Extractive bioconversion of 3-hydroxypropionic acid : limiting mechanisms and integrated process optimisation

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Publications and communications

Article publications in an international peer-reviewed journal

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[1] These authors contributed equally

Patent

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Nomenclature

Molecules	Significance
1,3-PDO	1,3-propanediol
2-HP	2-hydroxypropinic acid
3-HP	3-hydroxypropinic acid
3-HPA	3-hydroxypropionaldehyde
cFDA	Carboxyfluorescein diacetate
DDMA	N,N-di-dodecyl-methylamine
DOA	di-n-octylamine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISPR	*In situ* or in stream product recovery
MCR	Malonyl-CoA reductase
MSA	Malonate semialdehyde
OA	n-octylamnine
PI	Propidium iodide
PLA	Polylactic acid
R3N	Tertiary amine
TBP	Tributyl phosphate
TCA	Trichloroacetic acid
TMA	Trimethylamine
TOA	Tri-n-octylamine
TOPO	Tri-octyl-phosphine oxide
VFAs	Volatil fatty acids

Other abbreviations	Significance
AAB	Acetic acid bacteria
CDW	Cell dry weight
D	Diffusion model
DW	Dry weight
F	Freundlich isotherm
GC	Global convection model
GRAS	Generally recognised as safe
HPLC	High performance liquid chromatography
HFMC	Hollow fibre membrane contactor
IFT	Interfacial tension
LAB	Lactic acid bacteria
L	Langmuir isotherm
LF	Langmuir-Freundlich isotherm
LC	Local convection model
OD	Optical density
PTFE	Polytetrafluoroethylene
RO	Reverse osmosis
TCA	Tricarboxylic acids

Symbols	Significance	Units
A^-	Dissociated acid in the aqueous phase	kmol m^{-3}
AH	Non-dissociated acid in the aqueous phase	kmol m^{-3}
AH_{org}	Non-dissociated acid in the organic phase	kmol m^{-3}
C	Molar concentration of a generic chemical species	kmol m^{-3}
CPX	Acid-amine complex in the organic phase	kmol m^{-3}
OH^-	Hydroxide ion	kmol m^{-3}
H_3O^+	Hydronium ion	kmol m^{-3}
D	Diffusion coefficient	m^2 s^{-1}
EMR	Excess mortality rate	h^{-1}
IFT	Interfacial tension	N m^{-1}
J	Mass flux	mol s^{-1}
k	Kinetic rate constant	$kmol^{-n}$ m^{3n} s^{-1}
K	Equilibrium constant	$kmol^{-n}$ m^{3n}
m	3-HP partition coefficient in decanol	-
M	Molar mass	kg $kmol^{-1}$
n	Number of reacting molecules in Freundlich isotherm	-
n	Unit normal vector	1
N_g	Cell generation number	-
p	Pressure field	Pa
ΔP_C	Critical pressure difference	Pa
$q_{3\text{-HP}}$	Specific 3-HP productivity	$g_{3\text{-HP}}$ g_{DW}^{-1} h^{-1}
r_1, r_4	Volume reaction rates	kmol m^{-3} s^{-1}
r_2, r_3	Surface reaction rates	kmol m^{-2} s^{-1}
$r_{3\text{-HP}}$	3-HP productivity	$g_{3\text{-HP}}$ L^{-1} h^{-1}
R	Radius, radial position	M
R_g	Ideal gas constant	J $kmol^{-1}$ K^{-1}
S_{ad}	Free sites for acid-amine complex adsorption at the interface	kmol m^{-2}
S	Surface for mass transfer	m^2
t	Time	S
T	Absolute temperature	K
TOA	Tri-n-octylamine in the organic phase	kmol m^{-3}
u	Velocity field	m s^{-1}
v	Molar volume at the normal boiling temperature	dm^3 $kmol^{-1}$
z	Vertical position	m

