStar-internal-RV

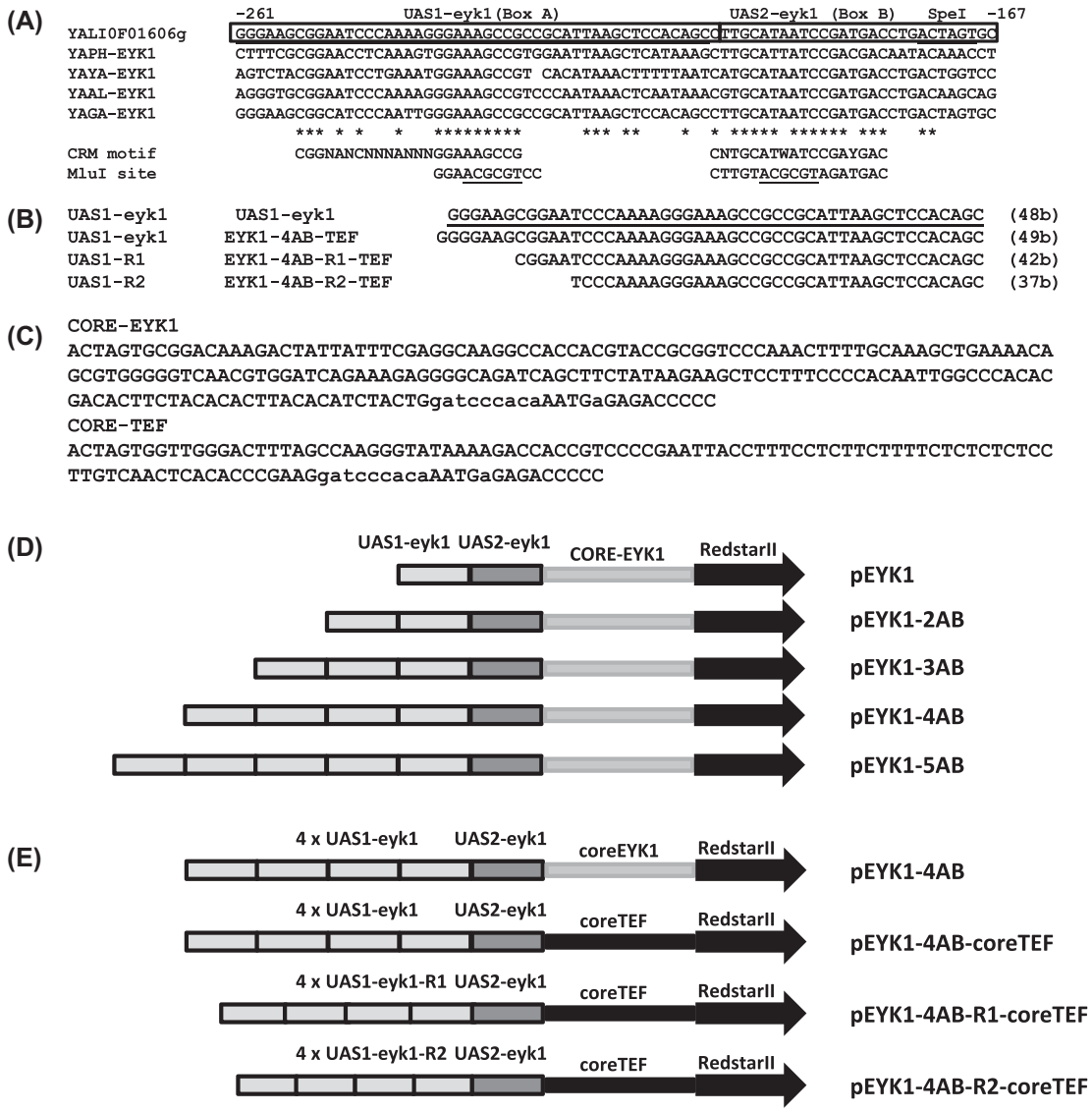

Figure 3. Multiple alignment of the EYK1 promoter containing the two CRMs and a schematic representation of the EYK1 hybrid promoters. **A**, Alignment of the *Y. lipolytica* EYK1 promoter region −261 to −167 containing the two CRMs that act as regulatory elements and control the expression of the *EYK1* gene in response to erythritol (the UAS1-eyk1 and UAS2-eyk1 motifs). The CRMs are indicated with asterisks, and the corresponding CRM consensus sequences are provided. The genomic sequences are from *Y. lipolytica* W29 (YALI; YALI0F01606g), *Y. phangngensis* (YAPH), *Y. yakushimensis* (YAYA), *Y. alimentaria* (YAAL) and *Y. galli* (YAGA). The *SpeI* site is underlined. The underlined *MluI* sites used in the mutation of the CRMs are shown in panel A. The region containing the UAS1-eyk1 motif used for tandem repeat construction is boxed. **B**, Sequence of the UAS1-eyk1 motif (48 bp) used for EYK3AB hybrid promoter construction (Trassaert *et al.* 2017) and the reduced regions used in this study. **C**, EYK and TEF core sequences. D, Schematic representation of the hybrid promoters that contained varying numbers of copies of UAS1-eyk1, which controlled the expression of RedStarII. **E**, Schematic representation of the hybrid EYK-4AB promoter and the hybrid EYK-coreTEF promoters with the reduced UAS1-eyk1 motif that controlled the expression of RedStarII: pEYK1-4AB; pEYK1-4AB-coreTEF (4 tandem copies of 49-bp UAS1-eyk1 + UAS2-eyk1 + coreTEF); pEYK1-4AB-R1-coreTEF (4 tandem copies of 42-bp UAS1-eyk1 + UAS2-eyk1 + coreTEF); and pEYK-4AB-R2-coreTEF (4 tandem copies of 37-bp UAS1-eyk1 + UAS2-eyk1 + coreTEF). The sequences are provided in additional file 1: Table S1 (Supporting Information). N (any base), W (A or T) and Y (C or T) are defined according to IUPAC nucleotide codes (1970).

(Table 2). Plasmids were extracted and verified by PCR using the primer pair URA3-internal-FW/ZetaDOWN-internal-RV and *NotI* digestion. The resulting plasmids are depicted in Table S1 (Supporting Information). The expression cassettes digested by *NotI* were used to generate the *Y. lipolytica EYK1* WT strain (JMY1212) and *eyk1*Δ strain (JMY7126). Ura + transformants were selected on YNB medium supplemented with lysine when necessary. Twelve transformants were selected for further analysis. A representative clone was conserved for comparative studies (Table S1, Supporting Information).

Tandem repeats of $UAS1_{EYK1}$ increase promoter strength in both the EYK1 wild type (JMY1212) and the eyk1Δ strain (JMY7126)

We showed that promoter strength was increased with the hybrid promoter pEYK300A3B, which was composed of three repeats of the 48-bp UAS1-eyk1 (Trassaert *et al.* 2017). Four new hybrid promoters were generated by fusing two, three, four and five UAS1-eyk1 tandem elements taken from the EYK1 promoter, which were named EYK1-2AB, EYK1-3AB, EYK1-4AB and EYK1-5AB, respectively (Fig. 3D). The expression levels and strength

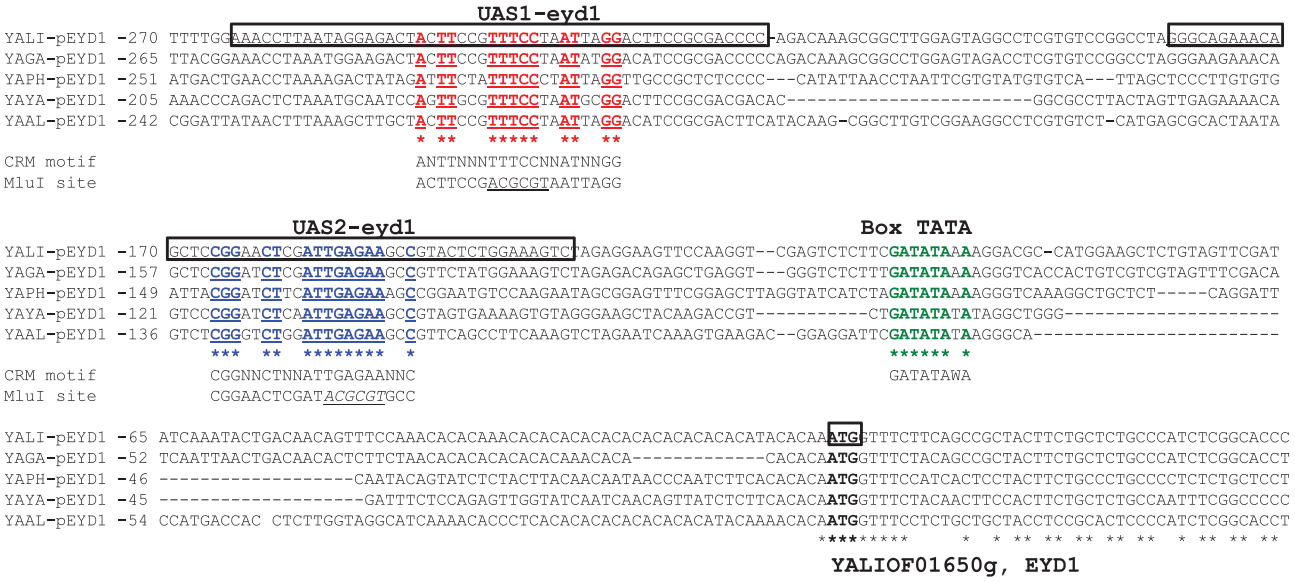

Figure 4. Multiple alignment of the *EYD1* promoter. The alignment of the region between YALI0F01650g (*EYD1*) and the upstream gene YALI0F01672g in *Y. lipolytica* and strains from the *Yarrowia* clade highlights the putative conserved cis-regulatory modules (CRMs) that represent putative regulatory elements for the expression and regulation of the *EYD1* gene by erythritol and erythrulose. The genomic sequences are from *Y. lipolytica* W29 (YALI-pEYD1), *Y. phangngensis* (YAPH-pEYD1), *Y. yakushimensis* (YAYA-pEYD1), *Y. alimentaria* (YAAL-pEYD1) and *Y. galli* (YAGA-pEYD1). The sequences are provided in additional file 1: Table S3 (Supporting Information). The region containing the UAS1-eyd1 and UAS2-eyd1 motifs used for tandem repeat construction is boxed. The nucleic acids that have been conserved in the five species are indicated by a star. The start codon of *EYD1* is indicated as a boxed ATG. The *MluI* sites used in the mutation of the CRMs are shown. In the CRM sequences, N represents any nucleotide.

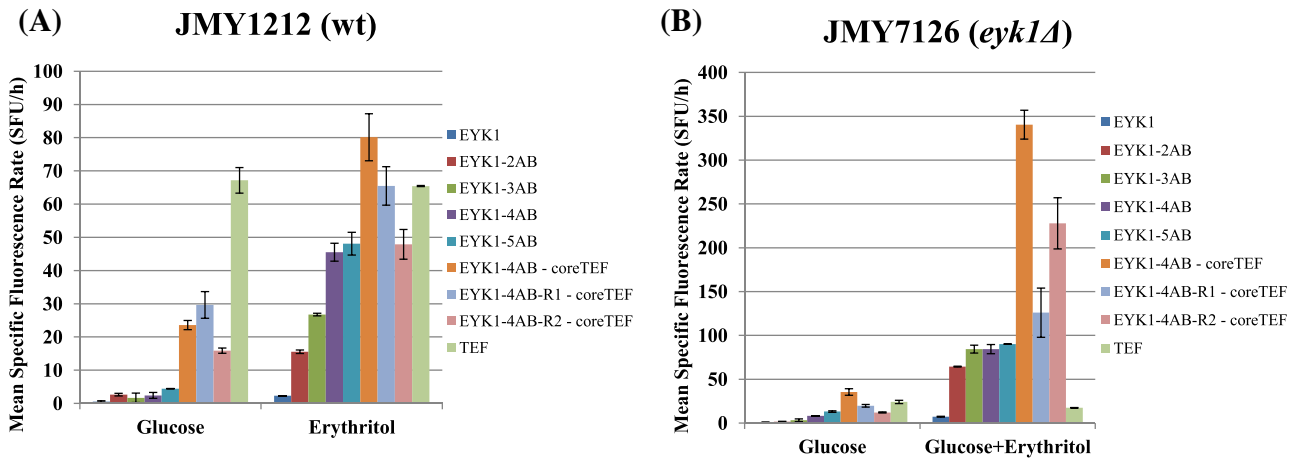

Figure 5. Hybrid EYK1 promoter expression and strength depending on the medium and strain *EYK1* wild type (JMY1212) and *eyk1*Δ mutant (JMY7126). **A**, Results for the *EYK1* wild type, which could use erythritol for growth and **B**, Results for the *eyk1*Δ mutant, which could not metabolize erythritol. Promoter strength was determined by quantifying RedStarII expression and comparing the mean rate of specific fluorescence (SFU/h) obtained when the *EYK1* wild type was grown on erythritol medium or the *eyk1*Δ mutant was grown on glucose + erythritol medium vs. when they were grown on glucose alone.

of the hybrid EYK1 promoters were determined by quantifying RedStarII expression: we determined the mean specific fluorescence rate (SFU/h) of the *EYK1* WT (JMY1212) grown on erythritol and of the *eyk1*Δ strain (JMY7126) grown on glucose + erythritol (results were compared to glucose-only medium; Fig. 5 and Table 3).

In the *EYK1* WT (JMY1212), activity increased slightly concomitantly with UAS1-eyk1 copy number and ranged from 0.54 to 4.42 SFU/h on the glucose medium (Table 3). The SFU rate increased significantly more on the erythritol medium, from 2.28 SFU/h for EYK1 (one copy) to 48.12 SFU/h for EYK1-5AB (five copies). Relative induction also increased, from 4.3 fold to 19.0 fold. Optimal levels were observed for EYK1-4AB. Under these growth conditions, EYK1 displayed low expression levels (0.54 SFU/h) compared to the TEF promoter (67.16 SFU/h). When erythritol was used as an inducer, TEF promoter strength (65.42 SFU/h) was equivalent to that on glucose medium; the strength of EYK1-4AB was comparable—48.12 SFU/h. Thus, when an inducer was present, the EYK hybrid promoter displayed similar activity to the TEF promoter and also had the significant advantage of being inducible.

In *eyk1*Δ strain (JMY7126), activity also increased concomitantly with UAS1-eyk1 copy number, ranging from 0.76 to 13.15 SFU/h on glucose medium (Table 3). The SFU rate increased significantly more on erythritol medium, from 7.13 for EYK1 (one copy) to 90.15 for EYK1-5AB (five copies). Relative

Table 3. Promoter expression and induction levels in the *EYK1* wild type (WT) and the *eyk1*Δ mutant.

	EYK1 WT (JMY1212)			*eyk1*Δ mutant (JMY7126)		
Promoter	Glucose[a]	Erythritol[a]	Fold change[b]	Glucose[a]	Glucose + Erythritol[a]	Fold change[b]
TEF	67.16 ± 3.87	65.42 ± 0.17	1.0	24.11 ± 1.88	17.45 ± 0.39	0.7
EYK1	0.54 ± 0.23	2.28 ± 0.04	4.3	0.76 ± 0.13	7.13 ± 0.51	9.4
EYK1-2AB	2.63 ± 0.38	15.55 ± 0.55	5.9	1.41 ± 0.57	64.48 ± 0.49	45.8
EYK1-3AB	1.68 ± 1.44	26.76 ± 0.38	15.9	3.23 ± 1.39	84.41 ± 4.55	26.1
EYK1-4AB	2.39 ± 0.88	45.50 ± 2.70	19.0	8.18 ± 0.07	84.29 ± 5.21	10.3
EYK1-5AB	4.42 ± 0.09	48.12 ± 3.43	10.9	13.15 ± 0.81	90.15 ± 0.30	6.9
EYK1-4AB-coreTEF	23.57 ± 1.37	80.14 ± 7.06	3.4	35.53 ± 3.73	340.52 ± 16.45	9.6
EYK1–4AB-R1-coreTEF	29.62 ± 4.01	65.50 ± 5.80	2.2	19.72 ± 1.54	125.94 ± 28.09	6.4
EYK1–4AB-R2-coreTEF	15.88 ± 0.76	47.89 ± 4.49	3.0	12.06 ± 0.68	227.84 ± 29.20	18.9

[a]Expressed in SFU/h as described in the materials and methods.
[b]Calculated by comparing the results on erythritol to those on glucose.

induction also increased, from 9.4 fold to 45.8 fold. Optimal levels were observed for EYK1-2AB. On glucose medium, EYK1 displayed low expression levels (0.76 SFU/h) compared to the TEF promoter (24.11 SFU/h). When erythritol was used as an inducer, the TEF promoter displayed slightly reduced strength (17.45 SFU/h), while EYK1-5AB remained strong (90.15 SFU/h). Under such growth conditions and for this strain background (deletion of *EYK1* gene), the performance of the EYK1 hybrid promoter surpassed that of the TEF promoter, as the former was 5.16-fold stronger.

Reduction of the UAS1-eyk1 region

Promoter strength also depends on the core promoter used (Shabbir Hussain *et al.* 2016). We tested hybrid promoters with a TEF core and examined the effect of reducing the size of the UAS1-eyk1 motif (Fig. 3E). We constructed synthetic promoters with different UAS sizes: UAS1-4AB-TEF (four copies of a 69-bp UAS1-eyk1), UAS1-4AB-R1-TEF (four copies of a 62-bp UAS1-eyk1r1) and UAS1—AB-R2-TEF (four copies of a 57-bp UAS1-eyk1r2). When erythritol was used as an inducer, the strength of the EYK1-4AB-coreTEF promoter increased 1.65 fold (80.14 SFU/h vs. 45.50 SFU/h for EYK1-4AB) in the *EYK1* WT (JMY1212) and, more surprisingly, that of the EYK1-4AB-coreTEF promoter increased 4.04 fold (340.52 SFU/h vs. 84.29 SFU/h for EYK1-4AB) in the *eyk1*Δ strain (JMY7126) (Fig. 5 and Table 3). Although we observed an increase in expression levels, induction levels declined (were just 9.6 fold). This result indicates that promoter strength declines when the size of the UAS1-eyk1 motif shrinks, which shows that CRM1 eyk1 extends all the way to the conserved CGG sequence, yielding a general consensus sequence of [**CGGNANCNNNANNGGAAAGCCG**].

Both $UAS1_{EYD1}$ and $UAS2_{EYD1}$ give rise to an inducible promoter in both the EYK1 wild type (JMY1212) and the eyk1Δ strain (JMY7126)

Two putative regulatory elements for the expression and regulation of the *EYD1* gene were found by comparing the upstream DNA sequences of *EYD1* homologs in the *Yarrowia* clade (Fig. 4). The two conserved motifs, CRMa and CRMb, were mutated by introducing a *MluI* site (Fig. 6A). The motif A [ACTTCCGTTTCCTAATTAGG] was replaced by [ACTTCCGACGCGTAATTAGG] and was named A*. The motif B [CGGAACTCGATTGAGAAGCC] was replaced by [CGGAACTCGATACGCGTGCC] and was named B*. This process yielded the EYD1A*B and EYD1AB* promoters, respectively. Promoter strength and induction levels were compared with those of the EYK1 and EYD1 promoters using the *EYK1* WT (JMY1212) and the *eyk1*Δ mutant (JMY7126) (Fig. 6A, B and Table 4).

In the *EYK1* WT (JMY1212), on glucose medium, the RedstarII expression levels allowed by pEYD1 (0.85 SFU/h) were similar to those allowed by pEYK1 (0.54 SFU/h). The former promoter was also induced by erythritol (11.5 SFU/h, as compared to 2.28 SFU/h for pEYK1) (Table 4 and Fig. 7). The mutation of Box A (EYD1A*B) completely abolished the expression of RedStarII on glucose medium. However, RedStarII continued to be slightly expressed on erythritol (0.16 SFU/h), indicating that CRMa is important for expression and induction. In contrast, the mutation of Box B (EYD1AB*) resulted in just a 2-fold reduction of RedStarII expression on glucose medium (0.43 SFU/h). RedStarII expression levels were higher on erythritol (2.57 SFU/h), indicating that CRMb is less important for expression and induction (Table 4).

In the *eyk1*Δ mutant (JMY7126), unexpected patterns of expression and relative induction were observed on glucose + erythritol medium (Table 4 and Fig. 7). All three promoters, including the mutated ones, showed low levels of expression on the glucose medium (0.5 SFU/h) but higher levels of expression on the glucose + erythritol medium (194.50 to 457.51 SFU/h); a tremendous induction was observed, ranging from 357.6 to 896.1 SFU/h. These results indicate that both CRMa and CRMb are important for expression and induction under these growth conditions and in this genetic background.

Both EYD1 UASA and UASB respond to erythritol

CRMa and CRMb appeared to be involved in *EYD1* expression. To determine their respective role in erythritol-based expression and induction, four hybrid promoters were designed. We used UAS1-eyd1 containing CRMa and UAS2-eyd1 containing CRMb (Fig. 6A). Two hybrid promoters EYK1/EYD1 were designed; they incorporated either four tandem repeats of UAS1-eyd1 or four tandem repeats of UAS2-eyd1 in the place of UAS-eyk1, which gave rise to EYK1/EYD1A-coreEYK1 and EYK1/EYD1B-coreEYK1, respectively (Fig. 6C). Two additional hybrid promoters were designed using a TEF core, which gave rise to EYK1/EYD1A-coreTEF and EYK1/EYD1B-coreTEF (Fig. 6C). These expression cassettes were introduced into the *EYK1* WT (JMY1212) and the *eyk1*Δ mutant (JMY7126) (Table S1, Supporting Information). In *EYK1* WT (JMY1212), UAS1-eyd1 allowed efficient expression of RedStarII in erythritol medium (66.94 SFU/h, with a 6.8-fold change

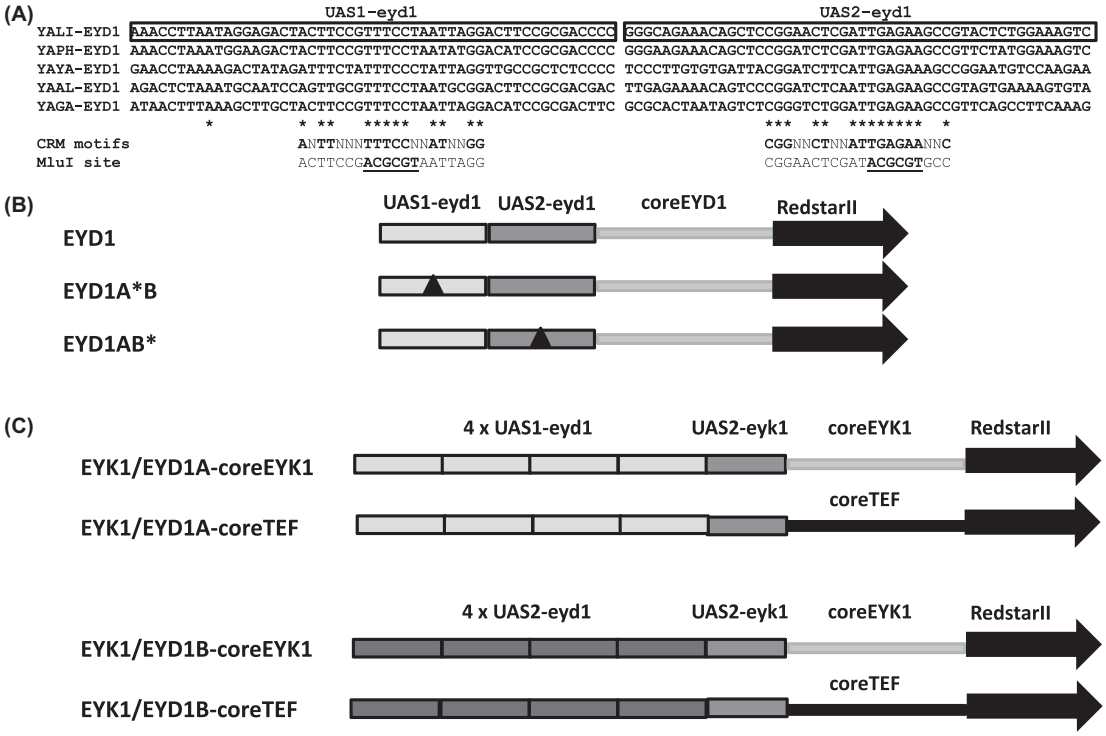

Figure 6. Multiple alignment of the *EYD1* UAS and a schematic representation of the mutated and hybrid promoters used in this study. **A**, Multiple alignment of the UAS1-eyd1 and UAS2-eyd1 motifs of the *EYD1* promoter for *Y. lipolytica* and strains in the *Yarrowia* clade. The CRMs are indicated with asterisks, and the corresponding CRM consensus sequences are provided. The region containing the UAS1-eyd1 and UAS2-eyd1 motifs used in tandem repeat construction is boxed. **B**, Schematic representation of the wild-type EYD1 promoter (EYD1) and the mutated EYD1 promoters within the UAS1-eyd1 (EYD1A*B) and the UAS2-eyd1 (EYD1AB*) motifs that controlled the expression of RedStarII. The *MluI* site used in the mutation of the CRM is shown in panel A. **C**, Schematic representation of the hybrid EYD1/EYK1 promoters containing either the EYK1 or the TEF core promoter; EYK1/EYD1A-EYK1, four tandem repeats of UAS1-eyd1 + UAS2-eyk1 + coreEYK1; EYK1/EYD1A-TEF, four tandem repeats of UAS1-eyd1 + UAS2-eyk1 + coreTEF; EYK1/EYD2-EYK1, four tandem repeats of UAS2-eyd1 + UAS2-eyk1 + coreEYK1; or EYK1/EYD1B-TEF, four tandem repeats of UAS2-eyd1 + UAS2-eyk1 + coreTEF.

Table 4. Strength of different promoters in the *EYK1* wild type (WT) and the *eyk1*Δ mutant.

	EYK1 WT (JMY1212)			*eyk1*Δ mutant (JMY7126)		
Promoter	Glucose[a]	Erythritol[a]	Fold change[b]	Glucose[a]	Glucose + Erythritol[a]	Fold change[b]
EYD1AB	0.85 ± 0.54	11.50 ± 0.25	13.4	0.67 ± 1.52	457.51 ± 11.37	682.5
EYD1A*B	– [c]	0.16 ± 0.32	–	0.54 ± 0.88	194.50 ± 11.50	357.6
EYD1AB*	0.43 ± 1.09	2.57 ± 0.66	5.9	0.27 ± 0.15	245.27 ± 14.56	896.1

[a]Expressed in SFU/h as described in the materials and methods.
[b]Calculated by comparing the results on erythritol to those on glucose.
[c]No fluorescence was detected.

between glucose and erythritol media). In contrast, in both media, low expression levels were observed for the promoters containing the four tandem repeats of UAS2-eyd1 (Fig. 7C and Table 5). In the *eyk1*Δ mutant (JMY7126), both UAS1-eyd1 and UAS2-eyd1 allowed expression of RedStarII in erythritol medium (91.15 SFU/h and 52.57 SFU/h, respectively). This result confirmed that both UAS1 and UAS2 are involved in erythritol induction (Fig. 7D and Table 5). In both strains, exchanging the EYK1 core with the TEF core had a drastic effect on erythritol induction (Table 4) but did not modify expression levels significantly. This result shows that, in this study, the use of a more efficient core promoter did not contribute to the development of inducible promoters.

Promoter expression depend on glucose and erythritol concentration in eyk1Δ

The best expression levels and greatest fold change were obtained with the EYK1, EYK3AB and EYD1 promoters in the *eyk1*Δ mutant. In a previous study (Trassaert *et al.* 2017), expression of EYK1 in the wild-type *EYK1* strain was shown to be modulated by erythritol and erythrulose concentrations in a glycerol medium. To examine how glucose and erythritol concentrations affected promotor expression patterns, RedStarII expression in the *eyk1*Δ mutant was characterized during strain growth on media with two concentrations of glucose, 0.25% and 0.50%, and three concentrations of erythritol, 0%, 0.25% and 0.50% (Figure S1, Supporting Information).