Greek letters	Significance	Units
γ	Interfacial tension between the aqueous and the organic phase	N m^{-1}
Γ	Excess surface concentration of the acid-amine complex	kmol m^{-2}
θ	Contact angle	°
ϕ	Association coefficient	-
μ	Dynamic viscosity	Pa s
μ_{max}	Maximum specific microbial growth rate	h^{-1}
φ	volumetric fraction of the active diluent	-

Subscripts	Significance
aq	Aqueous phase
d	Droplet
i	Interface
n	Needle
org	Organic phase
out	Outer boundary of the modelling region
S	Subsurface concentration

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Introduction

Fossil resources are nowadays the main raw material for chemical and energy industries, which in turn have come to play a dominant role in the global economic activity. Although great technological advances have been achieved thanks to these non-renewable supplies, they have worrisome disadvantages like unavoidable depletion and negative effects to the environment. There is a special worry about their greenhouse gases (GHG) emissions, because of their contribution to global warming (Cherubini, 2010). All this have led to a high volatility of the oil price, which have direct impact on the global economic activity (van Eyden *et al.*, 2019). This has awakened global concern to diminish our dependence on fossil resources.

Chemical industry has made an invaluable contribution to the current quality of life, but is one of the sectors under most pressure to reduce its excessive reliance on fossil resources (Gavrilescu and Chisti, 2005). Petrochemistry is the largest consumer of oil and gas, with 14% and 8% respectively of total primary demand and it is responsible for the 18% of CO_2 emissions from the industrial sector, and 5% of total combustion-related CO_2 emissions (International Energy Agency, 2018). Among the actions needed to tackle this problem, we have the challenge of turning to renewable resources exploitation, such as biomass, while meeting the ever increasing demand of goods and services. These concerns have been the driving force for the development of a wide range of technologies for disassembling biomass resources into their building blocks, and use them instead of fossil resources to produce biofuels and chemicals. In this context, concepts such as Green Chemistry and Biorefinery have emerged as a promising measure to confront these challenges (Anastas and Eghbali, 2010; Cherubini, 2010). Governments have encouraged the transition to a bio-based economy since the beginning of the 1990s with different public policies that have provided well defined guidelines for sustainable designs, *e.g.* the Paris Agreement (United Nations, 2015), and the European Bioeconomy Action Plan 2018 – 2030 (European Commission, 2018). Leading economies like the European Union (EU) are technologically poised to achieve ambitious objectives on bioeconomy development such as getting the 30% of chemicals to be produced from biomass by the year 2030 (Bio-based Industries Consortium, 2012), starting from 6.8% in 2015 (Piotrowski *et al.*, 2016). In terms of environmental impact, the EU objective is to reduce GHG emissions by at least 40% below 1990 levels, in order to limit global warming below 2°C above pre-industrial levels (Ohliger, 2019).

There has been a particular growth in biofuels industry since some Life Cycle Assessment (LCA) studies have found a net reduction in GHG emissions when bioethanol and/or biodiesel are used to replace conventional diesel and gasoline (ADEME, 2010; Cherubini, 2010). It generates a significant quantity of residues and by-products that could be used to give added value to biorefineries. As an initiative to support the development of integrated processing facilities to include the valorisation of these residues and by-products, the U.S. Department of Energy (DoE) published, in 2004 and 2010, a list of promising chemicals derived from biomass that could serve as an economic driver for a biorefinery (Bozell and

Petersen, 2010; Werpy and Petersen, 2004). Research from both the private initiative and scientific community have been oriented by these reports, and there has been remarkable growth on the industrial market for several compounds mentioned in the list. One case of success is succinic acid biorefineries, which are now economically mature and produce between 13 600 and 20 000 t per year of bio-based succinic acid (Ghayur *et al.*, 2019). In fact, this compound belongs to an attracting type of chemicals, the carboxylic acids, mainly used in food, pharmaceutical and chemical fields. Until recently, this type of components were almost exclusively produced by petrochemical means (Zacharof and Lovitt, 2013) and have a wide range of applications, making it a key group that represents the majority of the compounds in the original DoE list (Werpy and Petersen, 2004). However, more efforts are still needed to produce these compounds at an industrial level.