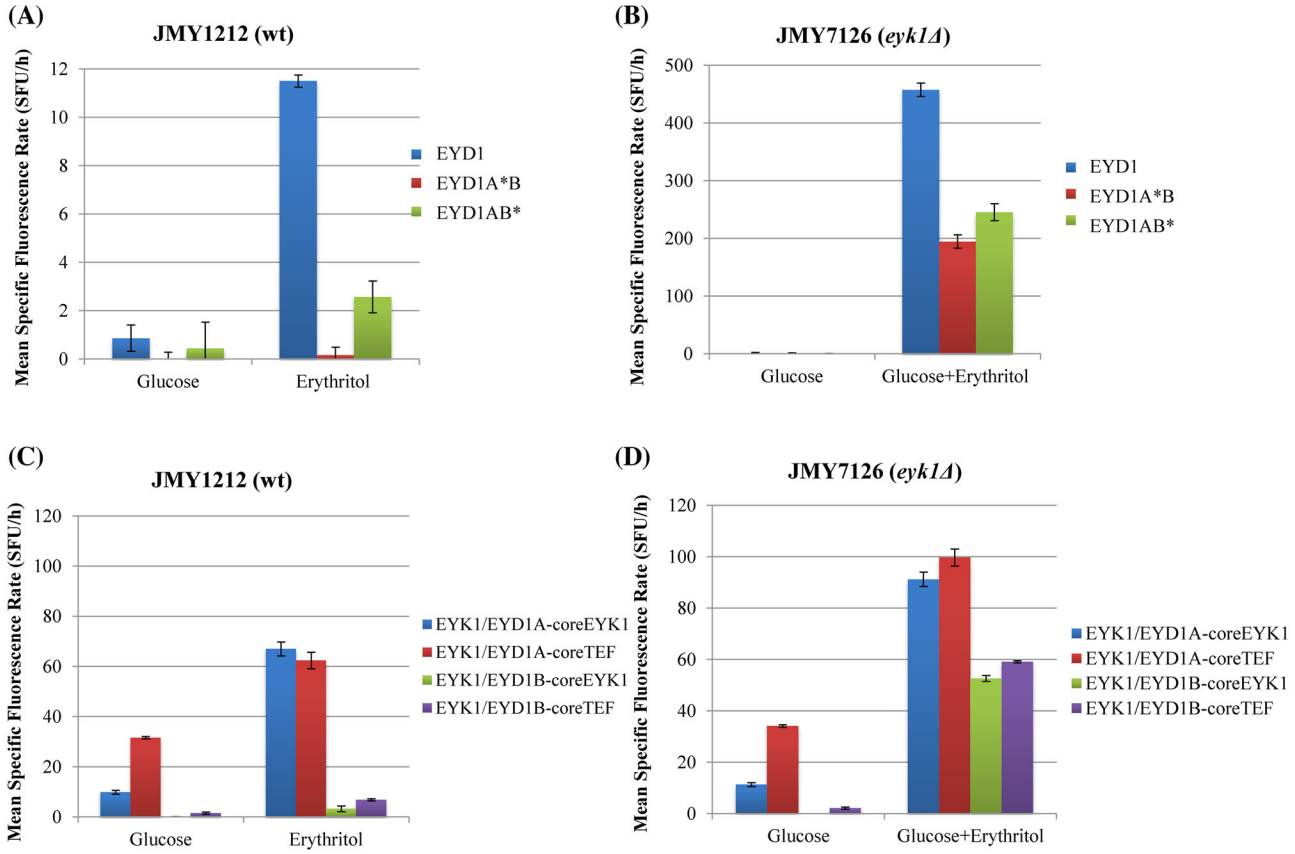

Figure 7. Hybrid EYD1 promoter expression and strength depending on medium and strain *EYK1* wild type (JMY1212) and *eyk1*Δ mutant (JMY7126). **A** and **C**, Results for the *EYK1* wild type, which could use erythritol for growth, and **B** and **D**, results for the *eyk1*Δ mutant, which could not metabolize erythritol. Promoter strength was determined by quantifying RedStarII expression and comparing the mean rate of specific fluorescence (SFU/h) obtained when the *EYK1* wild type was grown on erythritol medium or the *eyk1*Δ mutant was grown on glucose + erythritol medium vs. when they were grown on glucose alone.

Table 5. Promoter strength in the *EYK1* wild type (WT) and the *eyk1*Δ mutant depending on the *EYD1* upstreaming activating sequence (UAS) and core promoter.

	EYK1 WT (JMY1212)			*eyk1*Δ mutant (JMY7126)		
Promoter	Glucose[a]	Erythritol[a]	Fold change[b]	Glucose[a]	Glucose + Erythritol [a]	Fold change[b]
EYK1/EYD1A-coreEYK1	9.85 ± 0.78	66.94 ± 2.81	6.8	11.24 ± 1.82	91.15 ± 8.46	8.1
EYK1/EYD1A-coreTEF	31.57 ± 0.50	62.38 ± 3.30	2.0	34.06 ± 1.65	99.70 ± 17.14	2.9
EYK1/EYD1B-coreEYK1	0.10 ± 0.00	3.23 ± 1.62	32.3	–[c]	52.57 ± 0.76	
EYK1/EYD1B-coreTEF	1.51 ± 0.42	6.81 ± 0.42	4.5	2.11 ± 0.29	59.03 ± 6.00	28.0

[a]Expressed in SFU/h as described in the Materials and Methods.
[b]Calculated by comparing the results on erythritol to those on glucose.
[c]No fluorescence was detected.

On the 0.25% glucose medium containing no erythritol, the promoters were not induced; in contrast, when erythritol was present, there was dose dependent induction (Figure S1 A, C and E, Supporting Information). When the medium contained 0.25% erythritol, fluorescence at 120 h was 816 FU, 7956 FU and 6142 FU for EYK1, EYK3AB and EYD1, respectively. When the medium contained 0.50% erythritol, it reached 1378 FU, 15,018 FU and 11,883 FU, respectively. These results indicate that, in the 0.25% glucose medium, the higher erythritol concentration led to an approximately two-fold increase in fluorescence.

Similar results were observed on the 0.50% glucose medium, although the promoters responded differently. For EYK1 and EYD1, fluorescence at 120 h was lower, regardless of erythritol concentration (0.25% vs. 0.50% erythritol: 542 FU vs. 397 FU for EYK1 and 4512 FU vs. 5212 FU for pEYD1). In contrast, pEYK3AB was less affected by the increase in glucose concentration (0.25% erythritol—11 009 FU and 0.50% erythritol—13 394 FU).

The promoters' rate of fluorescence also varied depending on glucose and erythritol concentrations, making it possible to identify different growth phases (Fig. S1, Supporting Information). On the 0.25% glucose medium with 0.25% erythritol, EYK1, EYK3AB and EYD1 displayed constant fluorescence rates (13.99 FU/h, 136.26 FU/h and 102.92 FU/h, respectively) that lasted for 60 h, 52 h and 34 h, respectively. Duration was greater when the medium contained 0.50% erythritol: 100 h, 84 h and 100 h, respectively. In contrast, on the 0.50%

glucose medium, the fluorescence rate was drastically reduced for EYK1 at both erythritol concentrations (4.17 FU/h and 4.31 FU/h for 0.25% and 0.50% erythritol, respectively), while EYK3AB and EYD1 showed less pronounced differences during phases 2 and 3. These results demonstrate that promoter strength and expression can be modulated by varying glucose and erythritol concentrations.

DISCUSSION

UASs are essential for transcription in yeasts. They must be upstream from the TATA box and transcription start site, but they can be located at variable distances (Buratowski *et al.* 1988). Most often, promoters are studied and regulatory elements are identified by deleting promoters and measuring expression of reporter genes, as exemplified by the research in which the regulatory motifs of *XPR2*, *TEF1* and *POX2* promoters in *Y. lipolytica* were determined (Madzak, Treton and Blanchin-Roland 2000; Blazeck *et al.* 2011; Blazeck *et al.* 2013; Shabbir Hussain *et al.* 2016).

As the number of available genomes increases and the costs of sequencing decrease, researchers can more frequently employ strategies such as phylogenetic footprinting, which is a powerful tool for identifying CRMs with regulatory functions of interest. Recently, genes involved in the catabolism of erythritol were identified in *Y. lipolytica*, namely *EYD1*, which codes for erythritol dehydrogenase, and *EYK1*, which codes for erythrulose kinase. Using the N-terminus sequence of the erythritol dehydrogenase found in *Lipomyces starkeyi*, a BLAST search identified the coding gene ODQ69334.1 in the *L. starkeyi* genome, whose sequence was recently made available. A subsequent BLAST search of the *Y. lipolytica* genome using this gene revealed the *EYD1* gene, which is encoded by YALI0F01650g. Carly and Fickers confirmed that *EYD1* encodes erythritol dehydrogenase (Carly *et al.* 2018). However, *Y. lipolytica* genome mining did not lead to the identification of a gene coding for erythrulose kinase. Instead, this gene was discovered by screening a mutant library for strains unable to grow on erythritol. Sequencing of the mutagenesis cassette insertion site led to the identification of the *EYK1* gene, which is encoded by YALY0F01606g. Carly *et al.* confirmed that this gene encodes erythrulose kinase (Carly *et al.* 2017b). It has been shown that both genes are induced by erythritol (Carly *et al.* 2017b, 2018).

In this study, we employed phylogenetic footprinting within the *Yarrowia* clade to explore the CRMs of the *EYD1* and *EYK1* genes. We used the sequences of *Y. lipolytica* W29, *Y. phangngensis*, *Y. yakushimensis*, *Y. alimentaria* and *Y. galli*. This analysis detected two CRMs, -CRMA-eyd1 and CRMB-eyd1, that occurred within 300 bp of the *EYD1* promoter and two CRMs, CRMA-eyk1 and CRMB-eyk1, that occurred within 300 bp of the *EYK1* promoter; both pairs of CRMs may respond to erythritol. A restriction site was introduced into the most conserved region of the CRMs, leading to a mutation that functionally inactivated the CRMs, abolishing or reducing the response to erythritol. Consequently, the phylogenetic footprinting technique is a very powerful approach for rapidly identifying putative UASs and upstream regulatory sequences. However, it does not reveal the extent of the UASs. Here, when designing hybrid promoters, we defined the UAS as the region containing the CRM plus 5–17 bases to either side.

Thanks to our mutation test, we discovered that both UAS1-eyd1 and UAS2-eyd1 are important for effective expression and induction, regardless of genetic background. Between the conserved motifs A and B of the EYD1 promoter, motif A seemed to be more involved in erythritol-based induction. Trassaert *et al.* (2017) obtained similar results after introducing a mutation into the conserved motifs A (pEYK300aB) and B (pEYK300Ab) of the inducible EYK1 pEYK300 promoter. When grown in minimal YNB medium containing 1% erythritol, the strain carrying the pEYK300A*B-YFP cassette with the mutated motif A displayed a decreased level of YFP expression compared to that of the unmutated pEYK300 (683 and 3536 SFU after 60 h, respectively). In contrast, when motif B was mutated, induction levels were higher under the same conditions (8389 and 3536 SFU after 60 h, respectively).

Expression levels have been found to be dependent on UAS copy number, which have ranged from four tandem copies of UAS1B-xpr2 (Madzak, Treton and Blanchin-Roland 2000) to as many as 32 copies of UAS1B-xpr2 (Blazeck *et al.* 2011; Blazeck, Garg and Alper. 2012). However, this relationship was not observed for the EYK1 and EYD1 hybrid promoters examined in this study. Indeed, we found that an increased number of UAS1-eyk1 copies increased promoter strength when the *EYK1* wild type (JMY1212) was grown on glucose or erythritol (Fig. 6 and Table 3) and that four tandem repeats seemed optimal. Similar results were obtained for the *eyk1*Δ mutant (JMY7126); however, in that strain, optimal expression was reached with three tandem repeats. This result may reflect the titration of the transcription factor: the higher erythritol concentration may result in greater induction, leading to a saturation of expression.

For the hybrid promoter in which the core promoter was exchanged (i.e. EYK1-4AB-coreTEF vs. EYK1-4AB), expression levels were higher, while induction levels were lower. Indeed, when the strong core TEF hybrid promoter was used, expression increased 10 fold and 2 fold, respectively, in the *EYK1* WT (JMY1212) and *eyk1*Δ mutant (JMY7126) grown on glucose. When erythritol was used as an inducer, hybrid promoter strength increased less than when glucose medium was used (two fold in the *EYK1* WT [JMY1212], five fold in the *eyk1*Δ mutant [JMY7126]). It seems that while the core TEF is able to act similarly to the core elements of erythritol-inducible promoter, the strength of its inducible response is less than that of the native EYD1 promoter. The hybrid promoter could be further improved by exchanging the core promoters or by employing a combination of TATA boxes from other inducible promoters (Redden and Alper 2015, Shabbir Hussain *et al.* 2016). Some hybrid promoters of EYK1 and EYD1 promoters used in the *eyk1*Δ mutant (JMY7126) were functionally strong upon induction. For example, the response associated with EYK1/EYD1B-core EYK1 and EYK1/EYD1B-coreTEF displayed a 52-fold and 28-fold increase, respectively (Table 5).

These studies demonstrate that EYK1-4AB provided the best expression levels and the greatest relative induction in the *EYK1* wild type, while EYK1-2AB yielded more optimal expression in the *eyk1*Δ mutant. The EYD1 promoter is a very tight promoter with very low expression levels on glucose media. Its strength is tremendous: ten-fold that of the strong pTEF promoter, with nearly 500-fold greater induction in the *eyk1*Δ strain. Consequently, in the *eyk1*Δ strain, the strength and expression of the EYK1, EYK3AB and EYD1 promoters can be modulated by varying glucose and erythritol concentrations, which generates additional possibilities for promoter fine-tuning.

In this article, we have demonstrated how CRMs can be identified and used to design a broad range of hybrid promoters with applications in metabolic engineering and synthetic biology. These new promoters that respond to erythritol could be

very useful in metabolic engineering, fundamental research and protein expression, as is the case for the Gal1 promoter in *S. cerevisiae*. This may be especially true for the strain containing the deletion in the *EYK1* gene, which allows erythritol to be used as an inducer. This trait is advantageous because erythritol is a cost-effective inducer in the industry. Several industrially relevant proteins such as the Brazzein (a sweetener) and *Candida antarctica* lipase B (CalB) have been successfully expressed using erythritol-inducible hybrid promoters in *Y. lipolytica* (unpublished results). The development of synthetic expression systems will help further improve the production capacity of *Y. lipolytica* in industrial processes.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Efficient expression vectors and host strain for the production of recombinant proteins by *Yarrowia lipolytica* in process conditions

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Abstract

Background: The oleaginous yeast *Yarrowia lipolytica* is increasingly used as an alternative cell factory for the production of recombinant proteins. Recently, regulated promoters from genes *EYK1* and *EYD1*, encoding an erythrulose kinase and an erythritol dehydrogenase, respectively, have been identified and characterized in this yeast. Hybrid promoters up-regulated by polyols such as erythritol and erythrulose have been developed based on tandem copies of upstream activating sequences from *EYK1* ($UAS1_{EYK1}$) and *XPR2* (encoding extracellular protease, $UAS1_{XPR2}$) promoters.

Results: The strength of native (p*EYD1*) and engineered promoters (*pEYK1-3AB* and *pHU8EYK*) was compared using the extracellular lipase CalB from *Candida antarctica* as a model protein and a novel dedicated host strain. This latter is engineered in polyol metabolism and allows targeted chromosomal integration. In process conditions, engineered promoters *pEYK1-3AB* and *pHU8EYK* yielded 2.8 and 2.5-fold higher protein productivity, respectively, as compared to the reference *pTEF* promoter. We also demonstrated the possibility of multicopy integration in the newly developed host strain. In batch bioreactor, the CalB multi-copy strain RIY406 led to a 1.6 fold increased lipase productivity (45,125 U mL^{-1}) within 24 h as compared to the mono-copy strain.

Conclusions: The expression system described herein appears promising for recombinant extracellular protein production in *Y. lipolytica*.

Keywords: Promoter, Regulation, Induction, Synthetic promoter, Erythritol, Protein secretion, Upstream activating sequence, *Yarrowia lipolytica*, CalB

Background

The oleaginous yeast *Yarrowia lipolytica* is increasingly used as an alternative to model yeasts such as *Saccharomyces cerevisiae* or *Komagataella phaffii* (*Pichia pastoris*). for the production of recombinant proteins [1, 2]. More than one hundred heterologous proteins have already been produced at high yield, highlighting its potential utilization as a cell factory. *Y. lipolytica* has been also shown able to produce efficiently chemicals such as citric acid [3], itaconic acid [4], erythritol [5], erythrulose [6] and lipid for biodiesel and biojet fuel [7, 8]. In nature, *Y. lipolytica* can grow in environments rich in lipids and proteins due to its ability to synthesize and secrete hydrolytic enzymes such as proteases or lipases [9, 10]. Based on these metabolic features, several engineering tools such as strong promoters and efficient sorting signals, including the prepro sequence of the *LIP2* gene encoding an extracellular lipase, have been developed [1, 11]. The promoter from the *XPR2* gene encoding alkaline extracellular protease was the first developed for recombinant gene expression [12]. Its functional dissection led to the identification of an upstream activating sequence ($UAS1_{XPR2}$), later used to construct constitutive

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hybrid promoters [13]. These are based on $UAS1_{XPR2}$ repeats fused upstream of the minimal *LEU2* promoter (*mLEU2*) [14, 15]. The number of $UAS1_{XPR2}$ repeats was found to modulate the strength of the promoter, allowing thus a fine-tuning of gene expression. A similar strategy was also applied to design constitutive promoters derived from the *TEF1* gene encoding the translation elongation factor 1α [16]. Regulated promoters derived from the *LIP2* and *POX2* genes encoding an extracellular lipase and an acyl-CoA oxidase, respectively, have been also developed for recombinant gene expression [17]. Although these regulated promoters delivered strong gene expression, their utilization at industrial scale is impeded by the hydrophobic nature of their inducers (i.e. triglycerides and fatty acids).

Recently, we have characterized two genes, namely *EYD1* and *EYK1*, involved in erythritol catabolic pathway [6, 18]. Gene *EYD1* was found to encode an erythritol dehydrogenase able to convert erythritol to erythrulose, while gene *EYK1* was suggested to encode an erythrulose kinase. Induction of *EYK1* native promoter (*pEYK300*) is significantly increased, in a dose-dependent manner, in the presence of erythritol or erythrulose, and drastically reduced in the presence of glycerol and glucose. Moreover, we demonstrated that erythrulose is a better inducer than erythritol [19]. The dissection of the *EYK1* promoter highlighted the existence of two UAS, namely $UAS1_{EYK1}$ and $UAS2_{EYK1}$. Using a reporter system based on yellow fluorescent protein (YFP) and mutated promoter, $UAS1_{EYK1}$ was identified as essential for promoter induction by both erythritol and erythrulose. By contrast, $UAS2_{EYK1}$ was found involved in repression by glucose. Synthetic promoters were constructed by addition of multiple copies of $UAS1_{EYK1}$ or $UAS1_{XPR2}$ upstream of the native *pEYK300* promoter. These promoters yielded, respectively, 3.2 and 15.6-fold higher expression levels of YFP-encoding gene than those obtained with wild-type *pEYK300* promoter [19]. Carly et al. [6, 18] found that the disruption of the *EYK1* gene impairs the ability of yeast cells to fully metabolize erythritol, since an *eyk1Δ* mutant could only convert erythritol into erythrulose. Therefore, with such a mutant, erythritol and/or erythrulose could be used as a free inducer as demonstrated previously [19]. Promoter engineering efforts were pursued with a reporter system based on RedStar fluorescent protein [20]. It concerned the influence of $UAS1_{EYK1}$ repeats and core element (*EYK1*, *TEF*) on promoter strength in wild type and *eyk1Δ* mutant.

Based on previous results, three promoters, namely *pEYK1-3AB* which comprises three repeats of $UAS1_{EYK1}$, *pHU8EYK* which comprises eight copies $UAS1_{XPR2}$ from *XPR2* gene and the native promoter of gene *EYD1* seemed to be promising to drive the production of recombinant proteins [19, 20]. Herein, these promoters were challenged for the production in process conditions of a protein of industrial interest, the lipase CalB from *Candida antarctica*. For that purpose, the pro-CalB nucleic acid sequence was codon-optimized for *Y. lipolytica*, fused with the secretion signal from the *LIP2* gene, and cloned under the control of promoters *pEYD1*, *pEYK1-3AB*, *pHU8EYK*, and of strong constitutive promoter *pTEF* used for comparison. The different CalB expression cassettes were then introduced in a novel host strain specifically developed for erythritol/erythrulose-inducible expression systems. For the different constructed strains, CalB gene expression and extracellular activity were monitored in process conditions, during cultures in bioreactor.

Methods

Media and culture conditions

Escherichia coli strains were grown at 37 °C in Luria–Bertani medium supplemented with kanamycin sulfate (50 µg mL^{-1}). *Y. lipolytica* strains were grown at 28 °C in YPD medium or in YNB medium (1.7 g L^{-1} yeast nitrogen base without amino acids and ammonium sulfate, YNBww (BD Difco, Franklin Lakes, NJ, USA), 50 mM phosphate buffer pH 6.8 supplemented with carbon and nitrogen sources, as described in Barth and Gaillardin [21]. For YNBD medium, glucose 10 g L^{-1} and NH_4Cl 5 g L^{-1} were added to YNB medium. For YNBE medium, erythritol 10 g L^{-1} and NH_4Cl 5 g L^{-1} were added to YNB medium. For YNBGE medium, glycerol 10 g L^{-1}, erythritol 10 g L^{-1}, yeast extract 5 g L^{-1} (Yeast extract UF, BD Difco), soytone 5 g L^{-1} (Select soytone, BD Difco) were added to YNB medium. $YNBG_2E$ medium is the same as YNBGE except it contained glycerol 20 g L^{-1}. Medium contained lysine (0.08%, w/v) and/or uracil (0.01%, w/v) to meet auxotrophic requirement. Hygromycin (200 µg mL^{-1}) was added for transformant selection. Solid media contained agar 1.5 % (w/v). Phosphate buffered saline (PBS) contained NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.44 g L^{-1} and KH_2PO4 0.24 g L^{-1}.

Cultures in bioreactor were inoculated at an initial optical density at 600 nm (OD_{600}) of 0.5 with PBS washed cells from a 16-h preculture in YPD medium. Cultures in 2Mag bioREACTOR (Munich, Germany) were performed in triplicate for 48 h in 10 mL of YNBGE medium with agitation set at 800 rpm. Cultures in DASGIP bioreactor (DASbox Mini Bioreactors SR0250ODLS, Eppendorf, Hamburg, Germany) were performed in duplicate for 48 h in 150 mL of $YNBG_2E$ medium supplemented with 500 µL L^{-1} antifoam (Tego KS911, Evonik, Essen, Germany). Airflow was set at 1 vvm, agitation was ranged from 800 to 950 rpm to ensure a dissolved oxygen level above 20% and pH was automatically adjusted to 6.8

Table 1 Strains and plasmids used in this study

Strain (plasmid)	Genotype	References
E. coli		
JME547 (JMP547)	pUB4-Cre-Hyg	[23]
JME1046 (JMP1046)	JMP62-*URA3ex-pTEF*	[34]
JME3267 (JMP3267)	PUT *lys5::URA3ex*	This work
JME3739 (JMP3739)	JMP62-*URA3ex-pTEF*-CalB	This work
RIE124 (RIP124)	PUT *eyk1::URA3ex*	[33]
JME4001 (JMP4001)	JMP62-URA3ex-pHU8EYK-YFP	Unpublished
JME4123 (JMP4123)	pUC57-*pEYK1-3AB*	[19]
JME4230 (JMP4230)	JMP62-*URA3ex-pHU8EYK*	This work
JME4243 (JMP4243)	JMP62-*URA3ex-pHU8EYK* CalB	This work
RIE132 (RIP132)	pGEMT-easy-Cre-*EYK1*	[33]
JME4266 (JMP4266)	JMP62-*URA3ex-pEYK1-3AB*	This work
JME4365 (JMP4365)	JMP62-*URA3ex-pEYK1-3AB*-CalB	This work
JME4579 (JMP4579)	JMP62-*LYS5ex-pEYK1-3AB*-CalB	This work
JME4590 (JMP4590)	JMP62-*URA3ex-pEYD1*-CalB	This work
RIE279 (RIP279)	JMP62-*LYS5ex*	This work
Y. lipolytica		
JMY1212	*MATA ura3-302 leu2-270-LEU2-Zeta, xpr2-322, lip2Δ, lip7Δ, lip8Δ*	[30]
JMY5207	*MATA ura3-302 leu2-270-LEU2-Zeta, xpr2-322, lip2Δ, lip7Δ, lip8Δ, lys5::URA3ex*	This work
JMY7121	*MATA ura3-302 leu2-270-LEU2-Zeta, xpr2-322, lip2Δ, lip7Δ, lip8Δ, lys5Δ*	This work
JMY7123	*MATA ura3-302 leu2-270-LEU2-Zeta, xpr2-322, lip2Δ, lip7Δ, lip8Δ, lys5Δ, eyk1::URA3ex*	This work
JMY7126	*MATA ura3-302 leu2-270-LEU2-Zeta, xpr2-322, lip2Δ, lip7Δ, lip8Δ, lys5Δ, eyk1Δ*	This work
JMY7536	JMY7126 + *pTEF*-CalB-*URA3ex*	This work
JMY7539	JMY7126 + *pEYK1-3AB*-CalB-*URA3ex*	This work
JMY7544	JMY7126 + *pHU8EYK*-CalB-*URA3ex*	This work
JMY7548	JMY7126 + *pEYD1*-CalB-*URA3ex*	This work
RIY368	JMY7539 + *LYS5ex*	This work
RIY394	JMY7536 + *LYS5ex*	This work
RIY406	JMY7126 + *pEYK1-3AB*-CalB-*URA3ex* + *pEYK1-3AB*-CalB-*LYS5ex*	This work

by addition of H_3PO_4 8 M or NaOH 12.5 M. Culture in Duetz deepwell plate (24-well plate with pyramidal bottom, Kühner AG, Birsfelden, Switzerland) were performed in 2 mL of $YNBG_2E$ medium for 48 h; they were inoculated with of 200 µL of a 24 h precultures carried out in 400 µL YPD in 48-well microplates (multiwell cell culture plates, flat bottom, TC-treated, VWR, Radnor, PA, USA) for 24 h.

Strains and plasmids construction

Standard molecular genetic techniques were used in this study [22]. Restriction enzymes were obtained from New England Biolabs (MA, USA). PCR amplifications were performed in an Applied Biosystems 2720 thermal cycler with GoTaq DNA polymerases (Promega, WI, USA) or Q5 High-Fidelity DNA Polymerase (New England Biolabs). PCR fragments were purified with a QIAgen Purification kit (Qiagen, Hilden, Germany) and plasmids DNA were isolated with a QIAprep Spin Miniprep kit (Qiagen). All strains and plasmids used in this study are listed in Table 1.