Plastics industry is a sector that has generated remarkable environmental concern in the past few decades. The great versatility and convenient properties of these synthetic polymers has made them indispensable materials used by practically every industry in the world. However, 90% of these products are used just once and then discarded, being a worrying source of pollution that can take hundreds of years to degrade ("The future of plastic," 2018). Bio-based polymers production could be a key measure to alleviate this problem. One success case is polylactic acid (PLA), which is the most widely used compound for polymers fabrication thanks to its unique properties like good processability, biocompatibility and biodegradability (Luckachan and Pillai, 2011). Examples like this have motivated researchers to look for other platform molecules, in order to produce bio-based polymers with diversified properties.

3-Hydroxypropionic acid (3-HP) is a platform molecule from the original DoE list (Werpy and Petersen, 2004) that has attracted attention from chemical industry. It is an positional isomer of lactic acid (2-HP) and it can also be used for bio-based polymers fabrication (Sabet-Azad, 2015), although at the moment its most attractive valorisation line is the production of bio-based acrylic acid. Its production by microbial processes has made remarkable advances in the last few years, but its industrial commercialisation is still limited by low productivities caused by product inhibition (Gopal Ramakrishnan *et al.*, 2015; Vidra and Németh, 2017; Zhao *et al.*, 2015). Several microorganisms capable of producing 3-HP have been discovered and studied. Further understanding of their metabolic pathways have given valuable insights for 3-HP production processes design and the development of more efficient microbial agents through genetic engineering (de Fouchécour, 2019). Better 3-HP production performances are being achieved within time, getting close to those necessary for industrial-scale commercialisation.

Product recovery and purification is a very important aspect to consider for commercialisation of a chemical product. Downstream processes contribution to the total cost of the final product are comprised between the 20 and 60% (Hoppe *et al.*, 2015; Straathof, 2011). Moreover, 3-HP recovery from bioconversion media at high concentration and purity is particularly challenging. This acid has a highly hydrophilic nature, has tendency to form oligomers at high concentrations and decomposes at high temperatures (Hoppe *et al.*, 2015). These characteristics make traditional downstream

processes to be less efficient and more costly than for other carboxylic acids with industrial commercialisation, *e.g.* lactic acid and citric acid. In addition, the problem of end-product inhibition needs to be alleviated and a promising approach is the continuous removal of the acid from the bioconversion medium, also known as *in situ* product recovery (ISPR) (López-Garzón and Straathof, 2014). Among the different methods for ISPR, reactive liquid-liquid extraction has been widely studied for carboxylic acids recovery from aqueous media. An organic phase that contains a molecule able to react with the acid is used to form an acid-amine complex that is soluble in the organic phase (Tamada *et al.*, 1990). In addition, devices known as hollow fibre membrane contactors (HFMC) can be used to put both liquid phases in contact, avoiding their mutual dispersion. Simultaneous acid extraction and back-extraction can be achieved by using two HFMC modules: both modules are fed with the same organic phase which is put in contact with the acid to be extracted and with a stripping aqueous phase at the same time, permitting continuous acid removal, organic phase regeneration and to obtain a second aqueous phase enriched with the recovered acid (Gabelman and Hwang, 1999). This is also known as reactive pertraction (Schlosser *et al.*, 2005). Despite its many advantages, this strategy has not been successfully implemented yet for 3-HP ISPR. However, it has been implemented for its positional isomer, lactic acid (2-hydroxypropionic acid) (Chen and Lee, 1997).

Within this framework, the joint research unit for Microbiology and Food Process Engineering (UMR 782 GMPA, AgroParisTech, INRA, Thiverval-Grignon, France), has set its eye on 3-HP. In 2012, a project started in collaboration with the research and development unit for Industrial Agro-Biotechnologies (URD ABI, AgroParisTech, Pomacle, France) to develop an integrated process for 3-HP microbial production and simultaneous recovery from the bioconversion medium. Valuable advances have been made, starting from glycerol bioconversion into 3-HP by the strain *Lactobacillus reuteri* DSM 17938 (Burgé *et al.*, 2015b), at bioreactor level. An ISPR approach consisting of reactive extraction assisted by a hollow fibres membrane contactor (HFMC) was developed, which permits selective and continuous 3-HP removal from model aqueous phase (Burgé *et al.*, 2016; Moussa *et al.*, 2016). Chemical equilibrium and mass transfer mechanisms during 3-HP reactive extraction have been meticulously studied, resulting in a predictive mathematical model that could be used as an optimisation tool (Chemarin, 2017; Chemarin *et al.*, 2017b, 2017a, 2019b). However, the first attempts to couple bioconversion with reactive extraction revealed some challenges that need to be overcome in order to demonstrate the feasibility of the integrated process:

i) the organic phase used for reactive extraction presented a high toxicity towards *L. reuteri* DSM 17938

ii) 1,3-propanediol (1,3-PDO) is an obligate by-product from glycerol bioconversion, limiting the final yield to a maximum of 0.5

iii) bioconversion optimal pH is higher than the pKa of 3-HP (4.51 (Haynes *et al.*, 2017)), limiting the extraction performance.

Furthermore, better understanding of the physicochemical mechanisms of 3-HP reactive extraction from bioconversion media is necessary to improve the previously developed

mathematical model and used it as a simulation tool to explore further optimisation strategies for the integrated process.

In 2016, three PhD theses were launched for further development of an integrated process for bio-based 3-HP production. Such a process can integrate three different unitary operations: i) glycerol bioconversion into 3-HP and 1,3-PDO by *L. reuteri* DSM 17938, ii) 1,3-PDO oxidation into 3-HP by the acetic acid bacterial strain *Acetobacter* sp. CIP 58.66, and iii) continuous 3-HP recovery from the bioconversion medium by membrane assisted reactive extraction in a HFMC. The present PhD work focuses on this last challenge.

The general objective is to demonstrate the feasibility of an integrated process including 3-HP reactive extraction assisted by membrane contactors, that could be directly coupled to bioconversion and achieve continuous 3-HP removal. To this end, the following specific objectives were set out:

- ✓ To perform a model-based analysis of 3-HP reactive extraction mechanisms at the liquid-liquid interface, through observation of the dynamic interfacial tension during the acid-base complex formation and mass transfer.
- ✓ To optimise an organic phase composition for 3-HP reactive extraction, in terms of extraction performance and biocompatibility with the 3-HP producing strain *Lactobacillus reuteri* DSM 17938.
- ✓ To evaluate the experimental feasibility of extractive bioconversion with two different 3-HP producing strains: *Lactobacillus reuteri* DSM 17938 and *Acetobacter* sp. CIP 58.66, using reactive pertraction for ISPR.
- ✓ To identify the limiting mechanisms for implementation of extractive bioconversion using reactive pertraction, and to explore further optimisation strategies using a predictive mathematical model as a simulation tool.

This manuscript is divided into **5 chapters**. It starts with a Literature review in **Chapter 1** describing the production and recovery of some bio-based carboxylic acids that are produced at significant scale in chemical industry. The following section highlights the potential of 3-HP as a promising platform molecule. Advances in 3-HP microbial production are also reviewed in this chapter, with discussion focussed on the challenges to overcome in order to achieve industrial-scale production. Downstream processes are also described as a key step for an economically viable production and reactive pertraction is highlighted as a promising technique for 3-HP recovery from bioconversion media. The chapter ends with some examples of successful extractive fermentations of carboxylic acids and the strategies that could be applied for the case of 3-HP. Then, a description of the overall experimental approach is given in **Chapter 2**. The methodology followed to achieve the different specific objective and the results obtained are presented in Chapters 3, 4 and 5. First, **Chapter 3** describes the model-based analysis of 3-HP reactive extraction mechanisms through observation of the dynamic interfacial tension. The insights obtained about mass transfer near the interface are presented. **Chapter 4** illustrates the strategy followed for the selection of an organic phase composition that displays a compromise between a good extraction performance and biocompatibility towards the strain

Lactobacillus reuteri DSM 17938. Insights obtained about the impact of reactive extraction assisted by a HFMC on the physiological state and bioconversion ability of cells are also detailed. The experimental demonstration of extractive bioconversion with the strains *L. reuteri* DSM 17938 and *Acetobacter* sp. CIP 58.66, using the selected organic phase, is described in **Chapter 5**. This chapter ends with the identification of the limitation mechanisms of the process and a preliminary exploration of further optimisation strategies. To this end, a predictive mathematical model was used as a simulation tool. Finally, a general conclusion integrates the main results and remarks obtained from this PhD work, together with perspectives for further research.