For the construction of JMY7126 (Fig. 1), *LYS5* and *EYK1* were disrupted using PUT cassettes according to Fickers et al. [23]. For *LYS5* disruption, the promoter (P) and terminator (T) regions of the gene were amplified by PCR with primer pairs LYS5-P1/LYS5-P2 and LYS5-T1/LYS5-T2, respectively. Primers LYS5-P1 and LYS5-T2, contained sequence of *Not*I restriction site while primers LYS5-P2 and LYS5-T1 contained the sequence of the I-*Sce*I restriction (Table 2). The corresponding amplicons were purified and used as a template for the second PCR step resulting PT fragment, that was subsequently cloned to pCR4Blunt-TOPO plasmid after purification (Invitrogen, CA, USA). Finally, *URA3ex* marker from JMP1046 was introduced at the I-*Sce*I site of this plasmid, yielding plasmid JMP3267. The *LYS5* PUT cassette obtained by *Not*I digestion of JMP3267 was used to transform *Y. lipolytica* strain JMY1212 to yield JMY5207 (Fig. 1). The

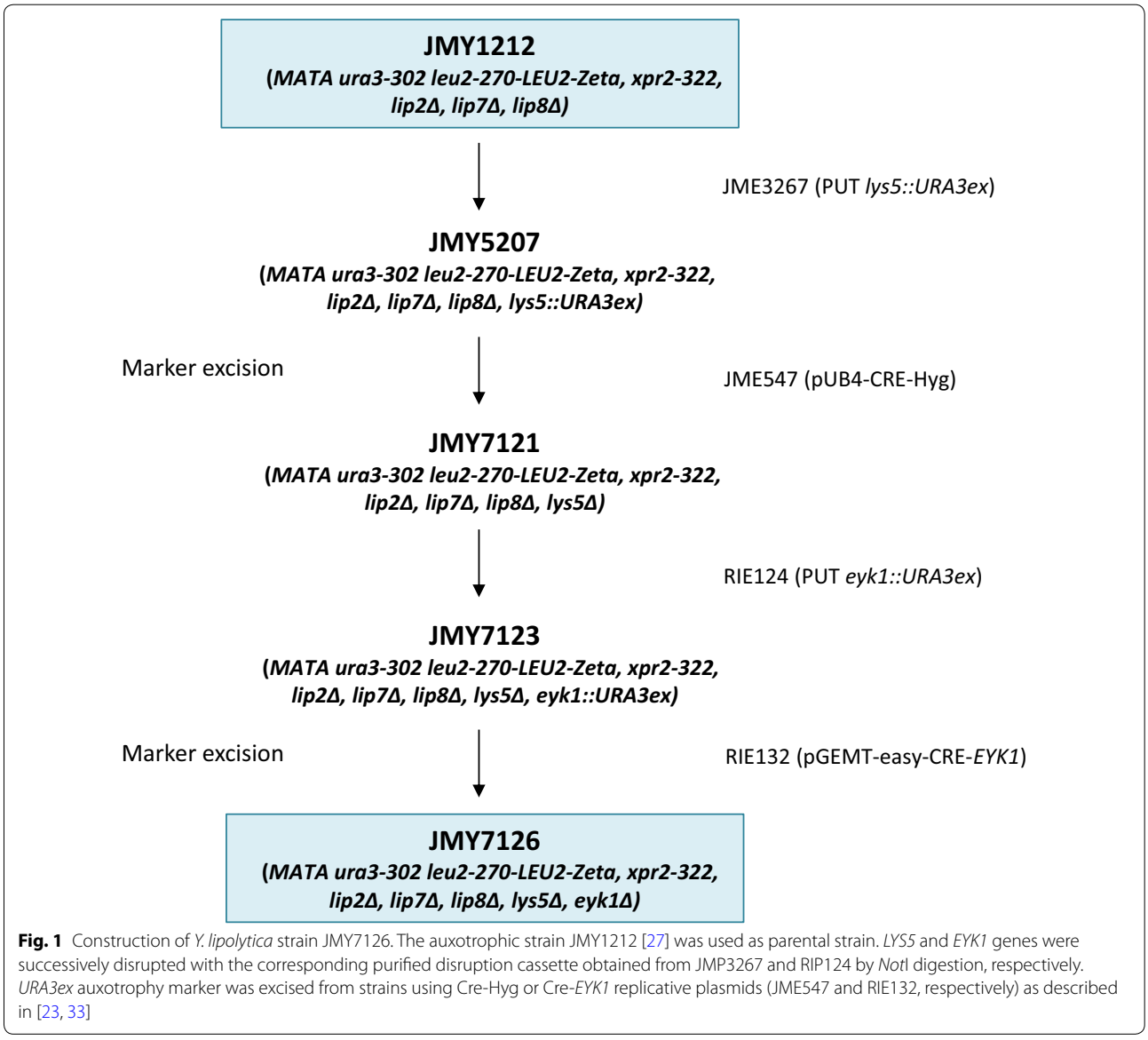

Fig. 1 Construction of *Y. lipolytica* strain JMY7126. The auxotrophic strain JMY1212 [27] was used as parental strain. *LYS5* and *EYK1* genes were successively disrupted with the corresponding purified disruption cassette obtained from JMP3267 and RIP124 by *Not*I digestion, respectively. *URA3ex* auxotrophy marker was excised from strains using Cre-Hyg or Cre-*EYK1* replicative plasmids (JME547 and RIE132, respectively) as described in [23, 33]

disruption of *LYS5* was verified by auxotrophy check on YNBD and YNBD-lysine. In that strain, *URA3ex* marker was rescued by transient expression of Cre recombinase using the replicative plasmid JMP547 as described previously [23]. In the resulting strain JMY7121, *EYK1* was disrupted using a PUT cassette obtained from plasmid RIE124 by *Not*I digestion. The disruption of *EYK1* was verified by colony PCR with primers pair preTEYK Fw/postPEYK Rv and growth on YNBD-lysine and YNBE-lysine. This yielded to strain JMY7123 that was further transformed with plasmid RIE132 in order to excise the *URA3ex* marker. This yielded to the final strain JMY7126 (Table 1, Fig. 1). The loss of the replicative plasmid was checked by replica plating on YPD supplemented or not with hygromycin for JME547, or on YNBD and YNBE for RIE132. To restore *LYS5* prototrophy, strains were transformed with the expression cassette of plasmid RIE279 obtained by *Not*I digestion, and gene integration was verified by colony PCR using primer LPR-R and LYS5PR.

The erythritol-inducible plasmids were constructed from JMP1046 by replacing the *pTEF* by inducible promoters (namely *pEYK1-3AB*, *pHU8EYK1*, plasmid JMP4123 and JMP4001, respectively) by digestion with *Cla*I and *Bam*HI and subsequent ligation. Here, *pEYK300A3B* described in Trassaert et al. [19] was renamed *pEYK1-3AB* according to Park et al. [20]. The *Y. lipolytica LIP2 pre-CalB pro-CalB* gene was codon-optimized by Biocatalysts LTD,

Table 2 Primers used in this study

Primers	Sequence (5′ to 3′)	Restriction site/utilisation
LYS5-P1	ATAAGAAT**GCGGCCGC**CGACTAAATTTCGACCCAC	*Not*I, *LYS5* disruption
LYS5-P2	CG**ATTACCCTGTTATCCCTA**GCGTAACTCGCTACTAGGCCGCCACC	I-*Sce*I, *LYS5* disruption
LYS5-T1	CG**TAGGGATAACAGGGTAAT**ATAGCGTAACTATAACGGTCCTAAGGTAGCGAA GGCGTTGGTGCTCTCTCGGAAGTAG	I-*Sce*I, *LYS5* disruption
LYS5-T2	ATAGTTTA**GCGGCCGC**AAAAATGTCCGCCATTGAGTGTTG	*Not*I, *LYS5* disruption
LPR-R	GCTAGATAGAGTCGAGAATTACCCTG	*LYS5* prototrophy
LYS5PR	TCGGTGCGTGTGAAAGACAC	*LYS5* prototrophy
preTEYK Fw	GTGTTTGACATTTTGTTTTGTGTGAGT	*Verification of EYK1 disruption*
postPEYK Rv	TACACACTCACACTCACCAGAACATC	*Verification of EYK1 disruption*
ClaI-pEYD1-Fw	CCCATCGATGGAAACCTTAATAGGAGACTACTTCC	*pEYD1* cloning
no AvrII-pEYD1-Fw	CCTCGTGTCCGGGCTAGGGCAGAAACAGCTC	*pEYD1* cloning, *pEYD1* verification
no AvrII-pEYD1-Rev	GAGCTGTTTCTGCCCTAGCCCGGACACGAGG	*pEYD1* cloning
BamHI-pEYD1-Rev	TGTGTATGTGTGTGTGTGTGTGTGTGTTTG	*pEYD1* cloning
pTEF-internal-Fw	TCTGGAATCTACGCTTGTTCA	*pTEF* verification
EYK300-Fw	GCATCTACTTTTCTCTATACTGTACGTTTCAATCTGGG	*pEYK1-3AB*, *pHU8EYK* verification
CalB-prepro-Fw	ATGAAGCTGCTGTCTCTGACC	CalB verification
CalB-internal-Rev1	CCACCTTAGATCGAATAGAAGGG	CalB verification
CalB-Rev	TTAAGGGGTGACAATACCAGAAC	CalB verification
ACT-F	TCCAGGCCGTCCTCTCCC	qPCR
ACT-R	GGCCAGCCATATCGAGTCGCA	qPCR
CalB-internal-Fw	TCTCTGCTCCTTCTGTGTGG	qPCR
CalB-internal-Rev2	GTCGAACAGAGGTCCACAGA	qPCR

(Cardiff, UK) and synthesized by Geneart (Regensburg, Germany). The sequence of the optimized synthetic gene (15ACCYPP_1762989_LIP2-CalB-Yl-Opt) is displayed in Additional file 1: Table S1. LIP2*pre-CalBpro-CalB* gene (CalB) was cloned into the vectors JME1046 (p*TEF*), JME4266 (p*EYK1-3AB*) and JME4230 (p*HU8EYK*) at *Bam*HI/*Avr*II restriction sites (Fig. 2 and Table 1). To obtain a p*EYD1*-CalB construct, promoter *pEYK1-3AB* from plasmid JMP4365 was exchanged by p*EYD1* obtained by PCR on genomic DNA of *Y. lipolytica* using primer ClaI-pEYD1-Fw and BamHI-pEYD1-Rev and subsequent enzyme digestion with *Cla*I and *Bam*HI. For the construction of plasmid JME4579, the *URA3*ex marker was exchanged with the *LYS5*ex marker by I-*Sce*I digestion and ligation. Gene expression cassettes were obtained by *Not*I digestion of the corresponding plasmid and used to transform *Y. lipolytica* strains JMY7126 by the lithium acetate method as described previously [24].

Analytical methods

Cell growth monitoring

Cell growth was monitored by optical density at 600 nm (OD_{600}). 1 unit of OD_{600} corresponds to 0.29 g of dry cell weight (DCW).

Quantification of CalB gene expression

RNA extraction and cDNA synthesis were performed as described previously [17] on 2.2×10^7 cells of strains JMY7536, JMY7539, JMY7544, and JMY7548 after 24 h of culture in 2Mag bioREACTOR. qPCR was performed with primer pairs CalB-internal-Fw/CalB-internal-R2 and ACT-F/ACT-R (Table 2) for CalB and actin genes, respectively. CalB gene expression levels were standardized relative to the expression level of the actin gene [17]. Experiments were performed in triplicate.

Lipase activity

Lipase activity in culture supernatants was determined by monitoring the hydrolysis of para-nitrophenyl butyrate (p-NPB), according to Fickers et al. [25]. p-NPB dissolved in acetonitrile (20% v/v) was added to a final concentration of 1 mM into 100 mM phosphate buffer, pH 7.2, containing 100 mM NaCl. The resulting solution was sonicated for 2 min on ice. The reaction was initiated by addition of 20 µL of culture supernatant to 1 mL of p-NPB solution. The release of para-nitrophenol (P-NP) was monitored for 3 min at 405 nm (A_{405}), considering the molar extinction coefficient of P-NP (εPNP) equal to 0.0148 μM^{-1} cm^{-1}. When necessary, supernatant samples were diluted to obtain initial velocities below OD_{405} of 0.3 U min^{-1}. All lipase activity assays

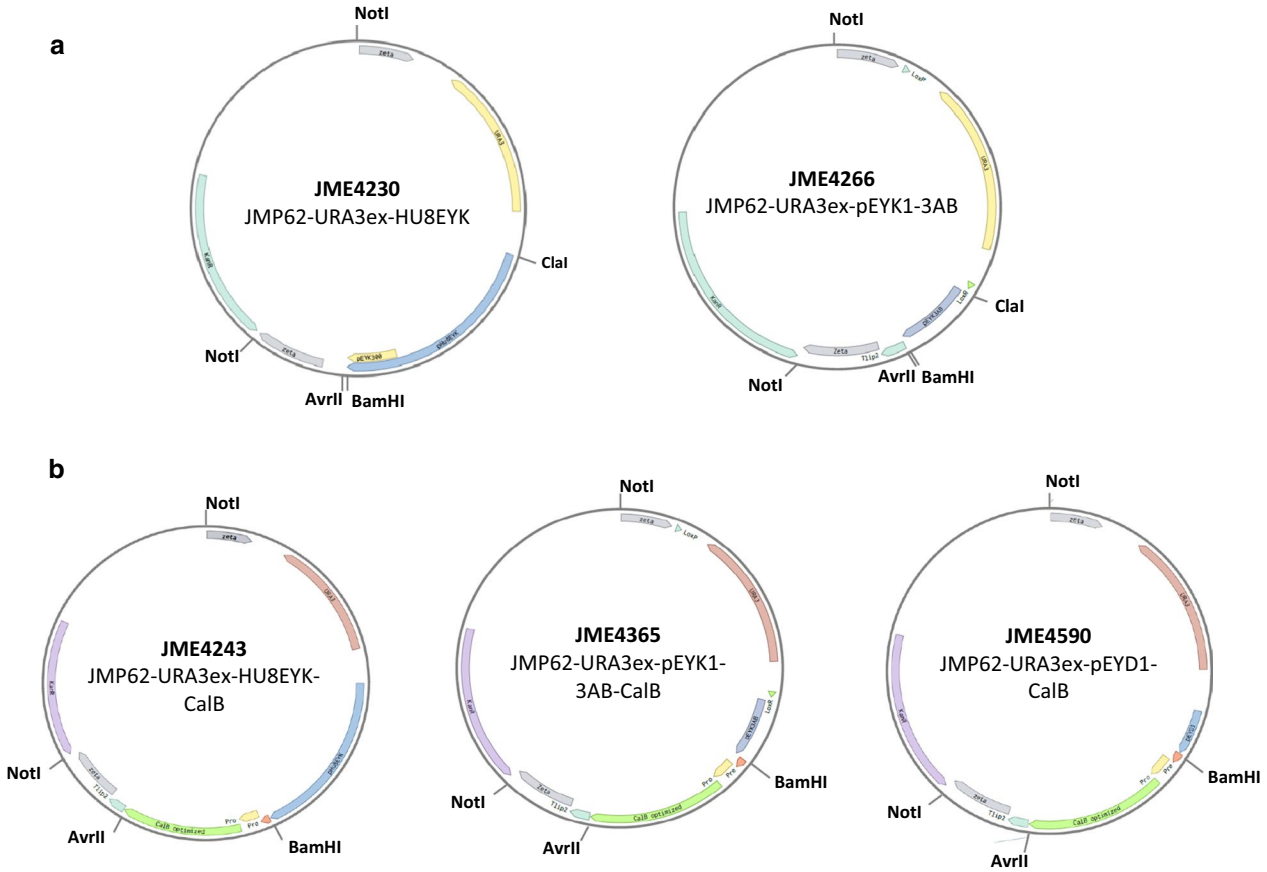

Fig. 2 Maps of expression plasmids. **a** The erythritol inducible plasmids JME4230 containing *pHU8EYK* and JME4266 containing *pEYK1-3AB*. **b** CalB expressing plasmids JME4243 (*pHU8EYK*), JME4365 (*pEYK1-3AB*), and JME4590 (*pEYD1*)

were performed at least in duplicate from two independent cultures. One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol p-NPB per minute at 25 °C and pH 7.2 (U mL^{-1}). Specific lipase activity was defined as lipase activity per gram of dry cell weight (U $gDCW^{-1}$). Lipase volumetric production rate was defined as lipase activity per hour of culture (U mL^{-1} h^{-1}), while lipase specific production rate was defined as unit of lipase activity per gram of DCW and per hour (U $gDCW^{-1}$ h^{-1}).

Protein electrophoresis

Proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a Novex™ 12% Tris–Glycine Mini Gel (Thermo Fisher Scientific), according to Laemmli [26]. Four milliliter of prestained Protein Marker IV (AppliChem GmbH, Darmstadt, Germany) were used as molecular weight standards, and a supernatant sample contained 40 U of lipase in a volume 5 µL.

Carbon source concentration

Polyol concentrations in culture supernatants were determined by HPLC (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA). Compounds were eluted from an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 65 °C, using a mobile phase consisting of 5 mM H_2SO_4 solution at a flow rate of 0.5 mL/min. Glycerol and erythritol were detected using refractive index and erythrulose was detected using UV absorption at 210 nm. Specific glycerol uptake rate was defined as gram of glycerol per gram of DCW and hour (g $gDCW^{-1}$ h^{-1}).

Results and discussion

Construction of the host strain JMY7126 for erythritol-inducible based expression system

Yarrowia lipolytica strain JMY1212 was previously developed to target integration of an expression cassette at a zeta docking platform located at the *LEU2* locus [27]. With such a strain, any variability of recombinant gene

expression related to the integration locus could be avoided, which is a prerequisite for this study. Moreover, such a feature leads to precisely genetically characterized recombinant strains, which is of importance when developing an industrial chassis. Strain JMY1212 is also disrupted for gene *XPR2* encoding AEP alkaline protease and for *LIP2*, *LIP7* and *LIP8* encoding the main extracellular lipases in *Y. lipolytica* [10, 28]. Despite strain JMY1212 has been used successfully for heterologous gene expression [27, 29–31], it suffers from several drawbacks: (i) it possesses only a single auxotrophy based on uracil metabolism (*ura3*), impairing thus multiple genome editions; (ii) it is able to metabolize erythritol and erythrulose, preventing thus their utilization as free inducer. To adapt the potentialities of JMY1212 to the requirements of a versatile, erythritol/erythrulose-inducible expression system, the strain was further genetically engineered. Firstly, gene *LYS5* encoding saccharopine dehydrogenase [32] was disrupted with the *URA3ex* cassette. The resulting lysine auxotroph strain was named JMY5207 (Fig. 1).

In order to use erythritol as a free inducer, it is requested to dispose of a host strain unable to metabolize this polyol. We demonstrated in a previous study that erythrulose, the first intermediate of the erythritol catabolic pathway [18] is a better inducer than erythritol [19]. Therefore, the second step of JMY1212 strain improvement was to disrupt gene *EYK1.* Practically, the gene was disrupted in strain JMY7121, an auxothroph derivative of JMY5207 (Fig. 1) using a PUT cassette obtained from RIP124 by *Not*I digestion. This yielded finally to strain JMY7126, after *URA3ex* marker excision in strain JMY7123. With such a strain, we obtained a useful system to generate autonomously erythrulose from low-cost erythritol, and allowing the use of these polyols as free inducers. Moreover, in that strain, a Cre-*EYK1* replicative vector can be used for transient expression of Cre recombinase [33], which faster delivers transformants without the drawbacks of using Cre-Hyg (hygromycin resistance gene) vector [23]. In summary, JMY7126 contains three auxothrophies (*Ura-*, *Lys-*, *Eyk-*), is compatible with Cre-*EYK1* (RIE132) marker rescue, and with the set of erythritol-inducible promoters deriving from *pEYK1* and *pEYD1* (Trassaert and Vandermies [19, 20], unpublished observations).

Construction of expression vectors and CalB production strains

In most of recombinant protein production processes, protein synthesis occurs in a growth phase decoupled manner. For that purpose, promoters based on *LIP2* and *POX2* genes, strongly induced by oleic acid, have been developed (*pLIP2* and *pPOX2*) [17, 34]. The main drawback of these systems is the utilization of water-insoluble inducers, which is not convenient at large scale due to the lower mixing efficiency of industrial bioreactors. To overcome this problem, we recently developed a set of strong and tightly regulated promoters derived from *EYK1* and *EYD1* genes and induced by hydrophilic substances such as erythritol and erythrulose (Trassaert and Vandermies [19, 20], unpublished observations). Some of these promoters, namely *pEYK1-3AB*, *pHU8EYK*, and *pEYD1*, were selected for this study based on previous experiments (Trassaert and Vandermies [19, 20], unpublished observations) (Fig. 2).

The industrially relevant lipase CalB from *C. antarctica* was used as a model protein to assess the ability of these promoters to drive protein production in process conditions (i.e. in bioreactor). For that purpose, the CalB gene sequence, together with its pro-region was codon-optimized and fused with the signal peptide of *LIP2* (pre-region). With the resulting construct (pre*LIP2*-pro*CalB-CalB*, here after CalB, Additional file 1) different expression vectors were obtained, namely JME3739 (*pTEF*-CalB), JME4365 (*pEYK1-3AB*-CalB), JME4243 (*pHU8EYK*-CalB) and JME4590 (*pEYD1*-CalB) and JME3739 (*pTEF*-CalB) used for comparison. They were used to transform strain JMY7126, after *Not*I digestion and purification of the expression cassette. The resulting mono-copy strains, respectively JMY7536, JMY7539, JMY7544, and JMY7548, harbor a single copy of CalB expression cassette integrated at their zeta-docking platform. As stated above, the docking system prevents variability caused by random genomic integration, allowing here to compare the three selected erythritol/erythrulose-inducible promoters with the constitutive promoter *pTEF* used as a reference.

Comparison of erythritol-inducible promoters

CalB gene expression and protein production in function of the erythritol-inducible promoters used

As a first characterization, CalB expression were determined after 24 h (i.e. at the end of the exponential growth phase) for strains JMY7536, JMY7539, JMY7544 and JMY7548 grown in YNBGE medium in 2Mag bioREACTOR. The culture medium employed here appears more industrially relevant, as compared to defined media previously used for CalB production [30] and more generally for recombinant protein production [35]. Rather than glucose, glycerol was selected as a main carbon source since it is a cheap by-product of the biodiesel industry that has been demonstrated suitable for recombinant protein production [36–43]. Yeast extract and soytone were added at a low concentration to the culture medium to enhance cell growth and protein production. Soytone, a peptone from soy origin, efficiently replaces casein

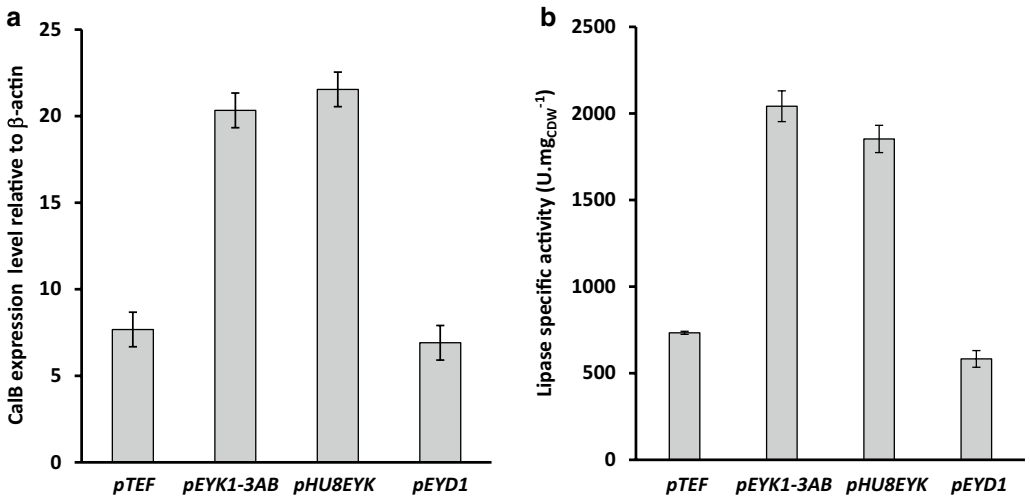

Fig. 3 CalB gene expression level and lipase specific activity of strains JMY7536 (*pTEF*), JMY7539 (*pEYK1-3AB*), JMY7544 (*pHU8EYK*), and JMY7548 (*pEYD1*). Cells were grown at 28 °C in YNBGE medium in 2Mag bioREACTOR. Values are mean and standard deviation of triplicate experiments. **a** CalB gene expression level at the end of the exponential growth phase (after 24 h). Expression levels were normalized to that the one of actin. **b** Specific lipase activity after 48 h

tryptone in bioprocesses mandatorily devoid of components of animal origin [44–47]. Moreover, erythritol is a perfectly affordable inducer, especially when it could be obtained in a bioprocess from glycerol with a *Y. lipolytica* metabolically engineered strain [5] and used in combination with a *eyk1Δ* derivatives that are no longer able to consume it [18, 19].

As shown in Fig. 3a, the highest expression were obtained for *pEYK1-3AB* and *pHU8EYK*. Although, the CalB expression level obtained with the two promoters were not significantly different, they were, respectively, 2.5 and 2.7-fold higher than the one obtained with *pTEF* considered as a strong constitutive promoter. By contrast, *pEYD1* led to a similar expression level than the one obtained with *pTEF*.

As a further characterization, biomass and extracellular lipase activity were determined in the same experimental conditions after 48 h of growth, and the specific activities were calculated. As shown in Fig. 3b, strain JMY7539 (*pEYK1-3AB*) and JMY7544 (*pHU8EYK*) yielded to the highest lipase specific activities (2041 ± 78 and 1852 ± 487 U mgDCW^{-1}, respectively). For those two strains, the specific enzymatic activities were, respectively, 2.8-fold and 2.5-fold higher than that obtained with strain JMY7536 (pTEF; 733 ± 88 U mgDCW^{-1}). By contrast, specific lipase activity of strain JMY7548 (*pEYD1*) was 1.3-fold lower than that of strain JMY7536. The enzymatic productivit**ies** obtained here with strain JMY7539 (*pEYK1-3AB*) and JMY7544 (*pHU8EYK*) were 1.7 and 1.6-fold higher than the lipase productivity obtained with strain JMY1105 (*pLIP2-LIP2*) in 20-L batch fermentation [48]. The results obtained with strains JMY7539 (*pEYK1-3AB*) and JMY7544 (*pHU8EYK*) were, however, somewhat unexpected. Indeed, in previous experiments performed with fluorescent reporter system, *pHU4EYK* [19] and *pHU8EYK* (Trassaert and Vandermies, unpublished observations), bearing respectively four and eight copies of UAS1$_{XPR2}$, significantly higher fluorescence level were obtained (4.9 and 9.8 fold, respectively) as compared to that obtained for *pEYK1-3AB*. However, these experiments were performed in microplate cultures that are known to be not representative of process conditions, notably in terms of oxygen transfer. Based on the results obtained for CalB gene expression and lipase specific activity, strain JMY7539 (*pEYK1-3AB-CalB*) was selected for further characterizations in batch bioreactor.

Culture of strain RIY368 in DASGIP bioreactor

To challenge the *pEYK1-3AB*-based expression system to process conditions, the strain RIY368, a prototroph derivative of strain JMY7539 was grown for 48 h in YNBG$_2$E medium in DASGIP bioreactor, with pH and pO_2 regulation. Samples were collected over time, and biomass together with lipase activity were determined. The exponential growth phase lasted for 12 h with specific growth rate of 0.29 ± 0.00 h^{-1} and final biomass of 6.96 ± 0.04 gDCW L^{-1} (Fig. 4, Table 3). Within the first 24 h, glycerol, the main carbon source, had been entirely consumed, and inducer (i.e. erythritol) assimilated by the cells (Additional file 2: Fig. S1). Lipase activity reached its highest titer (28,024 ± 743 U mL^{-1}) after 24 h of culture

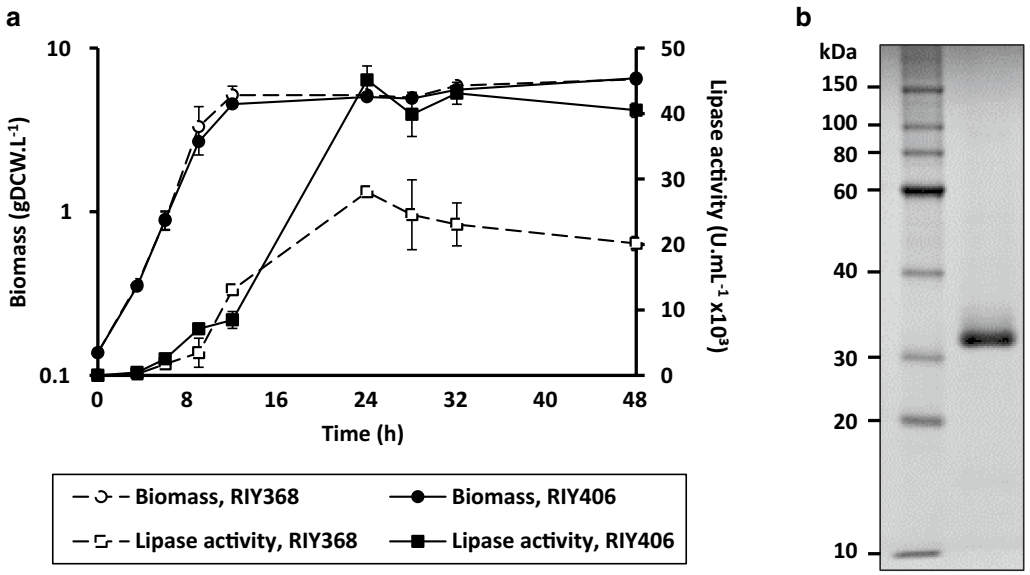

Fig. 4 Dynamics of culture of strains RIY368 (mono-copy) and RIY406 (multi-copy) in DASGIP bioreactors. Cells were grown for 48 h at 28 °C in $YNBG_2E$ medium. **a** Growth curve and lipase activity of strains RIY368 and RIY406. Values are means and standard deviations of duplicate experiments. **b** SDS-PAGE gel of 5 µL of supernatant (containing 40 U of lipase CalB, sample taken at 24 h). Protein sizes are indicated on the left-hand side

Table 3 Dynamics of CalB production for DASGIP bioreactor cultures of strains RIY368 (mono-copy) and RIY406 (multi-copy)

Parameters	Strains	
	RIY368	RIY406
Specific cell growth rate (h^{-1})	0.29 ± 0.00	0.27 ± 0.01
Specific glycerol uptake rate (g $gDCW^{-1}$ h^{-1})	0.37 ± 0.06	0.44 ± 0.00
Maximum lipase activity (U mL^{-1})	28,024 ± 743	45,125 ± 2144
Lipase volumetric production rate (U mL^{-1} h^{-1})	1357 ± 34	2179 ± 104

Cells were grown for 48 h at 28 °C in $YNBG_2E$ medium, in DASGIP bioreactors. Displayed means and standard deviations are the result of duplicate experiments

(Fig. 4). It decreased slightly then after until the end of the culture (until 20150 ± 1060 U mL^{-1}). Analysis of culture supernatant by SDS-PAGE clearly highlighted that CalB is the only secreted protein in those conditions (Fig. 4b). During the enzyme production phase (between 3.5 and 24 h of culture), the lipase volumetric productivity was of 1357 ± 34 U mL^{-1} h^{-1} (Table 3). Culture of strain RIY394 (*pTEF*-CalB prototroph) in the same experimental conditions yielded to a 6.2 fold lower lipase activity after 24 h (data not shown). Here, we demonstrated that a high CalB production level could be achieved in bioreactor by a combined strategy of codon optimization, and suitable inducible promoter and host strain use. In a previous bioreactor study, the native CalB sequence under the control of promoter *POX2* had been cloned in the *Y. lipolytica* strain JMY1212 (parent strain of JMY7126). The resulting lipase activity was about 5 U mL^{-1} after 102 h of cultivation [30]. Other studies in bioreactor using *K. phaffii* as a host strain for CalB production under the control of methanol-inducible promoter *pAOX1* yielded 83 U mL^{-1} in 68 h, without codon optimization [49], and 6100 U mL^{-1} in 110 h with codon optimization [50]. Here, about 28,000 U mL^{-1} of lipase CalB were obtained in 24 h. To our knowledge, this process is the most efficient identified for CalB production in yeast, and it can be safely hypothesized that such efficiency will be reflected in the production of other recombinant proteins.