Chapter 1
Literature review

This chapter aims to describe the current knowledge of bio-based carboxylic acids production and recovery, in order to provide a context for 3-hydroxypropionic acid (3-HP) production by biochemical processes. Focus is given to downstream processes, notably to *in situ* product recovery (ISPR), and their integration to fermentation/bioconversion processes. Membrane-based reactive liquid-liquid extraction is described as a promising recovery method, according to 3-HP physicochemical properties. The limiting mechanisms of reactive extraction used in this configuration are identified and discussed, as well as the main hurdles to address for extraction process integration with bioconversion.

1.1. Bio-based carboxylic acids industrial production and recovery

Carboxylic acids are low molecular weight organic acids that contain one or more carboxyl (-COOH) groups. The molecules included in this group are very versatile, presenting diverse properties that depend of their carbon-chain length, molecular configuration, and the presence of other functional groups. This has conferred them numerous applications in food, pharmaceutical, cosmetic and chemical industry. Their production at industrial scale is currently divided into chemical synthesis from petrochemical feed stocks, and biotechnological processes from carbohydrates. Currently, most of the industrial production of carboxylic acids is made by chemical synthesis, except for some carboxylic acids such as citric, gluconic and itaconic acid, which are produced solely by microbial processes (Yang *et al.*, 2007). This reflects the fact that generally conventional microbial processes are not economically competitive compared with petrochemical processes. The overall cost of microbial production is dominated by feedstock cost and downstream processing. In particular, downstream processes contribution represents between 20 and 60% of the selling price (Djas and Henczka, 2018; Straathof, 2011).

The increasing interest in a bio-based economy has generated favourable conditions for cost reduction of biomass as raw material. Although some traditional microbial processes require relatively expensive feedstocks – like pure glucose for bioethanol production and other desired materials (*i.e.* biopolymers), or vegetable oils for biodiesel production (Pott *et al.*, 2018) – the recent growth of biorefineries has led to the generation of large quantities of co-products and waste streams that could be further valorised through other bioprocesses. One example is glycerol generation as co-product of biodiesel manufacture, which could be used for carboxylic acids production. The US DoE had identified this compound as a promising building block, predicting its cost reduction thanks to the increased use of biodiesel. Indeed, the price of crude glycerol did fall from USD $1.30 – 1.90/kg in 2004 to USD $0.10 – 0.40/kg in 2018 (Oleoline®, 2018; Werpy and Petersen, 2004). At present, glycerol has become an abundant carbon source, especially for industrial microbiology, since it can replace feedstocks directly connected to human food supply. Among others processes for glycerol valorisation, research for carboxylic acids production processes have

been triggered. Such are the cases of citric acid, lactic acid and 3-hydroxypropionic acid (Russmayer *et al.*, 2019). Another way of producing carboxylic acids from low cost feedstock is the use of waste streams to obtain volatile fatty acids (VFAs) through microbial processes (Strazzera *et al.*, 2018) or direct recovery (Kumar and Babu, 2008).

In addition to being more eco-friendly, microbial production have technical advantages over chemical synthesis like working under mild conditions and obtaining a pure isomer of the carboxylic acid. This is very important for targeted applications and further processing purposes, such as biodegradable polymers manufacture. For example, polylactic acid (PLA) is produced using optically pure L-lactic acid and had market value of USD $1.2 billion in 2018 (Grand View Research, 2019). Therefore, research efforts have been focussed on alleviating the main disadvantages of these processes, such as low productivity and final product concentration that limit their industrial application. Ironically, there is a long history of using carboxylic acids for food preservation, since one of their functionalities is inhibiting microbial activity. End product inhibition represents the main challenge to achieve carboxylic acids microbial production with good enough performances to conceive an economically viable process (Jarboe *et al.*, 2013). The approaches that have been developed in order to tackle this problem are the traditional addition of a neutralising agent, genetic engineering of the microbial strains to increase their resistance towards carboxylic acids (Liu and Jarboe, 2012), and *in situ* product recovery (ISPR), to maintain a low acid concentration in the bioconversion medium (López-Garzón and Straathof, 2014; Yang *et al.*, 2007).

There are some successful cases of bio-based carboxylic acids produced at industrial scale. The current compounds of the bio-based carboxylic acids market are acetic acid, formic acid, citric acid, lactic acid, succinic acid, and gluconic acid (P&S Intelligence, 2019). There are also some emerging compounds that are taking an important place on the market, *e.g.* itaconic acid (De Carvalho *et al.*, 2018) and adipic acid (Skoog *et al.*, 2018). Three examples of bio-based carboxylic acids industrial production are developed below. The first is citric acid, which is currently the most important bio-based acid on the market and one of the oldest obtained by biotechnology route at an industrial level. After, succinic acid industrial production is detailed as an interesting example of industrial production achieved thanks to recent technologies development. Lactic acid (2-hydroxypropionic acid) production is also described, paying particular attention to its nature as isomer of 3-hydroxypropionic acid, the molecule of interest of this PhD work. And finally, volatile fatty acids (VFAs) production from waste streams is described as a challenging example for carboxylic acids recovery.

1.1.1. Citric acid

1.1.1.1. Production and applications of bio-based citric acid

Citric acid has been the most widely used organic acid in the field of food and pharmaceuticals. It is principally used as acidulant, antioxidant and detergent, having also other current and potential uses as building-block for methacrylic acid, 1,5-pentanediol,

pyrrolidones and polyesters production (Hilbold and Schab, 2013). Fermentation is the most economical and widely used way of obtaining this carboxylic acid. *Aspergillus niger* is the main microorganism used for citric acid industrial production (Vandenberghe *et al.*, 1999) because it is easy to handle and it is able to ferment a broad range of low-cost raw materials, providing high yields. Among the raw materials used for citric acid production are beet molasses, black strap molasses, cane molasses, carob pod extract, n-paraffin, glycerol, corn starch, hydrolysate starch, yam bean starch, wood hemicellulose, olive oil, rapeseed oil, palm oil and soya bean oil (Soccol *et al.*, 2006). Moreover, this microorganism is able to grow at low pH (around 2.5-3.5), conditions where citric acid production is favoured, allowing high yields and titres, up to 100% and 113 g L^{-1} in submerged fermentation and 883 g kg^{-1} in solid-state fermentation (Soccol *et al.*, 2006). Moreover, pKa values of this tricarboxylic acid are 3.30, 4.76 and 6.40, meaning that low pH is a convenient condition for further acid recovery in its undissociated form by downstream processes (Bar and Gainer, 1987).

1.1.1.2. Recovery of bio-based citric acid

Recovery of citric acid is currently achieved by precipitation. This is the traditional method for acids recovery from fermentation broth, including citric acid. It requires the initial removal of the microbial cells and suspended particles by filtration or centrifugation. Then, calcium hydroxide (known as milk-lime) is added to the medium to form tricalcium citrate tetrahydrate, which has a very limited solubility in water. The resulted precipitate is then filtered and washed with water, to subsequent addition of sulphuric acid to recover a more purified solution of citric acid. Calcium sulphate (gypsum) is formed as by product from this step and it is separated by filtration. The citric acid solution is treated with active carbon and passed through cation and anion exchangers, then concentrated by evaporation in a vacuum at 40°C. Finally, crystals of citric acid monohydrate are formed in a vacuum crystallizer at 20 – 25°C and anhydrous citric acid is formed at crystallization temperatures above 36.5°C (Show *et al.*, 2015; Soccol *et al.*, 2006; Vandenberghe *et al.*, 1999). This process generates two different wastes: one is the microorganism residues rich in organic matter and nutrients, which could be dried for use in feed factories (Dhillon *et al.*, 2011). The other is the low valuable gypsum, whose disposal represents a contamination problem (Pazouki and Panda, 1998). In general, one ton of citric acid obtained by this method produces 2.5 tons of waste (Bauer *et al.*, 1989), thus efforts have been made to develop alternative recovery methods.