Additional genome edition

Construction and screening of multi-copy CalB expressing strains

Another goal of this study was to endow the recipient strain with the possibility of additional genome editions. For that purpose an additional auxotrophy based on lysine metabolism (*lys5*) was introduced in strain JMY7126. Indeed, for some recombinant proteins, the process productivity could be enhanced by co-expression of specific chaperone as demonstrated for *K. phaffii* (see [51–53]) or by cloning an additional copy of the expression cassette [54]. To highlight this possibility of additional genome editions, strain JMY7539

(*pEYK1-3AB-CalB*) was transformed with the expression cassette of plasmid JMP4579 (*LYS5ex-pEYK1-3AB-CalB*, rescued beforehand by *Not*I digestion). Since the second expression cassette was integrated randomly in the yeast genome, six independent transformants were tested for their lipase activity. They were cultivated in $YNBG_2E$ medium in Duetz-System deepwell microplates, alongside with strain RIY368 (mono-copy). After 48 h of culture, supernatants were screened for lipase activity and it was found that the lipase productivity spanned over 3.2-fold of intensity (data not shown). The transformant presenting the highest specific lipase activity was named RIY406 and used for further experiments.

Culture of strain RIY406 in DASGIP bioreactor

Strain RIY406 was grown in DASGIP bioreactors under the same conditions as previously adopted for culture of mono-copy strain RIY368, in order to compare cell growth, carbon source uptake and lipase production. Cell growth kinetics of strain RIY406 was found similar to that of RIY368 (see Fig. 4 and Table 3), demonstrating that this additional recombinant gene expression did not affect cell growth capacity. Similarly to what was observed for strain RIY368, the exponential growth phase lasted for 12 h, with a specific growth rate and a final biomass of 0.27 ± 0.01 h^{-1} and 6.51 ± 0.02 gDCW L^{-1}, respectively. Growth and lipase production were sustained by similar glycerol consumption in strains RIY406 and RIY368 (Additional file 2). From these data, it can be concluded that the additional metabolic load resulting from the expression of a second recombinant gene does not alter host strain metabolism. As shown in Fig. 4 and Table 3, lipase activity of RIY406 reached its highest level ($45{,}125 \pm 2144$ U mL^{-1}) after 24 h of culture, again after glycerol entire consumption and erythritol entire assimilation (Additional file 2). At the maximal value, RIY406 lipase activity was 1.6-fold higher than the one of RIY368. This result is consistent with the ratios of volumetric and specific production rates, which are both of 1.6 (Table 3). In conclusion, the addition of a second expression cassette based on lysine auxotrophy properly increases protein production without negative effect on the host strain. Such results confirm the opportunity of co-expression of two genes, based on two selection markers (*URA3* and *LYS5*), under the control of an erythritol/erythrulose-inducible promoter in strain JMY7126.

Conclusions

An efficient expression system relies on four mains properties: it depends on the vector used to express the gene of interest, on the host strain used to produce the compound of interest, and on the production and downstream processing steps. The design of a given expression system arises from its use and intended versatility. Here, we developed an expression system suitable for recombinant protein production in the yeast *Y. lipolytica*. It is based on erythritol/erythrulose-inducible promoters and a dedicated host strain, JMY7126, which enables multi-copy integration of expression cassettes and prevents erythritol complete metabolization. As a proof of concept, lipase CalB was expressed in mono-copy in strain JMY7126 under the control of three types of inducible promoters. The most appropriate promoter (namely *pEYK1-3AB*) for the production of this given protein was selected for demonstration of multi-copy expression. Under these conditions, about 45,000 U mL^{-1} of lipase CalB were obtained in 24 h in batch bioreactor, which represents to date the most efficient process identified for CalB production in yeast. Process development shall expand the potentialities of the proposed expression system even further, and the combination may greatly improve the production of other recombinant proteins in *Y. lipolytica*.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-019-1218-6.

Additional file 1. Nucleic and amino acid sequence of codon optimized CalB. Bold sequences correspond to the pre sequence of the extracellular lipase Lip2p encoded by the *LIP2* gene, and underlined sequences correspond to the pro CalB targeting sequence.

Additional file 2. Carbon source consumption of strains RIY368 (mono-copy) and RIY406 (multi-copy). Cells were grown for 48 h at 28 °C in $YNBG_2E$ medium, in DASGIP bioreactors. Displayed means and standard deviations are the result of duplicate experiments.

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Authors' contributions

JMN, PF and MV designed the experiments. PS constructed strain JMY7126. StT and YKP constructed generic and CalB expression vectors. YKP constructed CalB-mono-copy strains, and performed the first screen of CalB transformants. MV constructed CalB prototroph and multi-copy strains. MV and SaT performed cultures in bioreactor. MV performed qPCR analysis, all enzymatic and biochemical tests, and analyzed CalB production. JMN, PF, YKP and MV wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Strain JMY7126 and plasmids JMP4266, JMP4230, and JME4365 are available upon MTA with INRA transfer and are patent pending.

Ethics approval and consent to participate

Not applicable.

Consent for publication
All authors have given their consent for publication.

Competing interests
The authors declare that they have no competing interests.

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SPECIAL ISSUE ARTICLE

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Screening a genomic library for genes involved in propionate tolerance in *Yarrowia lipolytica*

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Abstract

Microbial oils are regarded as promising alternatives to fossil fuels. For bio-oil production to be sustainable over the long term, utilizing low-cost substrates like volatile fatty acids (VFAs) is crucial. Increasing attention is being paid to one of the most common VFAs: propionate, a substrate that could be used to produce the odd-chain FAs of industrial interest. However, little is known about microbial responses to propionate-induced stress and the genes involved. Using genomic library screening, we identified two genes involved in propionate tolerance in *Yarrowia lipolytica*—*MFS1* and *RTS1*. Strains containing each of the genes displayed enhanced tolerance to propionate even when the genes were expressed in truncated form via a replicative plasmid. Compared with the control strain, the strain overexpressing *MFS1* under a constitutive promoter displayed greater tolerance to propionate: It had a shorter lag phase and higher growth rate in propionate medium (0.047 hr^{-1} versus 0.030 hr^{-1} for the control in 40 g/L propionate); it also accumulated more total lipids and more odd-chain lipids (10% and 3.3%, respectively) than the control. The strain overexpressing *RTS1* showed less tolerance for propionate than the strains harboring the truncated form (0.057 hr^{-1} versus 0.065 hr^{-1} in 40 g/L propionate medium) but still had higher tolerance than the control strain. Furthermore, the overexpression of *RTS1* seemed to confer tolerance to other weak acids such as lactate, formic acid, malic acid, and succinic acid. This work provides a basis for better understanding the response to propionate-induced stress in *Y. lipolytica*.

KEYWORDS

genomic library, propionate, stress response, tolerance, volatile fatty acids, *Yarrowia lipolytica*

1 | INTRODUCTION

Microbial oils (lipids and fatty acid (FA)-derived products) are viewed as promising alternatives to fossil fuels in the face of growing concerns over environmental issues and energy production. To ensure the long-term sustainability of bio-oils, much research has been dedicated to enhancing lipid production from microorganisms and minimizing the cost of substrates. As a result, there is a growing interest in using volatile FAs (VFAs) as carbon sources for lipid production (Beopoulos et al., 2008; Fei et al., 2011; Qiao et al., 2015). VFAs can be obtained from agro-industrial waste and several types of biodegradable organic waste (Papanikolaou, Galiotou-Panayotou, Fakas, Komaitis, & Aggelis, 2008); in theory, they should have higher conversion efficiencies than sugar-based carbon sources because of the shorter metabolic pathways involved (Gao, Li, Zhou, Cheng, & Zheng, 2017).

Abbreviations: DCW, dry cell weight; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; gDNA, genomic DNA; MFS, major facilitator superfamily; ORF, open reading frame; PCR, polymerase chain reaction; TFAs, total fatty acids; VFA, volatile fatty acid; WT, wild type

Propionate, one of the most common VFAs, can be used by *Yarrowia lipolytica* as its sole carbon source in lipid production (Fontanille, Kumar, Christophe, Nouaille, & Larroche, 2012; Kolouchová, Schreiberová, Sigler, Masák, & Řezanka, 2015; Gao et al., 2017). Recently, *Y. lipolytica* was metabolically engineered to produce unusual lipids, namely, odd-chain FAs, using propionate (Park, Dulermo, Ledesma-Amaro, & Nicaud, 2018). In the wild-type (WT) strain, lipid content ranged from 7.39% to 8.14% (w/w dry cell weight [DCW]), depending on the composition of the carbon source, and odd-chain FAs represented 22.9% to 36.5% of total lipids, corresponding to a concentration of 0.01–0.12 g/L. In the strain in which *PHD1* had been deleted (the gene codes for 2-methylcitrate dehydratase), lipid content was 8.1% (w/w DCW) higher than in the WT strain in minimal glucose and propionate media; odd-chain FAs also made up a greater percentage of total lipids (Δ*phd1* strain: 46.8% versus WT strain: 28.3%; corresponding to a concentration of 0.17 g/L in the former). Under the same conditions, in the obese Δ*phd1* strain, accumulated lipid content was 24.8% (w/w DCW) much higher than in the WT strain; the representation of odd-chain FAs among total lipids was slightly lower (41.9%) and corresponded to a concentration of 0.57 g/L.

Propionate and other weak acids have been used as food preservatives due to their potent inhibitory effects on microbial growth (Abbott et al., 2007). These effects have been observed in several studies of lipid production employing *Y. lipolytica* (Fontanille et al., 2012; Kolouchova et al., 2015; Park et al., 2018). Our previous study showed that propionate inhibited the growth of a WT strain: The growth rate was 0.236 hr^{-1} in 2 g/L propionate medium and dropped to 0.029 hr^{-1} in 100 g/L propionate medium (Park et al., 2018). For this reason, it will be necessary to improve microbial resistance to propionate and other weak acids if they are to be used as substrates in lipid production. Very few studies have looked at the tolerance and utilization of propionate by oleaginous yeast for lipid production. Consequently, it is crucial to have a better understanding of the molecular and regulatory responses of yeast to propionate if we wish to use the VFA as a carbon source.

Several research has investigated at stress response mechanisms in yeast, and efforts have been made to engineer strains with enhanced tolerance. Most studies aiming to improve tolerance to weak acids utilized acetic acid. For example, acetic acid tolerance in *Saccharomyces cerevisiae* was improved when the FA composition of the yeast's cell membrane was modified by overexpressing *ELO1* (Zheng et al., 2013). It was also improved by blocking aquaglyceroporin channels (Zhang et al., 2010) and by introducing the acetate consumption pathway (Wei, Quarterman, Kim, Cate, & Jin, 2013). In addition to traditional metabolic engineering approaches, such as overexpressing or knocking out one or more genes, genomic or global approaches are also proving increasingly successful in developing tolerant phenotypes (Santos & Stephanopoulos, 2008; Nicolaou, Gaida, & Papoutsakis, 2010). Borden and Papoutsakis (2007) screened for butanol-tolerant *Clostridium acetobutylicum* strains using genomic library enrichment. In *S. cerevisiae*, key genes involved in acetic acid tolerance were identified by screening deletion or overexpression libraries (Ding et al., 2013; Peña, Glasker, & Srienc, 2013). When researchers used transcript analysis to study the stress response of *S. cerevisiae* to a variety of weak organic acids (propionate, benzoate, sorbate, and acetate), it was revealed that acetate and propionate had a stronger impact on membrane-associated transport processes (Abbott et al., 2007). However, the mechanisms underlying propionate tolerance have remained elusive, regardless of the species examined (Guo & Olsson, 2014). As a result, it is necessary to carry out further research into the regulatory responses of yeast to propionate to develop more robust strains capable of employing propionate to produce odd-chain FAs.

In this study, a genomic library was constructed that contained the native promoters of *Y. lipolytica*. It was used to screen for propionate-tolerant strains, and two genes were identified. We also observed that the overexpression of the identified genes under the constitutive promoter enhanced propionate tolerance. Furthermore, tolerance to other organic acids was explored using the overexpression strains. This work gives insight into propionate stress responses and can help to develop more robust *Y. lipolytica* strains that exploit a wider range of substrates.

2 | MATERIALS AND METHODS

2.1 | Strains and media

The *Y. lipolytica* strains used in this study were derived from JMY7228 (Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1*-*URA3*ex), which was derived from JMY3776 (Park et al., 2018). All the *Escherichia coli* and *Y. lipolytica* strains used in this study are listed in Table 1.

Media and growth conditions for *E. coli* were as described by Sambrook and Green (2012), and those for *Y. lipolytica* have been described by Barth and Gaillardin (1996). Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described previously (Park et al., 2018). Minimal medium (YNB) contained 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 0.5% (w/v) NH_4Cl, and 50 mM KH_2PO_4-Na_2HPO_4 buffer (pH 6.8). The following carbon sources were added: 0.5% (w/v) glucose and 1–5% (w/v) propionate. Leucine was supplemented at 0.1 g/L when necessary. Solid media were prepared by adding 1.5% (w/v) agar.

2.2 | Construction of plasmids and strains (*E. coli* and *Y. lipolytica*)

Standard molecular genetic techniques were used in this study (Sambrook & Green, 2012). Restriction enzymes were obtained from New England Biolabs (MA, USA). The polymerase chain reactions (PCRs) were performed using an Applied Biosystems 2720 Thermal Cycler and employing GoTaq DNA Polymerase (Promega, WI, USA) and Q5 High-Fidelity DNA Polymerase (New England Biolabs).

The overexpression plasmids were constructed by ligating JMP62-LEU2ex-pTEF and the PCR fragments. The gene expression

TABLE 1 Strains used in this study

Strain name	Plasmid and genotype	Reference
Escherichia coli		
MGM collection	pINA240	(Barth & Gaillardin, 1996)
JME4010	pINA240-Pool 1 from MGM1221021	Fournier et al., unpublished
JME4014	pINA240-Pool 2 from MGM1221022	Fournier et al., unpublished
JME4018	pINA240-Pool 3 from MGM1221023	Fournier et al., unpublished
JME4022	pINA240-Pool 4 from MGM1221024	Fournier et al., unpublished
JME4026	pINA240-Pool 5 from MGM1221025	Fournier et al., unpublished
JME4030	pINA240-Pool 6 from MGM1221026	Fournier et al., unpublished
JME4034	pINA240-Pool 7 from MGM1221027	Fournier et al., unpublished
JME4038	pINA240-Pool 8 from MGM1221028	Fournier et al., unpublished
JME4042	pINA240-Pool 9 from MGM1221029	Fournier et al., unpublished
JME4046	pINA240-Pool 10 from MGM1221030	Fournier et al., unpublished
JME2563	JMP62-*LEU2*ex-pTEF	(Dulermo et al., 2017)
JME4569	JMP62-*LEU2*ex-pTEF-*MFS1i*	This study
JME4596	JMP62-*LEU2*ex-pTEF-*RTS1i*	This study
Yarrowia lipolytica		
JMY2900	Po1d *URA3 LEU2*	(Dulermo, Gamboa-Meléndez, Dulermo, Thevenieau, & Nicaud, 2014)
JMY3776	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2*-*LEU2*ex + pTEF-*GPD1*-*URA3*ex	Park et al., 2018
JMY7228	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* + hp4d-*LDP1*-*URA3*ex	This study
JMY7264	Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* + hp4d-*LDP1*-*URA3*ex + *LEU2*	This study
JMY7588	JMY7228 + pINA240-*RTS1r*	This study
JMY7589	JMY7228 + pINA240-*MFS1r*	This study
JMY7567	JMY7228 + pTEF-*RTS1i*-*LEU2*ex	This study
JMY7569	JMY7228 + pTEF-*MFS1i*-*LEU2*ex	This study

cassettes to be transformed were prepared via the *Not*I digestion of the expression plasmids. The transformation of *Y. lipolytica* was carried out using a Frozen-EZ Yeast Transformation Kit (Zymo Research, CA, USA). Transformants were selected on YNB medium and verified by colony PCR. The expression cassettes were integrated randomly in the genome as described previously (Bordes, Fudalej, Dossat, Nicaud, & Marty, 2007).

2.3 | Genomic library construction

Y. lipolytica W29 genomic DNA (gDNA) was partially digested using the *Sau*3A restriction enzyme, and the resulting fragments (of up to 5 kb) were cloned into a pINA240 plasmid at the *Bam*H1 site (Figure 1). The recombinant plasmid conferred resistance to ampicillin, but the gene for tetracycline resistance was lost. The efficiency of gDNA cloning was verified by measuring the percentage of clones that were only resistant to ampicillin. The ampicillin-resistant *E. coli* colonies were mixed to form 10 independent pools (Fournier et al., unpublished, Table 1). They were then stored in the Microbiology and Molecular Genetics Collection (*Microbiologie et Génétique Moléculaire*, MGM) under the reference codes MGM1221021–MGM1221030 at the INRA Centre of Thiverval-Grignon (France). Transformant pools from the MGM collection were grown in 200 ml of LB ampicillin for 24 hr; 1 ml of culture was stored in 20% glycerol and kept in our laboratory collection at −80°C under the reference codes JME4010 to JME4046 (Table 1). The rest of the cultures were used for DNA plasmid preparation, which was carried out using the QIAGEN Plasmid Midi Kit (Hilden, Germany).

To verify the quality of the DNA plasmid pools, *E. coli* was transformed using 2 µl of the DNA preps from reference pool MGM1221021. Twenty random transformants were selected for plasmid extraction. Plasmids from the different clones presented different profiles upon digestion with restriction enzymes (i.e., *Eco*RI and *Bgl*II; Figure S1), indicating that each clone contained a different genomic insert.

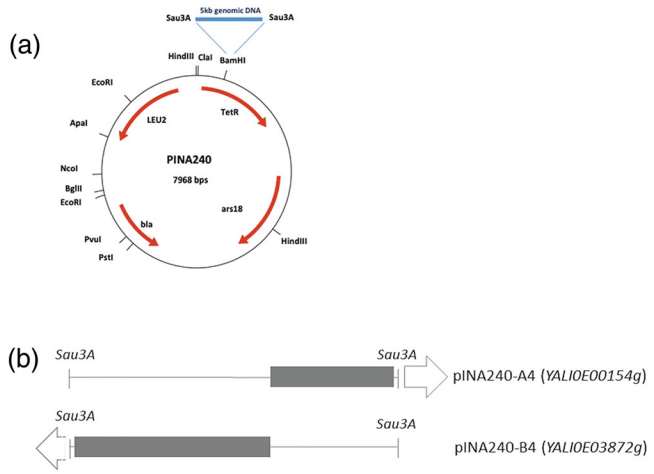

FIGURE 1 (a) Schematic map of the replicative plasmid pINA240 used to construct the gDNA library. The partial *Sau3*A genomic fragments were cloned at the *Bam*H1 dephosphorylated site. (b) Schematic map of the gDNA fragments inserted into JMY7588 (pINA240-A4) and JMY7589 (pINA240-B4). The grey bars indicate the genes' open reading frames (ORFs), and the white arrows show the ORFs of each gene that were not included in the genomic fragment

2.4 | *In silico* sequence analysis

Gene and protein sequences were obtained from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), UniproKB (the UniProt Knowledgebase) (http://www.uniprot.org/help/uniprotkb), and the yeast genomic database Génolevures (http://gryc.inra.fr/). The alignment of peptide sequences was performed using multiple alignment program MultAlin (multiple alignment program) (Institute of Biology and Protein Chemistry website: https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html). Blast searches were carried out on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Benchling software was employed for the gene sequence analysis and in silico plasmid construction (https://benchling.com/). Transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.5 | Screening for propionate-resistant transformants

Two plasmid library pools (MGM1221021 and MGM1221030) were used to transform *Y. lipolytica* JMY7228; the Frozen-EZ Yeast Transformation Kit was employed. The transformation mixture was incubated in liquid selection media (YNBD) overnight for the first screening corresponding to the selection of Leu+ transformants, then after the diluted cells (OD 0.005) were plated onto YNBD0.5P4 plate for the second screening (clones growing onto propionate media corresponding to a propionate stress). From the YNBD0.5P4 plates, we selected only big colonies, showing more tolerance to propionate. Around 150 colonies from each library pool (MGM1221021 and MGM1221030) were then transferred again onto propionate-containing medium (YNBD0.5P4) and grown for 3–5 days. The colonies showing better growth on propionate medium were subsequently transferred to YNBD medium and grown for 1–2 days to allow them to recover from propionate stress. The candidates were then assessed via colony PCR and propionate tolerance assays.

2.6 | Propionate tolerance assays

The tolerance of control and transformants strains was compared using spot assays or growth curves. In the spot assays, the cells were inoculated in 3 ml of YNBD1 and grown overnight. After their optical density (OD_{600}) values were adjusted to 1.0, these cell suspensions and three sequential dilutions (1:10, $1:10^2$, and $1:10^3$) were applied (3 µl) to the surface of YNB solid medium. The cultures were supplemented with adequate propionate or other organic acids and were incubated at 28°C for 3–5 days.

To test growth in liquid culture, precultures were inoculated in YNBD1 medium and grown overnight (28°C, 180 rpm). After their OD_{600} values were adjusted to 0.1, the precultures were transferred to 96-well plates containing fresh YNB medium with different concentrations of carbon sources. The strains were cultivated at 28°C with constant shaking for 120 hr. Growth was monitored by measuring the OD_{600} values every 30 min using a microtiter plate reader (Biotek Synergy MX, Biotek Instruments, Colmar, France). For each strain and set of conditions, we used two or three biological replicates.

2.7 | Quantifying lipid production

Lipid biosynthesis was performed in flasks using YNB medium containing 0.15% (w/v) NH_4Cl and 50 mM phosphate buffer (pH 6.8) with 0.5% (w/v) glucose and 4% (w/v) propionate. Lipids were extracted from 10 to 20 mg of freeze-dried cells and converted into their FA methyl esters (FAMEs) as per Browse, McCourt, and Somerville (1986). The FAMEs were analysed using gas chromatography (GC). More specifically, the GC analysis was carried out using a Varian 3900 GC equipped with a flame ionization detector and a Varian FactorFour VF-23ms column, where the bleed specification at 260°C was 3 pA (30 m, 0.25 mm, 0.25 µm). FAs were identified via comparison with commercial FAME standards (FAME32, Supelco). Their levels were quantified using the internal standard method, which involves adding 100 µg of commercial dodecanoic acid (Sigma-Aldrich). To determine DCW in a given flask, 10 ml of the culture was washed and lyophilized in a preweighed tube. The difference in mass was defined as the DCW, in milligram, of the cells found in 10 ml of culture. We used at least two biological replicates and calculated the mean and standard deviation.

3 | RESULTS

3.1 | Genomic library construction

The gDNA of *Y. lipolytica* was partially digested using the *Sau3*A restriction enzyme, and fragments of up to 5 kb in size were selected to construct the library. These fragments were cloned at the *Bam*HI position of the replicative plasmid (pINA240, [Barth & Gaillardin, 1996]) and used to form the library's 10 reference pools (Figure 1a). As a control, we used *Y. lipolytica* strain JMY7228 (*phd1*Δ). This strain is unable to use propionate as its sole carbon source; it is also more sensitive to propionate and thus reveals the compound's effects more distinctly (Park et al., 2018). The absence of the *PHD1* expression cassette in the genomic library was verified by PCR to avoid screening for positive clones arising from *PHD1* complementation (Figure S2).

3.2 | Screening for propionate-tolerant strains

A recent study showed that the deletion of *PHD1* in the methylcitrate pathway caused a severe growth defect on propionate: The engineered *phd1*Δ strain accumulated large amounts of odd-chain lipids (Park et al., 2018). To further increase lipid accumulation, we overexpressed the lipid droplet protein (*LDP1*), which enhances the storage of large amounts of triacylglycerol in intracellular lipid droplets. The *LDP1* gene was expressed under the control of the strong hp4d promoter (Madzak, Tréton, & Blanchin-Roland, 2000). The resulting obese *phd1*Δ *hp4-LDP1 (Leu-)* strain JMY7228 (Table 1) was complemented for leucine auxotrophy via transformation with the *LEU2* genomic fragment, giving rise to the prototrophic strain JMY7264.

At 120 hr of culture, compared with the control strain, the strain overexpressing *LDP1* had 9.7% higher biomass production, 1.83-fold greater total lipid content, and two-fold greater total lipid production (equivalent to a concentration 6.51 g/L; growth in YNBD6 medium; Table S2). These results were consistent with those from the study by Bhutada et al. (2018). We used the JMY7228 strain (Po1d *phd1 mfe1 tgl4* + pTEF-*DGA2* pTEF-*GPD1* hp4d-*LDP1-URA3*ex) as the starting strain in the library screening process since the strain had displayed much higher propionate sensitivity than other strains. To test propionate tolerance, glucose should always be supplemented together with propionate because the propionate-consumption pathway is blocked, as described above.

To find the optimal medium to use as we screened for propionate tolerance, growth of the control strain JMY7264 was monitored under different conditions, which varied based on (a) the cell amounts plated as determined via colony-forming units (OD_{600} of cell suspension from 0.002 to 0.1), (b) the glucose concentration (0.5–2% glucose combined with 0.4% propionate), and (c) the propionate concentration (1–4% propionate combined with 0.5% glucose; Figure S3). The results revealed that YNBD0.5P4 (0.5% [w/v] glucose and 4.0% [w/v] propionate) was the optimal screening medium because the control strain showed significantly less growth in this medium.

Because the transformation efficiency of the transformation kit was higher than that of the traditional LiAc method under our experimental conditions and because the same competent cells could be used later to transform other reference pools, we transformed two reference pools—MGM1221021 and MGM1221030—with the Frozen-EZ Yeast Transformation Kit. After transforming the same amount of DNA (308 ng) from each reference pool, Leu+ transformants on YNBD were obtained with transformation efficiency being 43,500 transformants/μg from MGM1221021 and 21,800 transformants/μg from MGM1221030, respectively. Propionate tolerant clones were selected as described in materials and methods. Among them, 150 colonies of transformants from each reference pool were transferred again onto propionate-containing plates (YNBD0.5P4) to screen for candidates displaying higher propionate tolerance. After 3–5 days of cultivation, 15 clones (seven from MGM1221021 and eight from MGM1221030) displayed better growth on propionate. These candidates were then transferred onto YNBD solid medium to allow them to recover from propionate stress; gDNA inserts were identified via colony PCR and sequence analysis. The inserts in the pINA240 plasmid were amplified with the primer pair P240-F1/P240-R1 or P240-F2/P240-R2 (Table S1) in the colony PCR.

Clearly defined PCR bands were amplified for clones A3 and A4 from among the seven MGM1221021 candidates and for clone B4 from among the eight MGM1221030 candidates (Figure S4). The PCR fragments were sequenced using primers P240-F2 and P240-R2 to determine the gDNA regions (Table S3). The sequence analysis showed that clone A3 contained 1-kb gDNA, which did not present any identifiable open reading frames (ORFs). The sequences of the gDNA in clones A4 (JMY7588) and B4 (JMY7589) contained partial sequences from the genes *YALI0E00154g* and *YALI0E03872g*, respectively (Figure 1b). In JMY7588, the plasmid contained a 2,610-BP genomic fragment that harboured 1,584 BP of the promoter region and 1,026 BP of *YALI0E00154g* (NCBI XP_503361.1), which codes for a 793-residue protein (UniProtKB/TrEMBL:Q6C7K1). In JMY7589, the plasmid contained a 2,135-BP genomic fragment that harboured 1,353 BP of the promoter region and 782 BP of *YALI0E03872g* (NCBI XP_503517.1), which codes for a 448-residue protein (UniProtKB/TrEMBL:Q6C745).