Beside precipitation, reactive liquid-liquid extraction has been also proven to be an effective approach with reduced environmental impacts, compared with precipitation. An acid-base reaction occurs between citric acid and tridodecylamine, a tertiary amine which is diluted in n-octanol. Citric acid extracted by this method has been recommended for the U.S. Food and Drugs Administration (FDA) and has declared that it could be used in food and drugs (U.S. Food and Drugs Administration, 1975). The principle of this method will be detailed in Section 1.3.

1.1.2. Succinic acid

1.1.2.1. Production and applications of bio-based succinic acid

Succinic acid is a dicarboxylic acid with many industrial applications in plastics, textiles, pharmaceuticals, solvents and as food additive. This carboxylic acid was mainly produced petrochemically in the past, for a relatively small specialty chemical market (15 000 tons/year at the end of the 1990s (Zeikus *et al.*, 1999)).

This molecule can be produced by several microorganisms, with the advantage that the greenhouse gas CO_2 is consumed in its acid production (Liebal *et al.*, 2018; Zeikus *et al.*, 1999), conferring environmental advantages to the process. Moreover, this molecule was identified as one of the most promising bio-based building blocks by the US DoE, becoming one of the rising stars of the list. At present, several microorganisms are being used and further developed by genetic engineering for succinic acid production. For example, the bacterial strains *Basfia succiniciproducens*, *Mannheimia succiniciproducens*, *Escherichia coli*, *Corynebacterium glutamicum*, and the yeast strain *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Akhtar *et al.*, 2014; Becker *et al.*, 2013; Cheng *et al.*, 2013; Tsuge *et al.*, 2014), with titres up to 190 g L^{-1} and yields up to 1.2 g g^{-1} (Becker *et al.*, 2015). Recently, several companies have been particularly active in commercial scale capacity development for this compound, achieving production capacities of 10 000 – 13 600 tons/year par plant site (Becker *et al.*, 2015; BioAmber Inc., 2015; Vaswani, 2010).

1.1.2.2. Recovery of bio-based succinic acid

Succinic acid recovery from bioconversion medium can be particularly challenging, even if there are processes with adequate titres, the usual presence of other carboxylic acids as by-products may affect the purity of the final product (Sosa *et al.*, 2016; Wang *et al.*, 2013a). In addition, most of the producing microorganisms need an optimal pH of 6 and 7, which is higher than the pKa values of this dicarboxylic acid (4.21 and 5.64 (Haynes *et al.*, 2017)). Thus, the produced succinic acid is mostly in form of an ionised salt (Vaswani, 2010). For all recovery methods used for commercially produced succinic acid, the first step is cells and insoluble particles removal from the bioconversion medium by centrifugation or filtration. Then, different recovery and purification steps are applied. Among them, crystallization stands out as the common method for succinic acid recovery and purification, thanks to its specific physicochemical properties. An alternative to the final succinic acid crystallisation is the conversion to another chemical products. For example, it can be subjected to catalytic hydrogenation to produce 1,4-butanediol (BDO), tetrahydrofuran (THF) and gamma-butyrolactone (GBL).

Reactive liquid-liquid extraction has also been proposed for the recovery of bio-based succinic acid, resulting in numerous studies in the literature with promising results (Alexandri *et al.*, 2019; Kurzrock *et al.*, 2011; Kurzrock and Weuster-Botz, 2010; Prochaska *et al.*, 2018). However, their application has not reached yet an industrial level.