YALI0E00154g is homologous to *S. cerevisiae RTS1* (YOR014W), which encodes the regulatory subunit of protein phosphatase 2A (PP2A); the latter is involved in cell growth control, cell division control, and the stress response in this yeast (Evangelista, Rodriguez Torres, Limbach, & Zitomer, 1996; Ronne, Carlberg, Hu, & Nehlin, 2015). It has been reported that the deletion of *RTS1* caused sensitivity to temperature, ethanol, sorbate, and osmotic pressure and increased accumulation of CYC7 RNA, which is involved in the global stress response in *S. cerevisiae* (Evangelista et al., 1996; Shu, Yang, Hallberg, & Hallberg, 1997; Mollapour et al., 2004). In *Y. lipolytica*, PP2A was found to act as a regulator of glycogen metabolism (Queiroz-Claret, Jolivet, Chardot, Bergeron, & Meunier, 2002). However, neither PP2A nor its RTS1 subunit have been observed to play a role in the stress response to weak acids (e.g., propionate) in *Y. lipolytica*.

YALI0E03872g is similar to YJR124C in *S. cerevisiae*, but gene function remains unknown in both yeasts. From a BLAST performed on amino acid sequences (Zhang & Madden, 1997), *YALI0E03872g* was similar (~48%) to a major facilitator superfamily (MFS, pfam07690) transporter found in several fungi (e.g., *Nadsonia fulvescens* and *Metarhizium album*). The MFS transporter facilitates the transport of a variety of substrates, including ions, sugar phosphates, drugs, amino acids, and peptides, across cytoplasmic, or internal membranes. In addition, it has recently been shown that the MFS transporter regulates the stress response machinery and controls membrane potential and/or internal pH (Dos Santos, Teixeira, Dias, & Sá-Correia, 2014). In *Y. lipolytica*, using MultAlin (Combet, Blanchet, Geourjon, & Deléage, 2000), we found three genes coding for a putative MFS transporter: *YALI0E03872g*, *YALI0C08228g*, and *YALI0A15774g*. They were named *MFS1*, *MFS2*, and *MFS3*, respectively. The three proteins contain the characteristic cd06174 conserved motif of MFS secondary transporters and present nine putative transmembrane domains (Figures S5 and S6).

3.3 | Overexpression of *RTS1r* and *MFS1r* improved propionate tolerance

For simplicity's sake, we gave the name *RTS1r* to strain JMY7588 and *MFS1r* to strain JMY7589, in reference to the replicative plasmids that

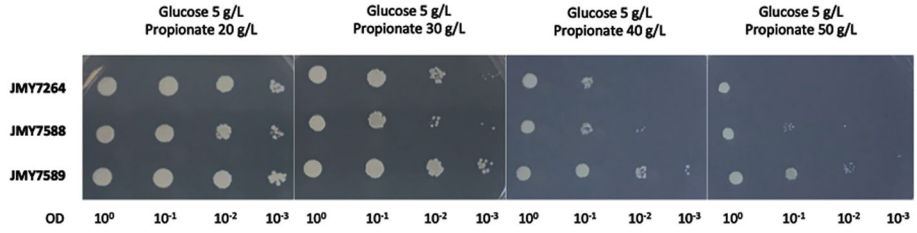

FIGURE 2 Results of the spot assay for the control strain (JMY7264), the *RTS1r* strain (JMY7588), and the *MFS1r* strain (JMY7589). Minimum media (YNBD0.5) containing different propionate concentrations were used. Pictures were taken after 3 days of growth at 28°C

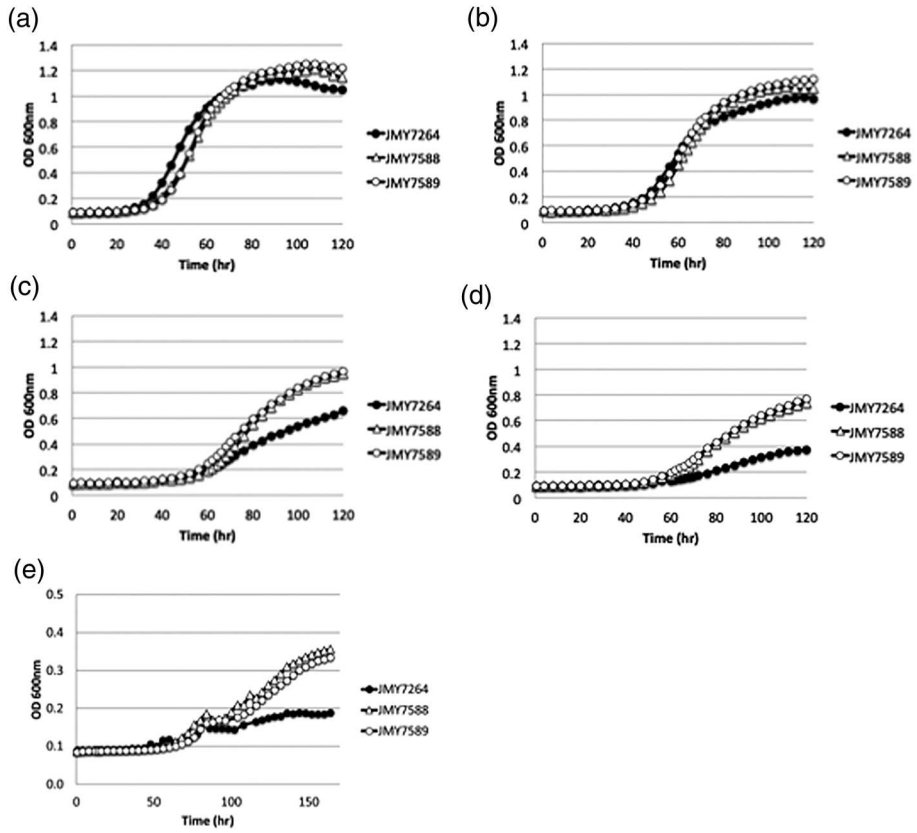

FIGURE 3 Growth curves of the control strain (JMY7264,), the *RTS1r* strain (JMY7588, Δ), and the *MFS1r* strain (JMY7589, ○) in YNBD0.5 liquid medium containing (a) 10 g/L of propionate, (b) 20 g/L of propionate, (c) 30 g/L of propionate, (d) 40 g/L of propionate, and (e) 50 g/L of propionate

expressed the truncated forms of the genes. The strains containing the full-length genomic genes were named *RTS1i* and *MFS1i* (i.e., they contained the integrated expression cassettes for the full-length genes).

To assess strain phenotype and propionate tolerance, the growth of the *RTS1r* and *MFS1r* strains on propionate media was compared with that of the control strain (JMY7264, derived from strain JMY7228 used in the library transformation process) by spot assays on agar plate and in liquid cultivation (Figures 2 and 3). As described above, the control—a leucine prototroph derivative—was not able to use propionate as its sole carbon source; we therefore added 5 g/L of glucose to the propionate media.

Compared with the control, both *RTS1r* and *MFS1r* displayed greater tolerance of propionate. However, *MFS1r* had higher tolerance than *RTS1r*, and it remained tolerant even at propionate levels of 50 g/L (Figure 2). In the liquid media, the growth curves of the two strains were similar, and their final OD_{600} values were slightly higher than that of the control strain in media containing 10 and 20 g/L of propionate (Figure 3). When the propionate concentration was more than 40 g/L, the difference in growth between the control and the two transformants was substantially greater. The final OD_{600} values of the two strains were almost twice of that of the control strain in YNBD0.5P4. Both *RTS1r* and *MFS1r* displayed improved growth rates under all the experimental conditions (Table S4). When the propionate concentration was 40 g/L, the maximal growth rates of *RTS1r* and *MFS1r* were 0.065 hr^{-1} and 0.054 hr^{-1} (increase of 2.17 and 1.8 fold over control), respectively. These results confirmed that the two strains displayed higher propionate tolerance than the control even though they were expressing truncated genes.

3.4 | Overexpression of *RTS1i* and *MFS1i* improved propionate tolerance

The library screening process identified two candidate strains that were found to display propionate tolerance even though they expressed truncated genes. To determine whether overexpression of the complete ORF under the control of a strong promoter could further increase propionate tolerance, the *RTS1* and *MFS1* genes were each cloned into the expression vector JME2563 under the pTEF1 constitutive promoter (JME2563; Figure S7). The gene expression cassettes were transformed into JMY7228, and gene integration was verified by colony PCR using the primer pairs pTEF-internal-Fw/RTS1-noBamHI-Rev and pTEF-internal-Fw/AvrII-E03872g-Rev for *RTS1* and *MFS1*, respectively.

To determine the effects of overexpression on propionate tolerance, two strains—JMY7567 and JMY7569—were constructed and evaluated under same conditions as described above (see Section 3.3.). As mentioned above, the strains were named *RTS1i* and *MFS1i*. The propionate tolerance of *MFS1i* was similar to that of *MFS1r*, as estimated from their growth rates, which means that *MFS1i* had

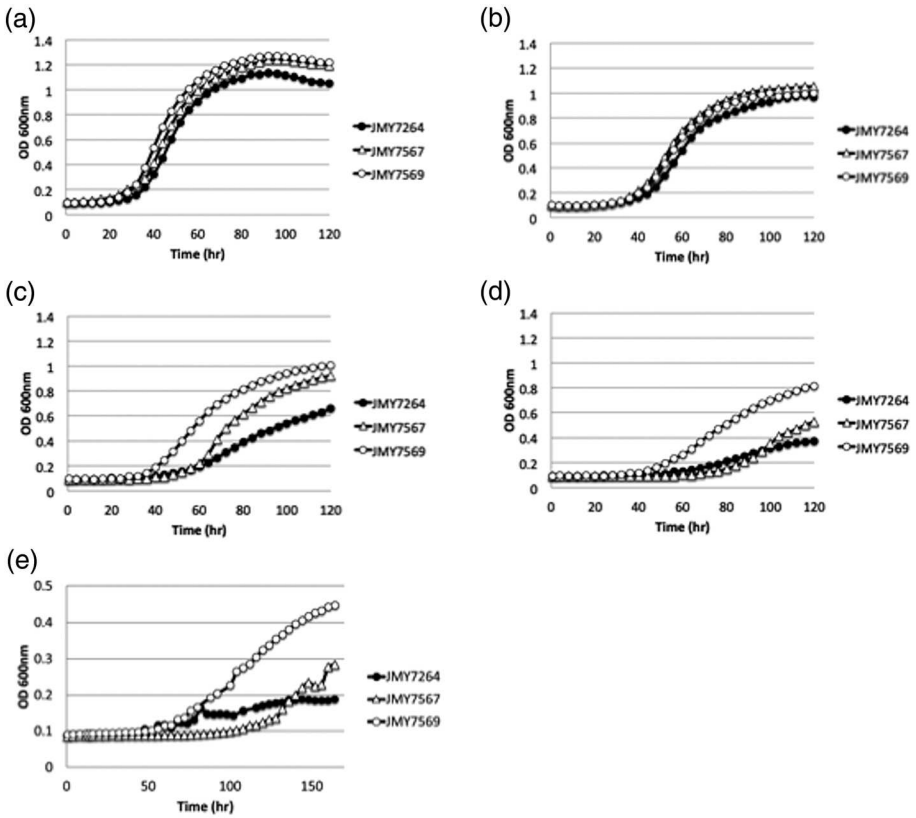

FIGURE 4 Growth curves of the control strain (JMY7264,), the *RTS1i* strain (JMY7567, Δ), and the *MFS1i* strain (JMY7569, ○) in YNBD0.5 liquid medium containing (a) 10 g/L of propionate, (b) 20 g/L of propionate, (c) 30 g/L of propionate, (d) 40 g/L of propionate, and (e) 50 g/L of propionate

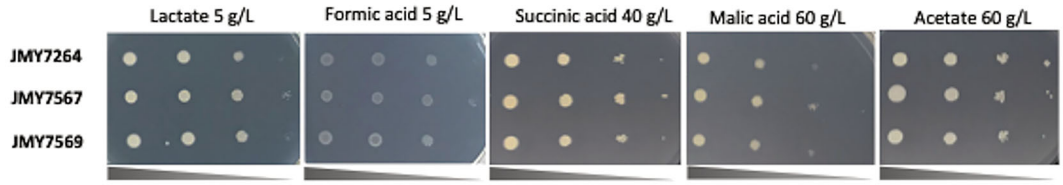

FIGURE 5 Results of the spot assay for the control strain (JMY7264), the *RTS1i* strain (JMY7567), and the *MFS1i* strain (JMY7569) with different weak acids. Pictures were taken after 5 days of growth at 28°C

greater propionate tolerance than control (Table S4). When the propionate concentration was 30 g/L, *MFS1i* had a shorter lag phase and a higher growth rate than the control (Figure 4). These results indicate that *MFS1* is involved in propionate tolerance in *Y. lipolytica*. The propionate tolerance of *RTS1i* was similar to that of *MFS1i* at propionate concentrations of up to 20 g/L. However, at 30 g/L, *RTS1i* had a longer lag phase than *MFS1i*. At 40 and 50 g/L, its lag phase was even longer than that of the control strain, although it displayed a higher growth rate after the lag phase. When we compared the growth rates of *RTS1r* and *RTS1i* at 40 g/L, *RTS1r* appeared to display greater propionate tolerance than *RTS1i* (0.065 hr^{-1} versus 0.057 hr^{-1}). This difference between the two strains could be attributable to differences in gene length, promoter type, or plasmid type, as shown in another study (Nicaud, Fournier, La Bonnardière, Chasles, & Gaillardin, 1991).

We performed a spot assay to assess the general stress tolerance of *RTS1i* and *MFS1i*; we employed other weak organic acids, namely, acetate, lactate, formic acid, succinic acid, and malic acid, to determine whether overexpression increased tolerance more broadly (Figure 5). We observed the same differences in tolerance between *RTS1i* and *MFS1i* that we had already seen for propionate. JMY7567 (*RTS1i*) was more tolerant to lactate, formic acid, malic acid, and succinic acid than the control strain. In contrast, JMY7569 (*MFS1i*) did not show big difference on growth with organic acids in this condition. In the case of acetate, there were no differences in growth among strains, even at high concentrations (60 g/L). These findings suggest that *MFS1* is involved in a propionate-specific stress response. It also seems that, when *MFS1* is overexpressed, the general tolerance of weak acids is somehow sacrificed for increased propionate tolerance. In comparison, the overexpression of full-length *RTS1* increased tolerance not only to propionate but also to other weak acids. It has been found that the deletion or overexpression of *RTS1* resulted in different levels of tolerance, depending on parental strain and stressor type (Shu & Hallberg, 1995, Evangelista et al., 1996, Shu et al., 1997). Taken together, our results and those of previous studies suggest that PP2A probably has a functional role in more than one cell pathway.

3.5 | Overexpression of *RTS1* and *MFS1* improve odd-chain FA production

As shown in a previous study (Park et al., 2018), propionate is an important substrate for lipid synthesis in *Y. lipolytica*, especially when it comes to the production of odd-chain FAs. To determine whether increased propionate tolerance could improve total lipid accumulation and the production of odd-chain FAs, lipid synthesis by the *RTS1*- and *MFS1*-expressing strains was evaluated. After 120 hr of cultivation in a minimal glucose medium (YNBD0.5P4) containing a high concentration of propionate (40 g/L), the experimental strains had produced less biomass than the control strain (by 6.4–11.5%). Total lipid content was lower in the *RTS1*-expressing strains (by 4.5% and 23.1% for *RTS1r* and *RTS1i*, respectively), whereas it was higher in the *MFS1*-expressing strains (by 7.1% and 10.4% for *MFS1r* and *MFS1i*, respectively). Despite these low biomass and similar lipid content, the ratio of odd-chain lipids to total lipids was higher for all the experimental strains (Table S5).

4 | CONCLUSION

The objective of this study was to identify genes potentially involved in propionate tolerance in *Y. lipolytica*. To this end, we screened a plasmid-based genomic library harboring native promoters for propionate tolerance allowing identifying two genes of potential interest: *RTS1 (YALI0E00154g)* and *MFS1 (YALI0E03872g)*. We discovered that the initial transformants were expressing truncated genes. As a result, we then compared the phenotypes associated with the expression of the partial and full-length genes.

Two strains expressed *RTS1*, which encodes a regulatory subunit of the PP2A. They had different growth patterns on propionate that depended on gene length and promoter type. Growth was stronger, and lipid accumulation was greater for the strain expressing the truncated gene under a native promoter (*RTS1r*) than for the strain expressing the whole gene (including the ORF) under a strong promoter (*RTS1i*). Both strains (*RTS1r* and *RTS1i*) had higher levels of odd-chain lipid production than did the control strain. Further research should focus on whether these phenotype differences stem from differences in expression levels or differences in sequence conservation between the partial and full-length genes. Interestingly, the overexpression of *RTS1* seems to enhance tolerance to other weak acids, such as lactate, formic acid, malic acid, and succinic acid. Given that *RTS1* encodes a single subunit of PP2A—and that there is another regulatory subunit (encoded by *CDC55*) and a catalytic subunit (encoded by four genes)—the relationship among these subunits and its contribution to the tolerance of weak acids must be explored further to better understand the mechanisms at hand.

On propionate media, the *MFS1*-expressing strains (*MFS1r* and *MFS1i*) showed greater propionate tolerance, shorter lag phases, and higher growth rates than the control strain. They also accumulated

more lipids and more odd-chain lipids. On the basis of the sequence alignment results, we identified two more genes coding for a putative MFS transporter (named *MFS2* and *MFS3*). Further, characterization of the proteins encoding these genes would be helpful in clarifying the mechanisms underlying propionate tolerance in *Y. lipolytica*.

The role of these genes in the stress response to propionate and other weak acids remains unclear. Therefore, we need more studies that carry out expression analysis at the transcriptional level or metabolic flux analysis under conditions of propionate-induced stress to gain insight into the regulatory mechanisms. These are also crucial steps to engineer strains with improved tolerance for use in industry. Library enrichment and evolutionary engineering are promising strategies that employ pre-existing libraries or strains (Borden & Papoutsakis, 2007; Wright et al., 2011). In addition, combining computational and experimental approaches may also help to improve tolerance. The effects of overexpressing or disrupting multiple genes at the same time can be predicted using a computational model that has incorporated the experimentally determined effects of overexpressing or disrupting individual genes (Goodarzi et al., 2010).

Our results have laid the foundation for future research aimed at further improving propionate tolerance, which is crucial to use propionate as a substrate in the industrial production of valuable biochemicals such as odd-chain lipids. Furthermore, studies on propionate tolerance should augment *Y. lipolytica*'s ability to employ a wider range of substrates, including waste products and inexpensive materials, which will help make microbial production more competitive than petroleum-based production.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Biotechnology for Biofuels

RESEARCH

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Optimization of odd chain fatty acid production by *Yarrowia lipolytica*

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Abstract

Background: Odd chain fatty acids (odd FAs) have a wide range of applications in therapeutic and nutritional industries, as well as in chemical industries including biofuel. *Yarrowia lipolytica* is an oleaginous yeast considered a preferred microorganism for the production of lipid-derived biofuels and chemicals. However, it naturally produces negligible amounts of odd chain fatty acids.

Results: The possibility of producing odd FAs using *Y. lipolytica* was investigated. *Y. lipolytica* wild-type strain was shown able to grow on weak acids; acetate, lactate, and propionate. Maximal growth rate on propionate reached 0.24 ± 0.01 h^{-1} at 2 g/L, and growth inhibition occurred at concentration above 10 g/L. Wild-type strain accumulated lipids ranging from 7.39 to 8.14% (w/w DCW) depending on the carbon source composition, and odd FAs represented only 0.01–0.12 g/L. We here proved that the deletion of the *PHD1* gene improved odd FAs production, which reached a ratio of 46.82% to total lipids. When this modification was transferred to an obese strain, engineered for improving lipid accumulation, further increase odd FAs production reaching a total of 0.57 g/L was shown. Finally, a fed-batch co-feeding strategy was optimized for further increase odd FAs production, which generated 0.75 g/L, the best production described so far in *Y. lipolytica*.

Conclusions: A *Y. lipolytica* strain able to accumulate high level of odd chain fatty acids, mainly heptadecenoic acid, has been successfully developed. In addition, a fed-batch co-feeding strategy was optimized to further improve lipid accumulation and odd chain fatty acid content. These lipids enriched in odd chain fatty acid can (1) improve the properties of the biodiesel generated from *Y. lipolytica* lipids and (2) be used as renewable source of odd chain fatty acid for industrial applications. This work paves the way for further improvements in odd chain fatty acids and fatty acid-derived compound production.

Keywords: *Yarrowia lipolytica*, Oleaginous yeast, Biolipid, Propionate, Odd chain fatty acids, Pentadecanoic acid, Heptadecanoic acid, Heptadecenoic acid, Metabolic engineering

Background

With the increasing environmental and energy concern, microbial oils (lipids and fatty acid-derived products) are regarded as promising alternatives to fossil fuels that can be used for the production of biofuels and oleo-chemicals. Microbial oils present multiple advantages over plant oils or animal fats, because they are not competitive with food, are less susceptible to seasonal availability, and they can be engineered to tune their composition and, therefore, properties [1]. For these reasons, many attempts have taken place to enhance lipid production from microorganisms with diverse metabolic engineering approaches. Despite the enhancement of microbial oils production, costs are not low enough to make the process economically feasible. One way to reduce production costs is to use low-cost carbon substrates [2]. Another is to produce value-added lipids or chemicals not readily obtainable via traditional petrochemical processes [3]. An example of this would be the production of odd chain fatty acids.

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Microbial lipids contain mostly fatty acids from 12 to 22 carbon atoms, with the prevalence of the even-numbered of 16–20 [4]. The availability of fatty acids with odd carbon number is scarce, although they are more valuable for commercialization because of their various applications [3]. For example, *cis*-9-heptadecenoic acid is known to have an anti-inflammatory effect and actives on psoriasis, allergies, and autoimmune diseases [5]. Pentadecanoic acid and heptadecanoic acid can be used as biomarkers for dietary food intake assessment, coronary heart disease (CHD) risk, and type II diabetes mellitus risk [6–9]. The chemical properties and potential biological activities of odd chain fatty acids are now being more extensively studied [4], so novel nutritional and pharmaceutical application could be discovered. The biodiesel properties directly depend on the fatty acid composition of biodiesel fuel [10]. Importantly, and although most effect is caused by the saturated/unsaturated fatty acid ratio, odd chain fatty acids also have a positive impact in the quality of biofuels enhancing transesterification reactions or storage conditions. In addition to fuels, the odd chain fatty acids and derivatives are precursors for manufacturing agricultural chemicals like biocides, flavor and fragrance intermediates, hydraulic fluids, plasticizers, coatings, and industrial chemicals [11–14].

Despite the wide range of application, studies aimed to produce odd chain fatty acids in microorganisms are limited because most of microbial cells normally produce even chain fatty acids. It is reported that exogenous propionate (C3) can be used as a primer for synthesis of odd chain fatty acids: Propionate can be converted to propionyl-CoA by propionyl-CoA synthase, and propionyl-CoA is condensed with malonyl-CoA in the first step of odd chain fatty acid synthesis [15]. A metabolic engineering strategy with propionate supplementation achieved a production of 0.276 g/L odd chain free fatty acids in *E. coli* [16]. In addition, further engineering of *E. coli* showed an increased percentage of odd chain free fatty acids in total free fatty acids by 6.25-fold with propionate supplementation [17]. Odd chain fatty acids have also been produced, with propionate supplementation, in both oleaginous yeasts (*Candida* sp., *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Y. lipolytica*, *Cryptococcus curvatus*) and non-oleaginous yeast (*Kluyveromyces polysporus*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*) [18, 19]. In the best performing yeast, *Y. lipolytica*, odd chain fatty acid did not exceed about 30% of total lipid with a maximum total lipid production of 0.31 g/L, and the highest lipid content of 8.9% g/g of cell dry weight (CDW) [18]. The studies on odd chain fatty acids production in yeast have been done, so far, by optimizing fermentation conditions [19] or evaluating capacity of producing lipids among several strains [18]. More research on propionate utilization and metabolic engineering approaches for enhancing odd chain fatty acids production are, therefore, needed.

Propionic acid is an abundant volatile fatty acid (VFA) which can be obtained from agro-industrial lignocellulosic wastes, sludge and several biodegradable organic wastes [20]. Recently, VFAs is gaining interests as a substrate for lipid production by oleaginous microorganisms since it can be produced from wastes with low-costs [18, 21, 22]. Additionally, VFAs including propionate could lead to higher theoretical conversion efficiencies to lipids compared to other sugar-based carbon sources such as glucose and glycerol due to their shorter metabolic pathways [19, 23]. Studies on tolerance and utilization of propionic acid by oleaginous yeast for lipid production are still limited in the literature, it is sure that further research are necessary to use VFAs, either propionate alone or mixture of VFAs, as more feasible carbon sources.

Yarrowia lipolytica is a widely recognized oleaginous yeast known for its superior characteristics in the production of lipids and fatty acid-derived compounds, as well as other biotechnological products such as organic acids, nutraceuticals, emulsifiers, and surfactants [2, 24]. In addition, *Y. lipolytica* can grow in a broad range of substrates and it has been recently engineered for expanding the substrates range of this yeast including renewable biomass. Several strategies by overexpressing genes or deleting competitive pathway have also been used for improving even lipid accumulation in *Y. lipolytica* [25–27].

In this work, we investigated the ability of *Y. lipolytica* to produce odd chain fatty acids from propionate either as sole carbon and energy source or in combination with glucose. To increase the propionyl-CoA pool for the synthesis of odd chain fatty acids, we disrupted *PHD1* encoding 2-methylcitrate dehydratase in the methyl citrate cycle. We also engineered the strain to accumulate more fatty acid by enhancing the synthesis capacity and blocking the degradation of lipids. Additionally, a fed-batch co-feeding strategy with glucose and propionate further increased total odd chain fatty acids. This work paves the way to use *Y. lipolytica* as a platform microorganism for producing valuable biochemicals with odd-numbered carbon chain.

Results and discussion

Yarrowia lipolytica can grow on propionate as sole carbon source

It is known that *Y. lipolytica* can be grown using diverse carbon sources from hydrophobic substrates such as *n*-alkanes, fatty acids, and oils to hydrophilic ones such as sugars or organic acids [21]. It was also reported that

VFAs could be used as substrates for lipid production [18, 21, 28]. However, high concentration of VFA and weak acids inhibit cell growth which differs depending on strains [18, 21, 28, 29]. The *Y. lipolytica* strains used in this study were derived from the wild-type *Y. lipolytica* W29 strain (ATCC20460). The auxotrophic derivative Po1d (Leu− Ura−, Table 1, Additional file 1: Figure S1) was previously described by Barth and Gaillardin [30]. The Po1d prototroph derivative JMY2900 (Table 1) was used as wild-type reference strain for the comparison with engineered strains derived from Po1d [31]. Growth performance of our reference strain on weak acids and inhibitory effect of propionate were analyzed in microplate (Fig. 1). *Y. lipolytica* was able to grow on weak acids at similar growth rate, about 0.16 h^{-1}, lower than in glucose (0.25 h^{-1}) (Fig. 1a, Additional file 1: Table S1). As previously shown, *Y. lipolytica* can utilize propionate as a sole carbon source—a substrate that promotes odd chain

Table 1 *E. coli* and *Y. lipolytica* strains used in this study

Name	Relevant genotype/plasmid description	Source of references
E. coli strains		
DH5α	*Φ*80*lacZ*Δm15 Δ(*lacZ*YA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Promega
JME740	DH5α pKS-PUT *phd1*	[32]
JME547	DH5α p*UB4-CRE1*	[53]
JME1000	DH5α pKS-PLT *tgl4*	[37]
JME1077	DH5α pGEMT easy-PUT *mfe1*	[36]
JME1128	DH5α JMP62-pTEF-*GPD1-URA3*ex	[36]
JME1822	DH5α JMP62-pTEF-*DGA2-LEU2*ex	[38]
Y. lipolytica strains		
Po1d (JMY195)	*MAT*a *ura3-302 leu2-270 xpr2-322*	[30]
JMY2900	Po1d Ura+ Leu+	[31]
JMY1203	Po1d *phd1::URA3*ex	[32]
JMY3279	Po1d *Δphd1*	This study
JMY3348	Po1d *Δphd1 mfe1::URA3ex*	This study
JMY3350	Po1d *phd1::URA3ex* + *LEU2*	This study
JMY3396	Po1d *Δphd1 mfe1::URA3ex tgl4::LEU2ex*	This study
JMY3433	Po1d *Δphd1 Δmfe1 Δtgl4*	This study
JMY3576	Po1d *Δphd1 Δmfe1 Δtgl4* +*pTEF-DGA2-LEU2ex*	This study
JMY3776	Po1d *Δphd1 Δmfe1 Δtgl4* +*pTEF-DGA2-LEU2ex* + *pTEF-GPD1-URA3ex*	This study

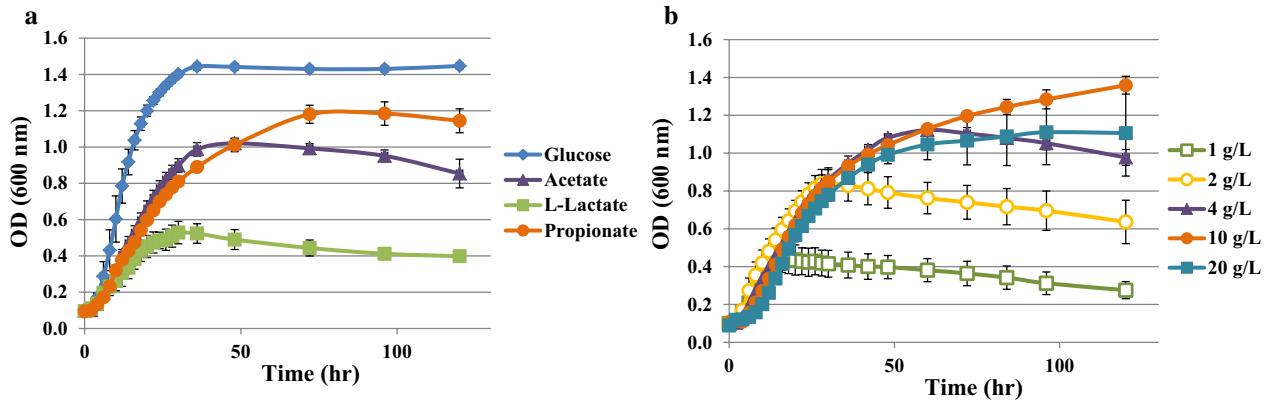

Fig. 1 Growth of wild-type W29 derivative strain JMY2900 on weak acids and on different propionate concentration. **a** Cell growth in minimal media containing 5 g/L of glucose, acetate, L-lactate and propionate as a sole carbon source. **b** Cell growth in propionate medium from 1 to 20 g/L. Growth curves are representative of three independent tests

fatty acid production. Our reference *Y. lipolytica* strain JMY2900 was able to grow on propionate although the growth rate and the final OD were lower than in glucose (Fig. 1a). This growth inhibition was also shown in other organic acids in the following order: L-lactate > propionate > acetate. In comparison to acetate, growth on propionate showed lower growth rate (0.16 h^{-1}) but higher final OD at same concentration (5 g/L). Although acetate has been regarded as preferable carbon source among VFAs because of their relatively lower growth inhibitory effect in previous studies [21, 28], our results showed that propionate can be also a potential carbon source for biomass and lipid production in our *Y. lipolytica* strain.

In a previous study, it is reported that propionate has an inhibitory effect on the cell growth at concentrations above 5 g/L [21]. To explore if our strain could grow on higher concentration than 5 g/L, JMY2900 was cultivated with different concentrations of propionate (Fig. 1b, Additional file 1: Figure S2). Our strain was able to grow up to 100 g/L of propionate as a sole carbon source, the highest growth rate was observed at 2 g/L of propionate (Additional file 1: Table S2). There was no big difference in initial OD trends between 4 and 10 g/L of propionate, but JMY2900 on 10 g/L of propionate was able to grow at higher cell density. The inhibitory effect of propionate to the cell growth was observed on concentration higher than 10 g/L (Additional file 1: Table S2). The growth test at higher concentration of propionate (100 g/L) showed a long lag phase of more than 48 h (Additional file 1: Figure S2), this shows propionate can be used as a carbon source in our strain. However, several *Y. lipolytica* strains behave very differently. For example, our strain appeared to be less sensitive to propionate than *Y. lipolytica* strain CICC31596 which shows an inhibitory effect of propionate on growth rate and lag phase already at 5 g/L [28], while *Y. lipolytica* strain ISA 1834 showed higher growth rate, 0.29 h^{-1}, on propionate [29]. This demonstrates important differences in propionate sensitivity depending on either strains, culture conditions or media composition.

Accumulation of odd chain fatty acids in propionate medium

To see whether propionate is a suitable carbon source for odd lipid production, flask cultures of JMY2900 with several compositions of carbon sources (YNBD1 glucose 1%, YNBD1P1 glucose 1% and propionate 1%, YNBP1 propionate 1%, YNBP2 propionate 2%) were carried out. It was revealed that lipid content obtained on YNBP1 (7.48%) was comparable to that on YNBD1 (7.86%) with significant difference of lipids composition (Table 2, Fig. 2). In YNBD1 media, oleic acid (C18:1) and linoleic acid (C18:2) were the major products with a percentage of

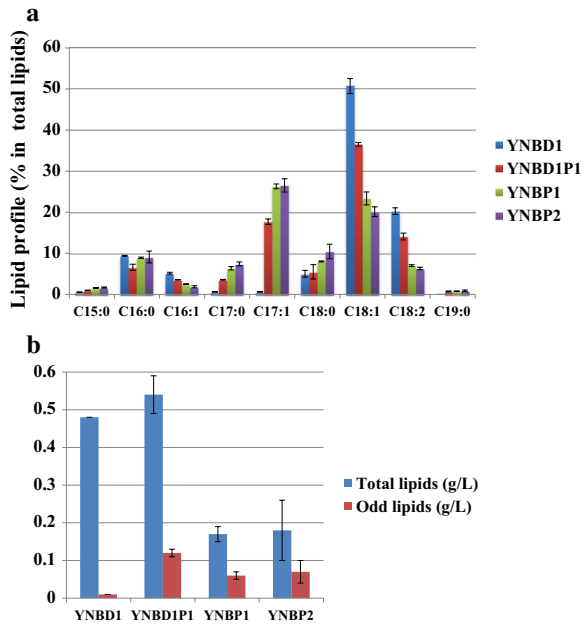

Fig. 2 Lipid profiles and lipid production in WT strain on different media. JMY2900 was grown in glucose (YNBD1), glucose and propionate (YNBD1P1), and in propionate media (YNBP1 and YNBP2) for 72 h at 28 °C, 180 rpm. The results presented are the mean values ± SD for three independent biological replicates. **a** Lipid profiles depending on carbon sources. **b** Total lipid (blue) and total odd lipid (red) production

Table 2 Biomass and lipid production by wild-type strain JMY2900 in minimal medium

	Biomass (g/L)	Lipid content % (g/g dry cell)		Odd lipids/total lipids (%)	Lipids (g/L)	
		Total	Odd		Total	Odd
YNBD1	5.37 ± 0.13	7.86 ± 0.13	0.14 ± 0.00	1.75 ± 0.02	0.48 ± 0.00	0.01 ± 0.00
YNBD1P1	6.80 ± 0.08	7.39 ± 0.15	1.70 ± 0.06	22.93 ± 0.68	0.54 ± 0.05	0.12 ± 0.01
YNBP1	2.60 ± 0.37	7.48 ± 0.69	2.61 ± 0.21	34.96 ± 0.45	0.17 ± 0.02	0.06 ± 0.01
YNBP2	2.71 ± 0.58	8.14 ± 1.51	3.01 ± 0.72	36.52 ± 2.18	0.18 ± 0.08	0.07 ± 0.03

Strain was grown for 72 h at 28 °C and 180 rpm

The mean value of three independent experiments is shown and the standard deviation is indicated

50.72 and 20.26%, respectively. Only 1.75% of odd chain fatty acids in total fatty acids were produced in this condition. However, in case of medium without glucose like YNBP1 and YNBP2, the ratio of odd chain fatty acid to total lipids increased to around 35%. In these conditions, the portion of oleic acid and linoleic acid in total lipids decreased in contrast to the increase of heptadecenoic acid (C17:1). In addition to heptadecenoic acid (C17:1), other odd chain fatty acids such as pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and nonadecanoic acid (C19:0) were also produced from all propionate-containing medium (Table 2, Fig. 2a). These results indicate that propionate can be used as a primer for the synthesis of odd chain fatty acids in *Y. lipolytica* as reported in other studies [18, 19]. Although the total lipid contents from YNBD1 and YNBP1 are similar, the biomass produced was significantly different (5.37 and 2.60 g/L, respectively). The difference in biomass production in YNBP1 and YNBP2 was already shown in Fig. 1b, and it might be due to a higher the inhibitory effect of higher concentration of propionate. In spite of lower ratio of odd chain fatty acids to total fatty acids in YNBD1P1 than that of YNBP1, 0.12 g/L of odd chain fatty acid was produced which showed the highest amount in this culture (Table 2). JMY2900 accumulated slightly higher odd and total lipids in YNBP2 than YNBP1, but it did not show significantly better performance for odd chain fatty acids production. In addition, higher concentration of propionate showed inhibitory effect from the beginning of culture (data not shown). From these results, YNBD1P1 is the best condition for the odd and total lipids production, and YNBP1 is also a suitable condition for high ratio of odd chain fatty acids to total fatty acids (Fig. 2b).

Inactivation of the propionate catabolic pathway improved odd chain fatty acid content and production

We previously reported the importance of the methylcitrate cycle on glycerol metabolism in *Y. lipolytica* [32]. *PHD1*, involved in the synthesis of 2-methylcitrate dehydratase is a mitochondrial protein, which catalyzes the conversion of 2-methyl citrate to 2-methyl-*cis*-aconitate in the methyl citrate cycle. It has been shown that, in *Y. lipolytica*, the deletion of *PHD1* results in the accumulation of 2-methyl citrate, which could potentially halt the TCA cycle and inhibit the entry of citrate into mitochondria [32]. Additionally, deletion of the *PHD1* gene coding for the 2-methyl-citrate dehydratase was shown to improved lipid accumulation. As described above, propionate can be converted to propionyl-CoA, which promotes the production of odd chain lipids. In *Y. lipolytica*, propionyl-CoA can be catabolized to form pyruvate and succinate through the methyl citrate cycle (Fig. 3). So the methyl citrate cycle can be regarded as competitive pathway for the synthesis of odd chain fatty acids. We hypothesize here that inhibition of 2-methyl citrate pathway by deleting *PHD1* increases the propionyl-CoA pool that could be used for further synthesis of odd chain fatty acids. To prove this, culture of JMY3350 (WT Δ*phd1*) was performed in the same condition as above. As expected, JMY3350 was not able to grown in YNBP1 since that propionate cannot be used as a sole carbon source. This confirmed that propionate cannot be metabolized through methyl citrate cycle to form pyruvate in JMY3350. In glucose media (YNBD1), we observed an increased of the ratio of odd chain fatty acid to total fatty acid even without propionate by 1.35 times (Additional file 1: Table S3). Inactivation of *PHD1* blocks the TCA cycle [32], which might cause growth defects and increase sensitivity of propionate to the cell. Therefore, we added lower amount of propionate (4 g/L) with glucose (10 g/L) after 16 h of the start of the culture with glucose (YNBD1). To compare the capability of the two strains, JMY2900 (WT) and JMY3350 (WT Δ*phd1*), for odd chain fatty acid production, we used an equivalent amount of metabolizable carbon with a C/N ratio 30, which is often found as the optimum condition for lipid production in *Y. lipolytica* [33–35]. Therefore, the glucose amount was adjusted in JMY3350 strain to compensate the lack of use of propionate as carbon source for biomass formation. The lipid content of JMY3350 increased by 17% comparing with the wild-type (8.01 and 6.85%). The ratio of odd chain fatty acids to total lipids was also higher (46.82%) than the wild-type (28.32%) (Table 3). JMY3350 produced 0.17 g/L of odd chain fatty acids 21.4% higher than control strain despite of its lower biomass. The lower biomass formation has been previously reported for this mutant [32]. In addition, the inactivation of *PHD1* modified the composition of lipids (Fig. 4). The percentage of heptadecanoic acid in total lipids increased 4 times. Heptadecenoic acid (C17:1) showed the highest portion (35.56%) of total lipids in WT Δ*phd1* (JMY3350), while JMY2900 produced mostly oleic acid (44.75%), likewise most of other *Y. lipolytica* strains. Stability of modified strains is a key parameter in a bioprocess. In this regard, Δ*phd1* strains is expected to be stable on time, since the gene was completely removed from genome and it is very unlikely that other enzymes evolve to consume propionate in a fermentation condition when glucose is the fed for growing.

Engineering higher accumulation of odd chain FA

Once we verified that strain carrying deletion of *PHD1* was able to produce more odd chain fatty acids, we wanted to engineer the strain to make it able to accumulate higher amount of total odd chain lipids. Therefore, we generated the strain named obese Δ*phd1*

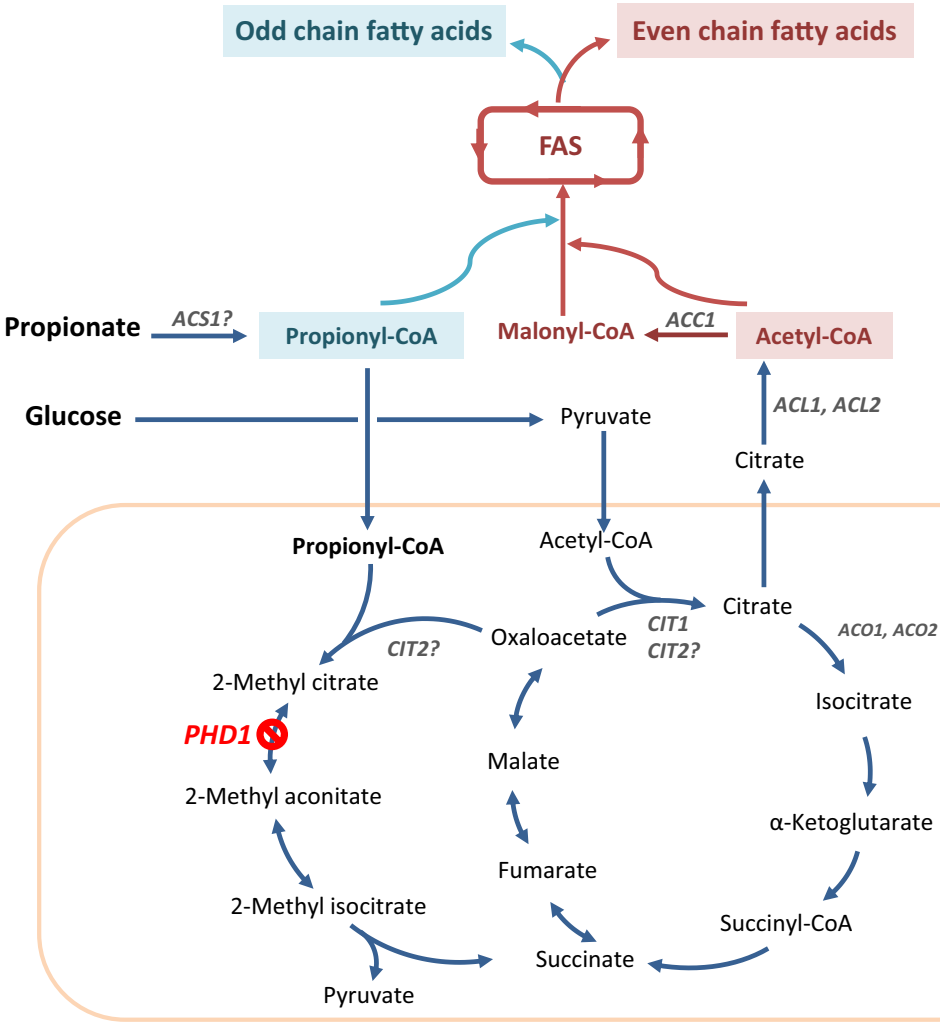

Fig. 3 Overview of the pathways involved in odd and even fatty acid production including the link with the TCA and methyl citrate cycles in *Yarrowia lipolytica*. Propionate is activated by the cytosolic acyl-CoA synthase (*ACS1*) to form the propionyl-CoA which is transported into mitochondria to enter the methyl citrate pathway. Propionate is condensed with oxaloacetate to form 2-methyl citrate by 2-methylcitrate synthase probably encoded by *CIT2* (*CIT2*, YALI0E02684g). 2-Methyl citrate dehydratase removes an H_2O to form 2-methyl aconitate by 2-methyl citrate dehydratase (*PHD1*, YALI0F02497), then is hydrated to form 2-methyl isocitrate probably by the aconitase (*ACO1*, YALI0D09361g; *ACO2*, YALI0E14949g), which is cleaved by 2-methyl isocitrate lyase (YALI0F31999g) to give succinate and pyruvate. Glucose undergoes glycolysis and enters the mitochondria as a form of pyruvate to be used in the TCA cycle. Mitochondrial pyruvate is condensed with oxaloacetate by citrate synthase (*CIT1*, YALI0E00638g) to form citrate which can be exported to cytosol. The cytosolic citrate is transformed by the ATP-citrate lyase (subunit a, *ACL1*, YALI0E34793g and subunit b, *ACL2*, YALI0D24431g) into acetyl-CoA. Acetyl-CoA is then converted into malonyl-CoA by the acetyl-CoA carboxylase (*ACC1*, YALI0C11407g) as the first step of fatty acid synthesis. Acetyl-CoA and malonyl-CoA are condensed by the fatty-acid synthase complex (FAS; subunit beta, *FAS1*, YALI0B15059g and subunit alpha, *FAS2*, YALI0B19382g) for the production of even fatty acids, while acetyl-CoA and propionyl-CoA are condensed for the production of odd fatty acids

(JMY3776) by multiple modifications (Additional file 1: Figure S1). First, to block β-oxidation, *MFE1* encoding the multifunctional enzyme, involved in the second step of β-oxidation, was deleted [36]. To inhibit triacylglycerols (TAG) remobilization, *TGL4* encoding a triglyceride lipase, was deleted [37]. In addition, to push and pull TAG biosynthesis, *DGA2* encoding the major acyl-CoA: diacylglycerol acyltransferase [25, 38], and *GPD1* encoding glycerol-3-phosphate dehydrogenase were overexpressed [36]. We then studied lipid production of engineered strain JMY3776 in the same conditions as before. As expected, odd chain lipid accumulation increased as well as total lipid accumulation, by 3.35 and 3.78 times, respectively (Table 3). The ratio of odd chain lipids to total lipids was slightly decreased in JMY3776, but still remained above 40%. The amount of odd chain

Table 3 Biomass and lipid production by WT, WT Δ*phd1* and obese Δ*phd1* strains in minimal glucose and propionate media

	Biomass (g/L)	Lipid content % (g/g dry cell)		Odd/total lipids (%)	Lipids (g/L)	
		Total	Odd		Total	Odd
JMY2900 (WT)	7.18 ± 0.25	6.85 ± 0.21	1.94 ± 0.03	28.32 ± 0.01	0.49 ± 0.01	0.14 ± 0.01
JMY3350 (WT Δ*phd1*)	4.50 ± 0.50	8.01 ± 0.64	3.75 ± 0.04	46.82 ± 0.03	0.36 ± 0.01	0.17 ± 0.02
JMY3776 (obese Δ*phd1*)	5.53 ± 0.32	24.76 ± 2.51	10.37 ± 0.49	41.9 ± 0.02	1.36 ± 0.06	0.57 ± 0.01

Strain JMY2900 (WT), JMY3350 (WT Δ*phd1*) and JMY3776 (obese Δ*phd1)* were grown for 72 h at 28 °C and 180 rpm

The mean value of three independent experiments is shown and the standard deviation is indicated

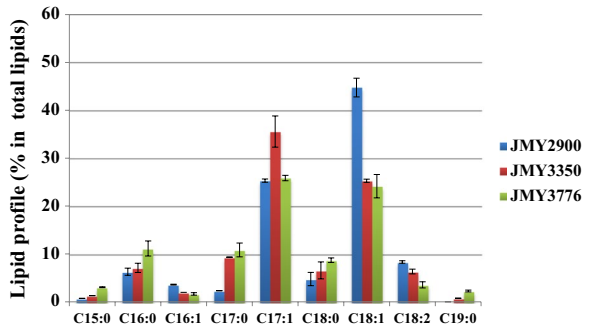

Fig. 4 Comparison of lipid profiles of JMY2900 (WT), JMY3350 (WT Δ*phd1*), and JMY3776 (obese Δ*phd1)* in glucose and propionate media. Cells were grown for 72 h at 28 °C, 180 rpm. The results presented are the mean values for three independent biological replicates

fatty acids was 0.57 g/L, the highest amount produced in *Y. lipolytica*, so far. The amount of all saturated fatty acids from C15:0 to C19:0 increased all together contrary to the unsaturated fatty acids moiety, which decreased (Fig. 4). This phenomenon was also shown in our previous study [39], the strains optimized for lipid accumulation (called obese strain) produced more C16:0 than wild-type and less unsaturated C16 and C18 commonly in different carbon sources (glucose, fructose, and sucrose).

Increase of accumulation of odd FA by fed-batch co-feeding carbon sources

As described above, propionate is a key carbon source for production of odd lipids meanwhile shows growth inhibitory effect in *Y. lipolytica*. Besides, the engineered strain is more sensitive to propionate allowing only small amount of propionate being used for odd chain lipid synthesis. Several fed-batch fermentation strategies have been used to improve yield and productivity by avoiding high level of inhibitory compounds in culture medium [40, 41]. To see whether fed-batch strategy could boost production of odd chain lipids while minimizing the inhibitory effect of propionate, we investigated fed-batch co-feeding of carbon sources during cultivation.

The obese Δ*phd1* strain was cultured in YNBD1 with addition of carbon sources (glucose 4 g/L and propionate 0.5 g/L) at four times points (Fig. 5a). As a result, the production of total lipid and odd chain lipid content, compare to batch culture, were increased by 50.35 and 12.64%, respectively (Table 4). However, the percentage of odd chain lipids in total lipids is diminished by 60% as compared to batch condition likely due to the co-feeding with glucose. Nevertheless, the amount of total odd chain fatty acids from fed-batch co-feeding reached 0.75 g/L, 31% higher than in batch, which represents the highest titer produced in *Y. lipolytica* so far (Additional file 1: Table S4). This represents a 395% increase of odd chain fatty acid production between wild-type JMY2900 and the obese Δ*phd1* deleted strain in the fed-batch condition (Fig. 5b, c). Fed-batch fermentation has been beneficial for the production of other compounds by *Y. lipolytica*, such as the production of lipids described for the obese strain JMY3501 on synthetic media [42] or the obese strain producing carotenoides with the concomitant production of 42.6 g/L of lipids on rich media [43]. The fermentation conditions can be further optimized by testing various feeding rate of glucose and propionate, which will allow to improve final biomass, higher lipid content and odd chain fatty acid content. Also, in future experiments it would be interesting to test other C/N ratios, such as C/N = 60 or C/N = 100 which are found better for certain strains and conditions [25, 39, 44, 45].

Additionally, one could envisage the production of different types of odd chain fatty acids and their derivatives such as shorter odd chain fatty acid by engineering the fatty acid synthase (FAS) as recently demonstrated in *Yarrowia lipolytica* [46], hydroxylated odd chain fatty acid by introducing Δ12-hydroxylase (FAH12) from *Claviceps purpurea* [47] and odd chain dicarboxylic acid by overexpression of the omega

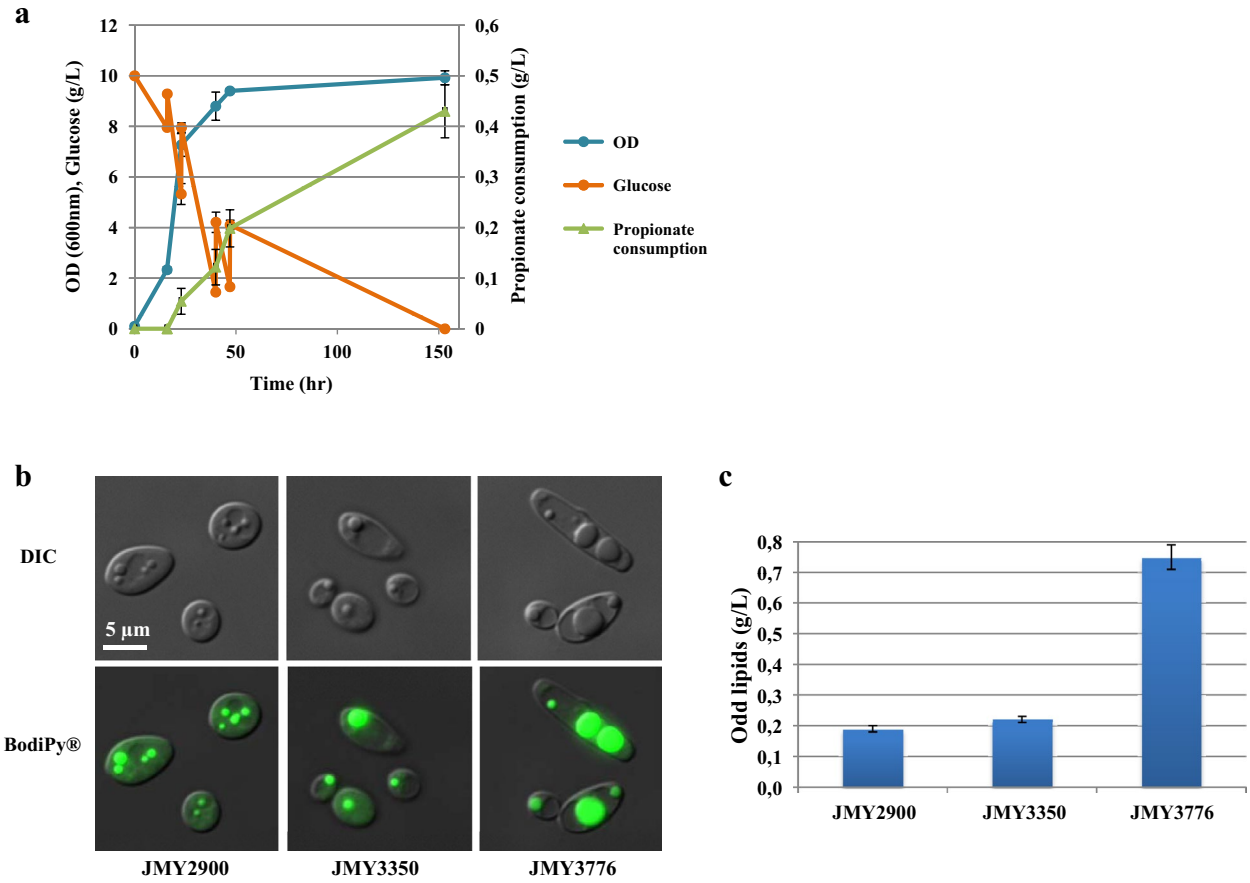

Fig. 5 Improvement of odd chain fatty acid production of JMY3776 by fed-batch co-feeding of propionate and glucose. **a** Increase of the accumulation of odd lipids by co-feeding of propionate and glucose. Cells were grown for 16 h in glucose media (YNBD1), then pulses of glucose + propionate (4 and 0.5 g/L, respectively) were added at $t = 16, 23, 40, 47$ h. The results presented are the mean values for three independent biological replicates. **b** Microscope image of cells (DIC) and lipid body visualization with Bodipy of JMY2900 (WT), JMY3350 (WT Δ*phd1*) and JMY3776 (obese Δ*phd1*). **c** Increase of odd lipids accumulation by pathway engineering in fed-batch co-feeding cultures

Table 4 Biomass and lipid production by WT, WT Δ*phd1* and obese Δ*phd1* strains by fed-batch co-feeding of glucose and propionate

	Biomass (g/L)	Lipid content % (g/g dry cell)		Odd/total lipids (%)	Lipids (g/L)	
		Total	Odd		Total	Odd
JMY2900 (WT)	8.20 ± 0.05	17.23 ± 0.31	2.30 ± 0.02	13.36 ± 0.01	1.41 ± 0.02	0.19 ± 0.01
JMY3350 (WT Δ*phd1*)	4.83 ± 0.13	17.71 ± 0.70	4.58 ± 0.05	25.90 ± 0.01	0.85 ± 0.01	0.22 ± 0.01
JMY3776 (obese Δ*phd1*)	5.93 ± 0.13	50.35 ± 1.99	12.64 ± 0.45	25.11 ± 0.17	2.99 ± 0.18	0.75 ± 0.04

oxidation pathway [48]. However, further basic knowledge is required to fully understand propionic acid catabolism, its transport and toxicity to the cells.

Conclusion

In this study, it is shown that *Y. lipolytica* produce odd chain fatty acids (mainly heptadecenoic acid,

heptadecanoic acid, and pentadecanoic acid) from propionate as a carbon source. By inactivating 2-methyl-citrate dehydratase in competing pathway utilizing propionyl-CoA, the amount of odd chain fatty acids is increased, the ratio of odd lipid to total lipids reached to 46.82%. Obese Δ*phd1* strain engineered to accumulate higher amount of lipid produced 3.35 times higher odd chain lipids together with increased total lipid accumulation. In addition, a fed-batch co-feeding strategy further improved production of odd chain fatty acids with amount of 0.75 g/L represents the highest titer produced in *Y. lipolytica* so far. Odd chain fatty acids are very important and versatile chemicals in both pharmaceutical and industrial fields. This work paves the way for further improvements in odd chain fatty acids and fatty acid-derived compound production.

Methods

Strains and media

Media and growth conditions for *E. coli* were as described by Sambrook et al. [49], and those for *Y. lipolytica* have been described by Barth and Gaillardin [30]. Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described previously [50]. Minimal medium (YNB) contained 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 0.5% (w/v) NH_4Cl, 50 mM phosphate buffer (pH 6.8). The following carbon sources were added: YNBD1 (1% (w/v) glucose, YNBD1P1 (1% (w/v) glucose, 1% (w/v) propionate), YNBP1 (1% (w/v) propionate), YNBP2 (2% (w/v) propionate). To complement auxotrophic processes, 0.1 g/L of uracil or leucine (Difco) was added as necessary.

Construction of strains

The overexpression and disruption cassettes were prepared as described previously [36–38], and were used for transformation by the lithium acetate method [51]. Transformants were selected on YNBUra, YNBLeu, YNBHyg, YNB media depending on their genotype. Then genomic DNA from yeast transformants was prepared as described in Querol et al. [52]. Positive transformants were checked by PCR. The removal of the selection marker was carried out via the LoxP-Cre system as previously used in *Y. lipolytica* [53].

Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). PCR amplifications were performed in an Eppendorf 2720 thermal cycler with GoTaq DNA polymerases (Promega) and Q5 High-Fidelity DNA Polymerase (New England Biolabs). PCR fragments were purified with a QIAgen Purification Kit (Qiagen, Hilden, Germany).

Growth test

Pre-cultures were inoculated into tubes containing 5 mL YPD medium, and cultured overnight (28 °C, 180 rpm). Pre-cultures were then centrifuged and washed with sterile distilled water, cell suspensions were standardized to an OD_{600} of 0.1. Stains were grown in 200 µL of minimal YNB medium (see above) in the presence of carbon sources (0.5% glucose, propionate, L-lactate, acetate as a carbon source) in 96-well plates, with constant shaking, at 28 °C. Growth was monitored by measuring the optical density (OD_{600}) every 30 min for 120 h with a microtiter plate reader (Biotek Synergy MX, Biotek Instruments, Colmar, France). For each strain and set of conditions, we used three biological replicates. The growth rate was calculated in the exponential phase for each strain and condition.

Culture conditions for lipid biosynthesis experiments

For lipid biosynthesis in minimal media, cultures were prepared as follows: an initial pre-culture was established by inoculating 10 mL of YPD medium in 50 mL Erlenmeyer flasks. This was followed by an overnight shaking step at 28 °C and 180 rpm. The resulting cell suspension was washed with sterile distilled water and used to inoculate 50 mL of YNB medium containing 0.15% (w/v) NH_4Cl and 50 mM phosphate buffer (pH 6.8) with various concentrations of carbon source in 250 mL Erlenmeyer flasks, at 28 °C and 180 rpm. For fed-batch co-feeding test, the strains were cultured in 20 mL of YNBD1 with addition 2 mL of mixture of carbon sources to a final concentration of glucose 4 g/L and propionate 0.5 g/L at four times points. The addition were performed at $T = 16$, 23, 40, 48 h after the inoculation.

Determination of glucose and propionate

Glucose and propionate were identified and quantified by HPLC. Filtered aliquots of the culture medium were analyzed by UltiMate 3000 system (Thermo Fisher Scientific, UK) using an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-RAD, USA) coupled to UV (210 nm) and RI detectors. The mobile phase used was 0.01 N H_2SO_4 with a flow rate of 0.6 mL/min and the column temperature was $T = 35$ °C. Identification and quantification were achieved via comparisons to standards. For each data point, we used at least two biological replicates and calculated average and standard deviation values.

Lipid determination

Lipids were extracted from 10 to 20 mg of freeze-dried cells, and converted into their fatty acid methyl esters (FAMEs) according to Browse et al. [54], and FAMEs

were analyzed by gas chromatography (GC) analysis. GC analysis of FAMEs was carried out on a Varian 3900 instrument equipped with a flame ionization detector and a Varian FactorFour vf-23 ms column, where the bleed specification at 260 °C is 3 pA (30 m, 0.25 mm, 0.25 µm). Fatty acids were identified by comparison to commercial FAME standards (FAME32, Supelco) and quantified by the internal standard method, involving the addition of 100 µg of commercial dodecanoic acid (Sigma-Aldrich). Commercial odd chain fatty acids (9 Odd carbon fatty acids, OC9, Supelco) were converted to their FAMEs with a same method for yeast samples, and analyzed by GC to identify and compare odd chain fatty acids from yeast samples.

To determine DCW in flask experiments, 2 mL of the culture were washed and lysophilized in a pre-weighed tube. The differences in weight corresponded to the mg of cells found in 2 mL of culture. For each data point, we used at least two biological replicates and calculated average and standard deviation values.

Microscopic analysis

Images were obtained using a Zeiss Axio Imager M2 microscope (Zeiss, Le Pecq, France) with a 100× objective lens and Zeiss filter sets 45 and 46 for fluorescence microscopy. Axiovision 4.8 software (Zeiss, Le Pecq, France) was used for image acquisition. To make the lipid bodies (LBs) visible, BodiPy® Lipid Probe (2.5 mg/mL in ethanol, Invitrogen) was added to the cell suspension ($OD_{600} = 5$) and the samples were incubated for 10 min at room temperature.

Additional file

Additional file 1: Figure S1. Schematic representation of strain construction. The auxotrophic strain Po1d (Leu − Ura −) was derived from the French wild-type strain W29. First, *PHD1* was disrupted with the *phd1*::*URA3*ex disruption cassette (JME740) yielding JMY1203 and JMY3350 after *LEU2* complementation. Uracil auxotrophe was restored by marker rescue yielding JMY3279. Second, *MFE1* and TGL4 were disrupted with the *mfe1*::*URA3*ex disruption cassette (JME1077) yielding JMY3348 and the *tgl4*::*LEU2*ex disruption cassette (JME1000) yielding JMY3396. Uracil and leucine auxotrophies were restored by marker rescue yielding JMY3433. Finally, p*TEF-DGA2-LEU2ex* (JME 1822) and p*TEF-GPD1-URA3ex* (JME1128) overexpression cassettes were introduced yielding JMY3776 (obese Δ*phd1*). For more information about construction, see "Methods" section and Table 1. **Figure S2.** Cell growth in different concentration of propionate. **Figure S3.** GC chromatogram of lipid profiles of JMY2900 (WT) and JMY3776 (obese Δ*phd1*). **Table S1.** Growth rate of JMY2900 on glucose and weak acids. **Table S2.** Growth rate of JMY2900 on propionate depending on concentration. **Table S3.** Lipid production of JMY2900 and JMY3350 in YNBD1. **Table S4.** Odd chain Fatty acid production in *Y. lipolytica*.

Abbreviations

DCW: dry cell weight; DIC: differential interference contrast; Odd FA: odd chain fatty acid; Even FA: even chain fatty acid; PCR: polymerase chain reaction.

Authors' contributions

YKP, RLA and JMN conceived the study and participated in its design. YKP, RLA designed the constructs, carried out all the experiments. TD designed and carried out the construction of the obese *PHD1* deleted strains. YKP drafted the manuscript. YKP, TD, RLA and JMN revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All data generated or analyzed in the present study are included in this published article and a supporting material "Additional file 1".

Consent for publication

All authors consent for publication.

Ethical approval and consent to participate

Not applicable.

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De novo Biosynthesis of Odd-Chain Fatty Acids in *Yarrowia lipolytica* Enabled by Modular Pathway Engineering

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Microbial oils are regarded as promising alternatives to fossil fuels as concerns over environmental issues and energy production systems continue to mount. Odd-chain fatty acids (FAs) are a type of valuable lipid with various applications: they can serve as biomarkers, intermediates in the production of flavor and fragrance compounds, fuels, and plasticizers. Microorganisms naturally produce FAs, but such FAs are primarily even-chain; only negligible amounts of odd-chain FAs are generated. As a result, studies using microorganisms to produce odd-chain FAs have had limited success. Here, our objective was to biosynthesize odd-chain FAs *de novo* in *Yarrowia lipolytica* using inexpensive carbon sources, namely glucose, without any propionate supplementation. To achieve this goal, we constructed a modular metabolic pathway containing seven genes. In the engineered strain expressing this pathway, the percentage of odd-chain FAs out of total FAs was higher than in the control strain (3.86 vs. 0.84%). When this pathway was transferred into an obese strain, which had been engineered to accumulate large amounts of lipids, odd-chain fatty acid production was 7.2 times greater than in the control (0.05 vs. 0.36 g/L). This study shows that metabolic engineering research is making progress toward obtaining efficient cell factories that produce odd-chain FAs.

Keywords: odd-chain fatty acids, propionyl-CoA, *Yarrowia lipolytica*, metabolic engineering, Golden Gate assembly, synthetic biology

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INTRODUCTION

Microbial oils (lipids and fatty acid-derived products) are regarded as promising alternatives to fossil fuels in the face of growing concerns over environmental issues and energy production. To lessen the cost of producing microbial oils, considerable effort has been dedicated to enhancing production yield (Dulermo and Nicaud, 2011; Tai and Stephanopoulos, 2013; Qiao et al., 2015); using low-cost carbon substrates (Papanikolaou et al., 2013; Lazar et al., 2014; Guo et al., 2015; Ledesma-Amaro and Nicaud, 2016); and targeting high-value lipids (Xue et al., 2013; Xie et al., 2015). Odd-chain fatty acids (FAs), a type of valuable lipid, are a product with potential because they can be used in a variety of applications. Notably, research has revealed that odd-chain FAs with chain lengths of 15 and 17 carbons may have functional importance for nutrition and medical field. For example, *cis*-9-heptadecenoic acid has anti-inflammatory effects and can help treat psoriasis, allergies, and autoimmune diseases (Degwert et al., 1998). Pentadecanoic acid and heptadecanoic

acid can be used as biomarkers of food intake in dietary assessments, the risk of coronary heart disease (CHD), and the risk of type II diabetes mellitus (Forouhi et al., 2014; Jenkins et al., 2015; Pedersen et al., 2016; Pfeuffer and Jaudszus, 2016). The chemical properties and potential biological activities of odd-chain FAs are now being more extensively studied (Rezanka and Sigler, 2009), so it is possible that novel nutritional and pharmaceutical applications could soon be discovered. In addition, odd-chain FAs and their derivatives are precursors for manufacturing substances such as pesticides, flavor and fragrance compounds, hydraulic fluids, plasticizers, coatings, and other industrial chemicals (Fitton and Goa, 1991; Avis, 2000; Clausen et al., 2010; Köckritz et al., 2010). Despite the broad range of applications for FAs, studies aiming to produce odd-chain FAs using microorganisms have had limited success because microorganisms produce a much greater proportion of even-chain FAs than odd-chain FAs.

Generally, *de novo* fatty acid synthesis in microorganisms begins with the condensation of acetyl-CoA and malonyl-CoA (**Figure 1A**). Then, the elongation step occurs: long-chain FAs are synthesized in a reaction catalyzed by fatty acid synthase (FAS). The resulting acyl-CoA products are esterified to generate lysophosphatidic acid (LPA), then phosphatidic acid (PA), and finally diacylglycerol (DAG) before forming triacylglycerol (TAG), the compound in which the lipids are stored. For odd-chain FAs, propionyl-CoA could be converted from propionate is a primer for the fatty acid synthesis. The condensation of both propionyl-CoA and malonyl-CoA results in the formation of 3-oxovaleryl-ACP, which is the launching point for odd-chain FA synthesis. This five-carbon compound goes through elongation, where two carbons are added in each cycle. Then odd-chain FAs can be synthesized as described in **Figure 1B**. However, most microorganisms require the presence of propionate in the medium to produce odd-chain FAs. Wu and San have shown that *E. coli* can produce odd-chain FAs—namely undecanoic acid (C11:0), tridecanoic acid (C13:0), and pentadecanoic acid (C15:0)—if grown with propionate-supplemented medium (Wu and San, 2014). They introduced a propionyl-CoA synthetase (*prpE*) from *Salmonella enterica* and acyl-ACP thioesterases (TEs) from *Umbellularia californica* and *Ricinus communis*. Additionally, propionate supplementation has allowed various yeasts (e.g., *Yarrowia lipolytica*, *Rhodotorula glutinis*, *Cryptococcus curvatus*, and *Kluyveromyces polysporus*) to produce odd-chain FAs (Zheng et al., 2012; Kolouchová et al., 2015). More recently, in *Y. lipolytica*, metabolic engineering and the optimization of propionate feeding helped boost the production of odd-chain FAs, namely heptadecenoic acid (C17:1) (Park et al., 2018).

Therefore, to date, most studies have been focused on processes that involve propionate supplementation. However, due to the high cost (Poirier et al., 1995; Aldor et al., 2002) and toxic effects of propionate (Fontanille et al., 2012; Park and Nicaud, 2019), it is crucial to find alternative pathways for generating propionyl-CoA to be able to produce odd-chain FAs on large scales. There have been a few studies in which odd-chain FAs have been produced using glucose. Tseng and Prather have shown that, in *E. coli*, the production of very short odd-chain FAs (i.e., propionate, trans-2-pentenoate, and valerate) can be increased through the upregulation of threonine biosynthesis (Tseng and Prather, 2012). Another study demonstrated that the overexpression of threonine biosynthesis in *E. coli* resulted in increased levels of odd-chain FAs (mainly C15:0): from 0.006 to 0.246 g/L. This study further modified the experimental strain by replacing the native β-ketoacyl-ACP synthase (encoded by *FabH*) with one from *Bacillus subtilis* (encoded by *FabHI*) so that there was a biochemical preference for propionyl-CoA over acetyl-CoA (Lee et al., 2013). However, to date, no one has reported the *de novo* production of odd-chain FAs in *Y. lipolytica* or in any other yeasts without propionate supplementation. Consequently, we need more research on metabolic engineering approaches for producing odd-chain FAs in this group of microorganisms.

Of the several microbial hosts used in such systems, the oleaginous yeast *Y. lipolytica* is the most studied and has been engineered to produce large amounts of lipids and lipid derivatives, such as ricinoleic acid (Beopoulos et al., 2014), conjugated linoleic acids (Imatoukene et al., 2017), cyclopropane FAs (Czerwiec et al., 2019), and cocoa butter-like oils (Papanikolaou et al., 2003). *Y. lipolytica* can naturally grow in a broad range of substrates and has been further engineered to use even more substrates (Ledesma-Amaro et al., 2016). In addition,

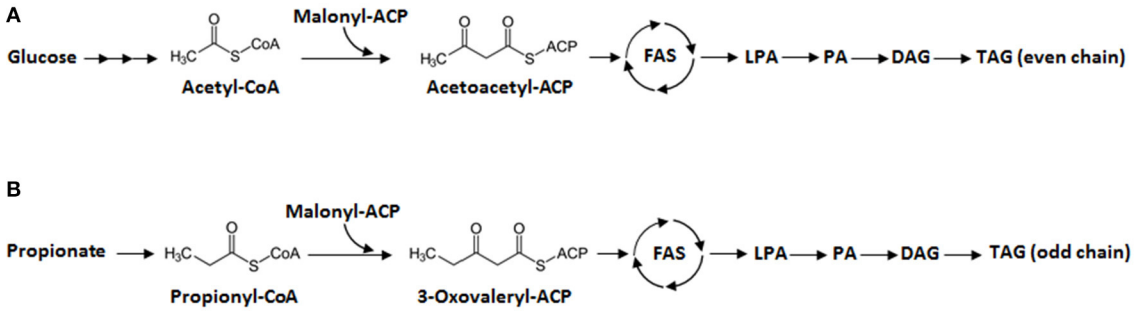


FIGURE 1 | Lipid synthesis in *Y. lipolytica*. **(A)** Synthesis of even-chain fatty acids (FAs) from glucose. **(B)** Synthesis of odd-chain FAs from propionate. First, there is elongation of fatty acyl-CoA by fatty acid synthase (FAS). Second, the resulting even- or odd-chain fatty acyl-CoA is transformed into lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG), in that order, before finally forming triacylglycerol (TAG).

many different synthetic biology tools have been created for and applied in *Y. lipolytica*, such as Gibson assembly, Golden Gate assembly, TALEN editing, and CRISPR/Cas9 editing [please see the recent review by Larroude et al. (2018) for more details]. These tools used in tandem with genetic and metabolic engineering strategies have boosted the capacities of *Y. lipolytica*, making the yeast into a promising host for biotechnological production processes.

The objective of this study was to biosynthesize odd-chain FAs *de novo* from glucose without propionate supplementation. We constructed a modular metabolic pathway for synthesizing propionyl-CoA from oxaloacetate in *Y. lipolytica* and confirmed that accumulation of odd-chain FAs was increased. We also investigated whether it could be competitive to produce odd-chain FAs from propionyl-CoA via the methylcitrate pathway using the engineered strain. Additionally, we overexpressed the pyruvate dehydrogenase (PDH) complex in the cytosol to see if it could improve the conversion of α-ketobutyrate to propionyl-CoA. This work demonstrates that our metabolic engineering strategy for directing metabolic fluxes through specific pathways can enhance odd-chain FA production.

MATERIALS AND METHODS

Strains and Media

Media and growth conditions for *E. coli* were previously described by Sambrook and Russell (2001); those for *Y. lipolytica* were previously described by Barth and Gaillardin (1997). Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described elsewhere (Milckova et al., 2004). The YNB contained 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww), 0.5% (w/v) NH_4Cl, 50 mM KH_2PO_4-Na_2HPO_4 (pH 6.8), and 2% (w/v) glucose. To complement strain auxotrophies, 0.1 g/L of uracil or leucine was added as necessary. To screen for hygromycin resistance, 250 μg/ml of hygromycin was added to the YPD. Solid media were prepared by adding 1.5% (w/v) agar.

Construction of Plasmids and Strains (*E. coli* and *Y. lipolytica*)

We used standard molecular genetic techniques (Sambrook and Russell, 2001). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). PCR amplification was performed in an Eppendorf 2720 Thermal Cycler with either Q5 High-Fidelity DNA Polymerase (New England Biolabs) or GoTaq DNA polymerases (Progema, WI, USA). PCR fragments were purified using a PCR Purification Kit (Macherey-Nagel, Duren, Germany), and plasmids were purified with a Plasmid Miniprep Kit (Macherey-Nagel).

The plasmids used in this study were constructed using Golden Gate assembly, as described in Celinska et al. (2017). The genes in the A, T, and H module were obtained via PCR using the genomic DNA of *Y. lipolytica* W29. Internal BsaI recognition sites were removed via PCR using the primers listed in **Supplementary Table 1**. The plasmids for each module included the Zeta sequence, the *URA3 ex* marker, and gene expression cassettes containing the *TEF1* promoter and *LIP2* terminator.

For the cytosolic PDH complex, all the genes were synthesized and cloned in the plasmid pUC57 by GenScript Biotech (New Jersey, US). Cytosolic *PDX1* was cloned into the expression plasmid (JME2563) using the BamHI and AvrII restriction sites. The other four genes were cloned into two plasmids (JME4774 and JME4775) using Golden Gate assembly.

To disrupt *PHD1*, the cassettes were constructed to include a promoter (*pPHD1*), a marker (*URA3* or *LEU2*), and a terminator (*TPHD1*), which allowed the ORF gene to be removed via homologous recombination, as described in Papanikolaou et al. (2013).

Gene expression and disruption cassettes were prepared by NotI digestion and transformed into *Y. lipolytica* strains using the lithium acetate method, as described previously (Barth and Gaillardin, 1997). Gene integration and disruption were verified via colony PCR using the primers listed in **Supplementary Table 1**. The replicative plasmid harboring the *Cre* gene (JME547; **Table 1**) was used for marker rescue (Fickers et al., 2003). After transformation with the *Cre* expression plasmid, the loss of the marker gene was verified on YNB with/without uracil. The loss of the replicative plasmid was checked using replica plating on YPD with/without hygromycin after culturing on YPD for 24 h. To construct the prototrophic strain, a *LEU2* fragment from plasmid JMP2563 was transformed. All the strains and plasmids used in this study are listed in **Table 1**.

Culture Conditions for the Lipid Biosynthesis Experiments

The lipid biosynthesis experiments were carried out in minimal media, and the cultures were prepared as follows: an initial pre-culture was established by inoculating 10 mL of YPD medium in 50 mL Erlenmeyer flasks. Then, the pre-culture was incubated overnight at 28°C and 180 rpm. The resulting cell suspension was washed with sterile distilled water and used to inoculate 50 mL of minimal medium YNBD6 containing 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate, YNBww, Difco), 0.15% (w/v) NH_4Cl, 50 mM KH_2PO_4-Na_2HPO_4 buffer (pH 6.8), and 6% (w/v) glucose. This medium had been placed in 250 mL Erlenmeyer flasks. The cultures were then incubated at 28°C and 180 rpm.

Lipid Determination

Lipids were extracted from 10 to 20 mg of freeze-dried cells and converted into FA methyl esters (FAMEs) using the procedure described by Browse et al. (1986). The FAMEs were then analyzed using gas chromatography (GC), which was carried out with a Varian 3900 instrument equipped with a flame ionization detector and a Varian FactorFour vf-23ms column, where the bleed specification at 260°C is 3 pA (30 m, 0.25 mm, 0.25 μm). The FAMEs were identified via comparisons with commercial standards (FAME32, Supelco) and quantified using the internal standard method, which involves the addition of 100 μg of commercial dodecanoic acid (Sigma-Aldrich). Commercial odd-chain FAs (Odd Carbon Straight Chains Kit containing 9 FAs, OC9, Supelco) were converted into their FAMEs using the same method employed with the yeast samples. They were then

TABLE 1 | The plasmids and strains used in this study.

Strain	Description	Abbreviation	References
Plasmid			
GGE0004	TOPO-P2-*TEF1*		Celinska et al., 2017
GGE0009	TOPO-P3-*TEF1*		Celinska et al., 2017
GGE0014	TOPO-T1-*LIP2*		Celinska et al., 2017
GGE0015	TOPO-T2-*LIP2*		Celinska et al., 2017
GGE0020	TOPO-T1-3-*LIP2*		Celinska et al., 2017
GGE0021	TOPO-T2-3-*LIP2*		Celinska et al., 2017
GGE0028	pSB1C3		Celinska et al., 2017
GGE0029	pSB1A3		Celinska et al., 2017
GGE0038	TOPO-ZetaDOWN-NotI		Celinska et al., 2017
GGE0067	TOPO-ZetaUP-NotI		Celinska et al., 2017
GGE0081	TOPO-T3-*LIP2*		Celinska et al., 2017
GGE0082	TOPO-P1-*TEF1*		Celinska et al., 2017
GGE0085	TOPO-M-*URA3* ex		Celinska et al., 2017
GGE0376	TOPO-*AAT2*		This study
GGE0377	TOPO-*THR1*		This study
GGE0378	TOPO-*THR4*		This study
GGE0379	pJET-*ILV1*		This study
GGE0380	TOPO-*HOM3*		This study
GGE0381	TOPO-*HOM2*		This study
GGE0382	pJET-*HOM6*		This study
JME0547	pUC-*Cre*		Fickers et al., 2003
JME0740	pGEM-T-*PHD1* PUT		Papanikolaou et al., 2013
JME1811	pGEM-T-*PHD1* PLT		Papanikolaou et al., 2013
JME2563	JMP62-*LEU2*ex-pTEF1		Dulermo et al., 2017
JME4478	GGV-*URA3* ex-pTEF1-*AAT2*	Module A	This study
JME4479	GGV-*URA3* ex-pTEF1-*THR1*-pTEF-*THR4*-pTEF-*ILV1*	Module T	This study
JME4632	GGV-*URA3* ex-pTEF1-*HOM3*-pTEF1-*HOM2*-pTEF1-*HOM6*	Module H	This study
JME4774	GGV-*URA3* ex-pTEF1-yl*PDA1*-pTEF1-yl*PDB1*	Module P	This study
JME4775	GGV-*URA3* ex-pTEF1-yl*LPD1*-pTEF1-yl*LAT1*	Module P	This study
JME4776	JMP62-*LEU2* ex-pTEF1-yl*PDX1*	Module P	This study
Y. lipolytica			
JMY195	MATa *ura3-302 leu2-270 xpr2-322*	WT	Barth and Gaillardin, 1997
JMY2900	JMY195 *URA3 LEU2*	WT	Dulermo et al., 2014
JMY7201	JMY195 + GGV-*AAT2-URA3* ex	WT-A	This study
JMY7202	JMY195 + GGV-*AAT2*	WT-A	This study
JMY7639	JMY195 + GGV-*AAT2-URA3* ex + *LEU2*	WT-A	This study
JMY7203	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1-URA3* ex	WT-AT	This study
JMY7204	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1*	WT-AT	This study
JMY7353	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex	WT-ATH	This study
JMY7357	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	WT-ATH	This study
JMY7374	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6-URA3* ex + *phd1*::*LEU2* ex	WT-ATH *phd1Δ*	This study
JMY7828	JMY195 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* +GGV-*HOM3-HOM2-HOM6*	WT-ATH	This study
JMY7640	JMY195 + GGV-*THR1-THR4-ILV1-URA3* ex	WT-T	This study
JMY7643	JMY195 + GGV-*THR1-THR4-ILV1-URA3* ex + *LEU2*	WT-T	This study
JMY7646	JMY195 + GGV-*HOM3-HOM2-HOM6-URA3* ex	WT-H	This study
JMY7649	JMY195 + GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	WT-H	This study
JMY7824	Y195ATH+ylPDHcyto	WT-ATHP	This study
JMY3501	*Δpox1-6 Δtgl4* pTEF-*DGA2-LEU2*ex pTEF-*GPD1-URA3*ex	Obese	Lazar et al., 2014

(Continued)

TABLE 1 | Continued

Strain	Description	Abbreviation	References
JMY3820	*Δpox1-6 Δtgl4* pTEF-*DGA2* pTEF-*GPD1*	Obese	Lazar et al., 2014
JMY7206	JMY3820 + GGV-*AAT2-URA3* ex	Obese-A	This study
JMY7207	JMY3820 + GGV-*AAT2*	Obese-A	This study
JMY7208	JMY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1-URA3* ex	Obese-AT	This study
JMY7267	JMY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1*	Obese-AT	This study
JMY7412	MY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* + GGV-*HOM3-HOM2-HOM6-URA3* ex + *LEU2*	Obese-ATH	This study
JMY7413	MY3820 + GGV-*AAT2* + GGV-*THR1-THR4-ILV1* + GGV-*HOM3-HOM2-HOM6*	Obese-ATH	This study
JMY7414	JMY7413 + *phd1::LEU2* ex	Obese-ATH *phd1Δ*	This study
JMY7417	JMY7413 + *phd1::LEU2* ex + *URA3*	Obese-ATH *phd1Δ*	This study
JMY7826	Y3820ATH+ylPDHcyto	Obese-ATHP	This study

identified using GC and compared with the odd-chain FAs from the yeast samples.

To determine dry cell weight (DCW), 2 mL of the culture was taken from the flasks, washed, and lysophilized in a pre-weighed tube. The differences in mass corresponded to the mg of cells found in 2 mL of culture.

RESULTS

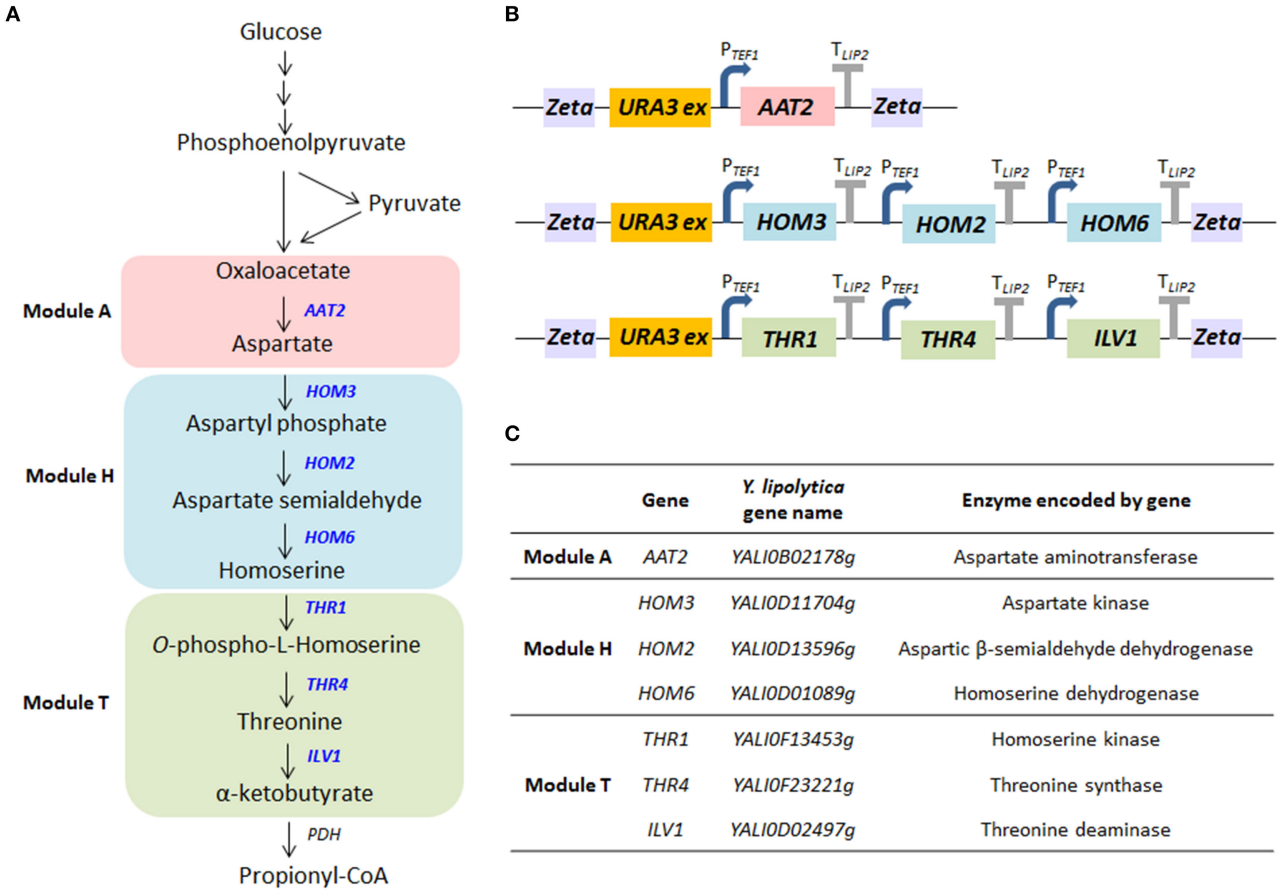
Modular Pathway Engineering Was Used for Odd-Chain Fatty Acid Synthesis

Propionyl-CoA is a key primer in the synthesis of odd-chain FAs. It can be synthesized using β-oxidation from direct precursors, propionate, or long-chain FAs. It can also be created from other metabolites via several metabolic pathways, such as the citramalate/2-ketobutyrate pathway, the aspartate/2-ketobutyrate pathway, the methylmalonyl-CoA pathway, the 3-hydroxypropionate pathway, and the isoleucine or valine degradation pathway (**Supplementary Figure 1**; Han et al., 2013; Lee et al., 2013). Here, we tested if the overexpression of the α-ketobutyrate pathway—which produces threonine as an intermediate—could increase levels of propionyl-CoA in *Y. lipolytica*. As shown in **Figure 2A**, the pathway eventually forms the amino acids aspartate, homoserine, and threonine from oxaloacetate. Then, threonine is deaminated to generate α-ketobutyrate, a reaction catalyzed by threonine dehydratase. Alpha-ketobutyrate is directly or sequentially converted into propionyl-CoA by the pyruvate dehydrogenase (PDH) complex or pyruvate oxidase, respectively. The upregulation of threonine has previously been used to boost propionyl-CoA availability in *E. coli*. Lee et al. showed that levels of odd-chain FAs could be increased by introducing the threonine biosynthesis pathway (which creates α-ketobutyrate from aspartate semialdehyde), especially when mutated homoserine dehydrogenase (*thrA**, reduced feedback inhibition) was also expressed (Lee et al., 2013). The percentage of odd-chain FAs out of total FAs increased from <1 to 18% by overexpressing the threonine pathway in *E. coli*. The predominant odd-chain FA produced was pentadecanoic acid (C15:0).

In this study, we enhanced the extended threonine biosynthesis pathway (the aspartate/α-ketobutyrate pathway)—from oxaloacetate to α-ketobutyrate—by overexpressing seven genes (**Figure 2A**). There were three modules (**Figures 2A,C**): the aspartate synthesis module (module A), which included *AAT2*; the homoserine synthesis module (module H), which included *HOM3*, *HOM2*, and *HOM6*; and the threonine synthesis module (module T), which included *THR1*, *THR4*, and *ILV1*. While threonine and α-ketobutyrate (module T) are synthesized in the mitochondria in *S. cerevisiae*, the same may not be true in *Y. lipolytica*. While the locations of the relevant enzymes are as yet unknown in *Y. lipolytica*, predictive analyses (**Supplementary Table 4**) suggest enzyme location may differ between *S. cerevisiae* and *Y. lipolytica*. Because the module T enzymes were predicted to occur in the cytoplasm in *Y. lipolytica*, we used the original sequence of each gene in this study, as described in **Supplementary Table 3**. However, more research is needed to confirm enzyme locations in *Y. lipolytica*. The genes in each module were cloned into one plasmid using Golden Gate assembly (**Figure 2B**). They were expressed under the constitutive promoter p*TEF1*, and the expression cassette of each module was randomly integrated into the genome. Each module (A, T, and H) in the pathway was overexpressed in *Y. lipolytica* both individually and in tandem. The strain with the full modular pathway (ATH) was constructed by removing and reusing the *URA3* marker (**Supplementary Figure 2**). We verified gene integration using colony PCR with the primer set of promoters and the ORF gene (**Supplementary Table 1**).

The Engineered Strain Could Produce Odd-Chain Fatty Acids Using Glucose as Its Sole Carbon Source

To determine whether the modular metabolic pathway was effective in producing odd-chain FAs, we evaluated the performance of the engineered strains overexpressing the individual modules and the entire pathway. The strains



	Gene	*Y. lipolytica* gene name	Enzyme encoded by gene
Module A	*AAT2*	*YALI0B02178g*	Aspartate aminotransferase
Module H	*HOM3*	*YALI0D11704g*	Aspartate kinase
	HOM2	*YALI0D13596g*	Aspartic β-semialdehyde dehydrogenase
	HOM6	*YALI0D01089g*	Homoserine dehydrogenase
Module T	*THR1*	*YALI0F13453g*	Homoserine kinase
	THR4	*YALI0F23221g*	Threonine synthase
	ILV1	*YALI0D02497g*	Threonine deaminase

FIGURE 2 | The biosynthetic pathway for propionyl-CoA. **(A)** The metabolic pathway by which propionyl-CoA is synthesized from glucose. The genes overexpressed in this study are shown in blue. Pyruvate produced by glycolysis is converted to oxaloacetate by pyruvate carboxylase, and oxaloacetate is converted to threonine through the biosynthesis of aspartate and homoserine. Threonine deaminase subsequently generates α-ketobutyrate from threonine. Then, α-ketobutyrate is converted to propionyl-CoA by the pyruvate dehydrogenase (PDH) complex. **(B)** Structure of the multigene modules encoding enzymes in the aspartate/α-ketobutyrate pathway that were constructed in this study. Each gene expression cassette included the native *TEF1* promoter and *LIP2* terminator. **(C)** The genes included in the modules and the enzymes they encode.

were cultivated in YNBD6 medium under nitrogen limitation conditions (C/N = 60), which have been found to positively influence lipid synthesis (Beopoulos et al., 2012; Ledesma-Amaro et al., 2016). For the WT-A strain, which overexpressed *AAT2*, and the WT-T strain, which overexpressed *THR*1, *THR4*, and *ILV1*, total lipid content (%, g/g DCW) was lower than in the wild-type (WT) strain; the percentage of odd-chain FAs out of total FAs was similar (**Table 2**). For the WT-H strain, which overexpressed *HOM3*, *HOM2*, and *HOM6*, this percentage was slightly greater (1.91%) than that in the WT strain (0.84%) (**Table 2** and **Figure 3A**). For the WT-ATH strain, which overexpressed the entire pathway, odd-chain FA synthesis was significantly greater, odd-chain FA content (%, g/g DCW) was 3.8 times higher, and odd-chain FA titers (g/L) were 3.6 times higher than in the WT strain; the percentage of odd-chain FAs out of total FAs was 3.86%, which was 4.6 times higher than the value seen in the WT strain. These results indicate that the engineered aspartate/α-ketobutyrate pathway can supply propionyl-CoA; they also show that the full pathway is needed for effective odd-chain FA synthesis.

WT-ATH primarily produced C17:1 FAs (**Figure 3B**). This profile resembles that of an engineered *Y. lipolytica* strain that received propionate supplementation (Park et al., 2018). This finding implies that enhancing carbon flux through the α-ketobutyrate pathway can boost propionyl-CoA availability and odd-chain FA synthesis the same way that propionate supplementation can.

Odd-Chain Fatty Acid Production Was Significantly Improved in a Lipid-Accumulating Strain

Once we had determined that the strain overexpressing the full modular pathway produced more odd-chain FAs, we wanted to boost their accumulation. Previously, we had engineered a *Y. lipolytica* strain, JMY3501, to accumulate large amounts of lipids (Lazar et al., 2014). We eliminated lipid degradation and

TABLE 2 | Fatty acid (FA) production in the wild-type (WT) strain and the engineered strains after growth on YNBD6 medium for 120 h.

Strain	DCW (g/L)	Lipid content %		Odd-chain FA /Total FA (%)	Lipid titer (g/L)	
		Total FA	Odd-chain FA		Total FA	Odd-chain FA
WT	18.65 ± 0.15	19.13 ± 2.22	0.16 ± 0.00	0.84 ± 0.09	3.571 ± 0.442	0.029 ± 0.000
WT-A	17.30 ± 0.15	16.60 ± 1.05	0.14 ± 0.01	0.87 ± 0.03	2.873 ± 0.207	0.025 ± 0.003
WT-T	16.50 ± 0.00	14.41 ± 0.21	0.15 ± 0.00	1.03 ± 0.01	2.378 ± 0.035	0.024 ± 0.001
WT-H	16.30 ± 0.55	10.96 ± 0.09	0.21 ± 0.03	1.91 ± 0.21	1.788 ± 0.075	0.034 ± 0.005
WT-ATH	16.90 ± 0.25	15.73 ± 0.95	0.61 ± 0.13	3.86 ± 0.57	2.656 ± 0.121	0.103 ± 0.020

The values represent the means and standard deviations for two independent experiments. DCW, dry cell weight.

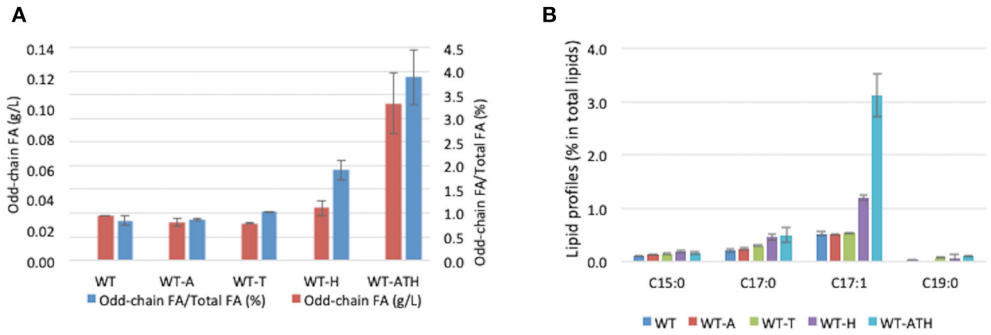


FIGURE 3 | Odd-chain fatty acid production and profiles for the wild-type and engineered strains. **(A)** Odd-chain fatty acid (FA) titers (g/L) and the percentage of odd-chain FAs relative to total FAs in the wild-type (WT) and engineered strains. **(B)** Odd-chain FA profiles (percentage of each FA out of total FAs) of the WT strain and engineered strains. WT, control strain; WT-A, strain expressing module A; WT-T, strain expressing module T; WT-H, strain expressing module H; WT-ATH, strain expressing the full pathway (all three modules). The results represent the means and standard deviations for two independent experiments.

remobilization in this obese strain by deleting the *POX* (*POX1-6*) genes (Beopoulos et al., 2008) as well as the *TGL4* gene, which encodes a triglyceride lipase (Dulermo et al., 2013). In addition, we overexpressed *DGA2*, which encodes the major acyl-CoA:diacylglycerol acyltransferase (Beopoulos et al., 2012), and *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, in order to push and pull TAG biosynthesis (Dulermo and Nicaud, 2011; Tai and Stephanopoulos, 2013). Therefore, using JMY3501, we built a new obese strain that overexpressed our full modular pathway. It was called the obese-ATH strain. We then studied lipid production under the same conditions as before. As expected, total lipid accumulation was 2.29-fold greater in the obese-ATH strain than in the WT-ATH strain (**Tables 2**, **3**). Interestingly, the obese-ATH strain also accumulated more odd-chain FAs: the percentage of odd-chain FAs out of total FAs was 5.64% in the obese-ATH strain vs. 3.86% in the WT-ATH strain. The obese-ATH strain produced 0.36 g/L of odd-chain FAs, which is 7.2 times greater than the amount produced by the regular obese strain. The obese-ATH strain and the WT-ATH strain differed in their even-chain FA profiles (**Table 4**). The obese-ATH had slightly higher levels of C16:0 and slightly lower levels of C18:1, a common pattern seen in strains with the obese background regardless of the carbon source (Lazar et al., 2014; Ledesma-Amaro et al., 2016).

The Disruption of *PHD1* in the Methylcitrate Cycle Did Not Increase Odd-Chain Fatty Acid Production

In a previous study, we inactivated *PHD1*, the gene that encodes 2-methylcitrate dehydratase, which catalyzes the conversion of 2-methyl citrate to 2-methyl-*cis*-aconitate in the methylcitrate cycle; we showed that the resulting higher levels of propionyl-CoA could be used to synthesize greater amounts of odd-chain FAs (Park et al., 2018). To investigate whether the inhibition of the methylcitrate cycle—via the deletion of *PHD1*—could further improve the accumulation of odd-chain FAs, we disrupted the *PHD1* gene in both the WT-ATH strain and the obese-ATH strain. The two *phd1*Δ strains displayed higher total lipid content compared to their relative controls (**Supplementary Table 2**), a result that was also demonstrated in Papanikolaou et al. (2013); however, they also displayed lower percentages of odd-chain FAs out of total FAs (**Figure 4**). This latter negative effect was significantly more pronounced in the obese-ATH *phd1*Δ strain than in the WT-ATH *phd1*Δ strain. For the obese-ATH *phd1*Δ strain, this figure dropped by 50%, and levels of odd-chain FAs were 67% of those seen in the relative control (0.24 vs. 0.36 g/L). These results suggest that disrupting the methylcitrate cycle does not provide the benefits seen previously (Park et al., 2018) when strains are already overexpressing the aspartate/α-ketobutyrate pathway.

TABLE 3 | Fatty acid (FA) production in the obese strain and the obese-ATH strain after growth on YNBD6 medium for 120 h.

Strain	DCW (g/L)	Lipid content %		Odd-chain FA /Total FA (%)	Lipid titer (g/L)	
		Total FA	Odd-chain FA		Total FA	Odd-chain FA
Obese	19.20 ± 0.04	37.11 ± 0.14	0.25 ± 0.00	0.68 ± 0.01	7.125 ± 0.012	0.049 ± 0.001
Obese-ATH	17.62 ± 0.03	36.02 ± 0.39	2.03 ± 0.05	5.64 ± 0.06	6.347 ± 0.058	0.358 ± 0.009

The values represent the means and standard deviations for two independent experiments. DCW, dry cell weight.

TABLE 4 | Comparison of the lipid profiles (% of each FA) of the WT-ATH strain and the obese-ATH strain.

Strain	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C19:0
WT-ATH	0.16 ± 0.03	8.31 ± 0.75	6.13 ± 0.00	0.50 ± 0.14	3.11 ± 0.40	5.96 ± 0.68	61.31 ± 2.43	11.02 ± 0.26	0.10 ± 0.00
Obese-ATH	0.40 ± 0.00	13.07 ± 0.01	7.25 ± 0.11	0.76 ± 0.00	4.26 ± 0.06	5.00 ± 0.17	50.12 ± 0.27	14.11 ± 0.16	0.22 ± 0.13

The values represent the means and standard deviations for two independent experiments.

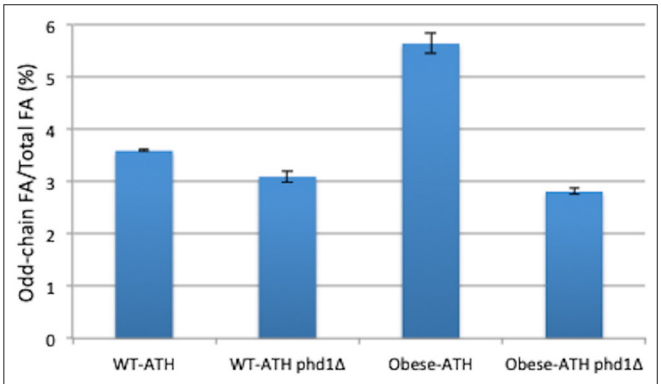


FIGURE 4 | Odd-chain fatty acid production in the *PHD1*-disrupted strains. The percentage of odd-chain fatty acids (FAs) relative to total FAs in the *PHD1*-disrupted strains and their respective controls. The results represent the means and standard deviations for two independent experiments.

The action of 2-methylcitrate dehydratase is restricted to the mitochondria. Consequently, disabling this enzyme does not directly improve cytosolic levels of propionyl-CoA. In the previous studies showing that the disruption of *PHD1* boosted odd-chain FA synthesis (Papanikolaou et al., 2013; Park et al., 2018), there was also propionate supplementation. Taken together, these findings suggest that, when the methylcitrate cycle is disrupted, the overexpression of the aspartate/α-ketobutyrate pathway has a weaker effect on propionyl-CoA levels than does propionate supplementation.

Overexpression of the Cytosolic Pyruvate Dehydrogenase (PDH) Complex Improved Lipid Synthesis but Reduced Odd-Chain Fatty Acid Production

The PDH complex consists of three main catalytic components: E1 (pyruvate dehydrogenase, encoded by *PDA1* and *PDB1*), E2 (dihydrolipoamide acetyltransferase, encoded by *LAT1*), and E3 (dihydrolipoamide dehydrogenase, encoded by *LPD1*). There is a fourth component (protein X, encoded by *PDX1*) that binds to and positions E3 relative to E2. In *Y. lipolytica*, the PDH complex is found in mitochondria and catalyzes the pathway from pyruvate to acetyl-CoA. A few studies have examined the functional expression of the PDH complex in *Y. lipolytica*. One study attempted to overexpress the direct pathway from pyruvate to acetyl-CoA in a coordinated manner (Markham et al., 2018), and another study showed that the individual overexpression of *PDA1* (shared subunits with α-ketoglutarate dehydrogenase) improved α-ketoglutarate production (Guo et al., 2014).

Here, we wanted to redirect the aspartate/α-ketobutyrate pathway to produce propionyl-CoA via the PDH complex, which has already been shown to be possible in *E. coli* (Danchin et al., 1984; Tseng and Prather, 2012; Lee et al., 2013). The PDH complex was built so it could be associated with the full modular pathway (ATH) and could provide a pool of propionyl-CoA in the cytosol where lipid synthesis takes place. The mitochondrial targeting sequences (MTSs) of each gene were predicted using MitoProt (Claros and Vincens, 1996), and the gene sequences used in this study are described in **Supplementary Table 3**. We then created the obese-ATHP strain by overexpressing the cytosolic PDH subunits in the obese-ATH strain. Next, to explore lipid accumulation dynamics, the strain was grown in YNBD6 with lipoic acid, which is required for cytosolic PDH activity in yeast (Kozak et al., 2014). Compared to the control strain, the obese-ATHP strain had significantly lower levels of odd-chain FAs (a 3.8 times lower percentage of odd-chain FAs out of total FAs); however, total lipid levels were higher (**Table 5**). The increase in lipid production might have resulted from the increased levels of cytosolic acetyl-CoA resulting from the overexpression of the PDH complex. A similar strategy was utilized in *Saccharomyces cerevisiae*, where PDH was introduced into the cytosol and was found to increase levels of acetyl-CoA (Kozak et al., 2014; Lian et al., 2014) and of the target compound of interest. Since acetyl-CoA is a key precursor in the production of both even- and odd-chain FAs, increasing levels of acetyl-CoA promotes lipid synthesis in general. However, the substantially lower levels of odd-chain FAs in the obese-ATHP strain implies that the PDH complex shows greater specificity for pyruvate than for α-ketobutyrate. The higher Km value of α-ketobutyrate

TABLE 5 | Fatty acid (FA) production in the obese-ATH strain and the obese-ATHP strain after growth on YNBD6 medium for 120 h.

Strain	DCW (g/L)	Lipid content %		Odd-chain FA /Total FA (%)	Lipid titer (g/L)	
		Total FA	Odd-chain FA		Total FA	Odd-chain FA
Obese-ATH	15.68 ± 0.02	29.23 ± 0.01	1.59 ± 0.03	5.44 ± 0.10	4.582 ± 0.010	0.249 ± 0.005
Obese-ATHP	15.25 ± 0.25	33.89 ± 1.06	0.48 ± 0.03	1.42 ± 0.03	5.171 ± 0.246	0.073 ± 0.005

The values represent the means and standard deviations for two independent experiments. DCW, dry cell weight.

compared to that of pyruvate has been seen elsewhere, such as in *E. coli* (Bisswanger, 1981), *Neurospora crassa* (Harding et al., 1970), and mammalian cells (Bremer, 1969). Therefore, it is important to explore enzyme engineering strategies that modify substrate specificity or that introduce other enzymes that can convert acetyl-CoA to propionyl-CoA with a view to further improving odd-chain FA production via a threonine-based upregulation strategy.

DISCUSSION

In this study, we developed a synthetic biological strategy for the *de novo* production of odd-chain FAs in *Y. lipolytica*. It is important to note that the wild-type *Y. lipolytica* strain produces only negligible amounts of odd-chain FAs even though it has an excellent capacity to accumulate large quantities of lipids. Several studies have shown that propionate supplementation can increase the production of odd-chain FAs (Fontanille et al., 2012; Kolouchová et al., 2015; Park et al., 2018). However, research has yet to explore the *de novo* production of odd-chain FAs from sugars in *Y. lipolytica*.

The overexpression of the aspartate/α-ketobutyrate pathway (from oxaloacetate to homoserine and threonine) via Golden Gate assembly resulted in higher levels of odd-chain FAs being produced from glucose. The best strain generated a level of odd-chain FAs, 0.36 g/L in flask that is the highest to date to be achieved in *Y. lipolytica* without propionate supplementation. Furthermore, it is comparable to the levels seen in a previous study of ours that employed propionate supplementation, where odd-chain FAs titers were 0.14 and 0.57 g/L in the wild-type strain and the obese strain, respectively (Park et al., 2018). To further increase the amount of propionyl-CoA produced via the overexpression of threonine synthesis, we constructed a cytosolic pyruvate dehydrogenase (PDH) complex. Because of the lower specificity of the PDH complex for α-ketobutyrate vs. pyruvate, the engineered strain generated lower levels of propionyl-CoA than did the relative control; however, the increased levels of acetyl-CoA in the engineered strain led to larger amounts of total FAs. This study is the first to describe the functional expression of the native PDH complex in the cytosol in *Y. lipolytica*, an approach that could also be employed to produce acetyl-CoA-derived compounds, such as polyhydroxybutyrates, isoprenoids, sterols, polyketides, polyphenols, alkanes, and alkenes (Nielsen, 2014).

Before the approach described here can be applied at industrial scales, certain issues must be resolved. It is necessary to perform research in which combinatorial pathway analysis (Lütke-Eversloh and Stephanopoulos, 2008), targeted-proteomics analysis (Redding-Johanson et al., 2011), and genome-scale metabolic network modeling (Xu et al., 2011) are used to identify bottlenecks and enhance metabolic fluxes to propionyl-CoA and odd-chain FAs. The results of the PDH overexpression experiment suggest that acetyl-CoA is a competitive precursor to propionyl-CoA in odd-chain FA synthesis and is also a precursor in lipid synthesis. Therefore, better balancing the pools of acetyl-CoA and propionyl-CoA could be key in further increasing odd-chain FA content. One way to improve odd-chain FA synthesis is to introduce enzymes—such as CoA transferase—that redirect CoA moieties from acetyl-CoA to propionyl-CoA (Yang et al., 2012) or that have greater specificity for 3-oxovaleryl-ACP than for acetoacetyl-CoA (Slater et al., 1998). Overall, this work has shown that applying synthetic biological engineering strategies in *Y. lipolytica* to improve odd-chain FA production could be useful in a wide range of pharmaceutical and industrial contexts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YP, RL-A, and J-MN planned the study. YP designed and carried out the experiments and drafted the manuscript. All the authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00484/full#supplementary-material

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université PARIS-SACLAY

Ecole doctorale n°581
Agriculture, alimentation, biologie, environnement et santé (ABIES)

Titre : Ingénierie métabolique de la levure *Yarrowia lipolytica* pour la production d'acides gras pairs et impairs

Mots clés : *Yarrowia lipolytica*, métabolisme des lipides, Acide gras, Acide gras à chaîne impair (AGI), Ingénierie métabolique, Biologie synthétique

Résumé : Les huiles microbiennes sont considérées comme des alternatives prometteuses aux combustibles fossiles qui suscitent de plus en plus de préoccupations environnementales et énergétiques. Les acides gras à chaîne impaires (AGI), un type de lipide inhabituel, sont des composés d'intérêt ayant diverses applications biotechnologiques. L'objectif de cette thèse est de développer *Yarrowia lipolytica* comme souche plateforme, par l'ingénierie métabolique, pour la production d'AGI.

Pour développer *Y. lipolytca*, l'identification et la caractérisation d'une nouvelle série de promoteurs érythritol-hybrides-inductible (pEYK1, pEYD1, et dérivés) ont été réalisées. La série de promoteurs hybrides a montré des forces variables, l'induction à base d'érythritol a augmenté de 2,2 à 32,3 fois dans la souche WT et de 2,9 à 896,1 fois dans la souche *eyk1*Δ. Ces promoteurs amélioreront la capacité de moduler l'expression de gènes chez *Y. lipolytica*.

Pour la production d'AGI, la tolérance au propionate a été étudiée. Deux gènes, *RTS1* et *MFS1*, améliorant la résistance au propionate ont été identifiés par le criblage d'une banque d'ADN génomique. Par des stratégies d'ingénierie métabolique, comme l'inhibition de la voie compétitive, l'augmentation des pools de précurseurs, et l'amélioration de l'accumulation de lipides totale, la production d'AGI a été augmentée de 0,14 g/L à 1,87 g/L. La production *de novo* des AGI sans supplémentation de propionate a également été explorée. Par surexpression des gènes dans la voie de synthèse de la thréonine, la production d'AGI été augmentée de 12 fois par rapport à la souche sauvage (0,36 *versus* 0,03 g/L).

En résumé, des souches de *Y. lipolytica* ont été développées pour produire efficacement des AGI, principalement l'acide heptadécénoïque. Ce travail ouvre la voie à la production microbienne d'AGI et de ses dérivés à plus grande échelle.

Title : Metabolic engineering of the yeast *Yarrowia lipolytica* for the production of even- and odd-chain fatty acids

Keywords : *Yarrowia lipolytica*, Lipid metabolism, Fatty acid, Odd-chain fatty acid, Metabolic engineering, Synthetic biology

Abstract : Microbial oils are regarded as promising alternatives to fossil fuels with growing environmental and energy concerns. Odd-chain fatty acids (OCFAs), a type of unusual lipids, are value-added compounds with various biotechnological applications. The objective of the thesis was to develop *Yarrowia lipolytica* as a platform strain for the production of OCFAs by metabolic engineering.

For developing *Y. lipolytica*, the identification and characterization of a new series of erythritol-hybrid-inducible promoters (pEYK1, pEYD1, and derivatives) were explored. The hybrid promoter series showed variable strengths, erythritol-based induction increased 2.2 to 32.3 times in the WT strain and 2.9 to 896.1 times in the *eyk1Δ s*train, which will improve the modulation of gene expression for metabolic engineering of *Y. lipolytica*.

For OCFA production, tolerance to propionate was studied. Two genes, *RTS1* and *MFS1*, were identified as propionate-tolerant genes by screening a genomic DNA library. Through metabolic engineering strategies, such as inhibiting competitive pathways, increasing precursor pools, and enhancement of total lipid accumulation, OCFA production was increased from 0.14 g/L to 1.87 g/L. *De novo* production of OCFAs without propionate supplementation was also explored by overexpression of the threonine synthesis pathway. OCFA production was increased by 12-times; 0.36 *versus* 0.03 g/L for WT.

In summary, *Y. lipolytica* strains were developed to produce high-amount of OCFAs, mainly heptadecenoic acid. This work paves the way for the microbial production of OCFAs and their derivatives at the industrial scale.

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