1.1.3. Lactic acid

1.1.3.1. Production and applications of bio-based lactic acid

Bio-based lactic acid is one of the best established carboxylic acids in industry. It is a bifunctional organic acid of 3 carbons (with an additional hydroxyl-group (–OH), conferring it valuable properties). This molecule has a wide range of applications, from the traditional uses in food industry as additive, acidulant, flavouring, emulsifier and preservative (Reddy *et al.*, 2008), to the more recently discovered applications as a building-block. It is especially suitable for polymers fabrication, used in the chemical, food and medical industries (Lee *et al.*, 2011). The most remarkable example is polylactic acid (PLA), a renewable and biodegradable polymer with a wide range of applications including packaging, paper coating, fibres, films and a variety of customised articles (Drumright *et al.*, 2000). PLA is expected to be the biggest driver for growth in bio-based lactic acid demand (Biddy *et al.*, 2016). Lactic acid is also widely used in pharmaceutical industry for cosmetics production, formulating ointments, anti-acne solutions and controlled-released drugs (Alves de Oliveira *et al.*, 2018). Because of these applications, enantiomeric purity of lactic acid is a crucial factor for its commercialisation. This molecule has a chiral carbon and can be found as L-lactic acid or D-lactic acid. This has led to microbial production being preferred over chemical synthesis, since it is well known that the latest produces racemic mixtures (Juodeikiene *et al.*, 2015). In fact, about 90% of the lactic acid produced worldwide comes from bacterial fermentation. Food, pharmaceutical and medical industries prefer L-lactic acid isomer, because D-lactic acid is not assimilated by the human body and is considered harmful at high doses (Vijayakumar *et al.*, 2008).

The microbial species capable of producing lactic acid are so widespread in nature that they form a group called lactic acid bacteria (LAB). However, only some species are used for the commercial production of acid lactic. The main species used nowadays are homofermentative microorganisms from the genus *Lactobacillus*, *Streptococcus*, and *Pediococcus*. They have the advantage of producing up to 85% of lactic acid from sugar, while heterofermentative species produce only 50% (Biddy *et al.*, 2016; Reddy *et al.*, 2008). Also, the development of genetically modified strains has resulted in processes using a wide variety of substrates, with interesting final titres of 102 – 226 g L^{-1}, and high productivities (1 – 5.27 g L^{-1} h^{-1}) (Alves de Oliveira *et al.*, 2018). The pH range most of the bacterial strains used is between 5 and 9, which is inconvenient for lactic acid recovery. This has been addressed with the use of genetically modified yeast and fungus strains, such as *Saccharomyces cerevisiae* and *Monascus ruber* who are capable of high titres and productivities at pH as low as 3.5 and 2.8 respectively (Baek *et al.*, 2017; Weusthuis *et al.*, 2017).

1.1.3.2. Recovery of bio-based lactic acid

Lactic acid (2-hydroxypropionic acid) is an isomer of 3-hydroxypropionic acid (3-HP), the molecule of interest in this PhD work. Lactic acid recovery from bioconversion medium at

a high purity is challenging, mainly because of its high affinity with water and the tendency to decompose at elevated temperatures (Komesu *et al.*, 2017). Contrary to succinic acid or citric acid, calcium salts of lactic acid are soluble in water (85 g L^{-1} in pure water at 30°C (Vavrusova *et al.*, 2014)), rendering the traditional calcium precipitation method even more problematic, yielding a low technical grade lactic acid solution (22 – 44%). Traditionally, higher purity is obtained by esterification of the technical grade lactic acid with methanol or ethanol. The ester is recovered by distillation and separated again in lactic acid and the corresponding alcohol by hydrolysis. Alcohol is recycled to the esterification step and the lactic acid solution is evaporated to obtain the final product (Datta and Henry, 2006). Research has been dedicated to reduce the high costs of this processes and the large amounts of waste water and gypsum production. With the development of strains able to perform at low pH, neutralisation is avoided. For bioconversions at higher pH, substitution of lime for other neutralising agents, such as ammonium salts, gives an alternative to the low value gypsum produced.

The company Cargill, Inc. (USA) developed an alternative process for lactic acid purification using liquid-liquid reactive extraction. Bioconversion broth is neutralised with Na_2CO_3 during lactic acid production, thus a solution of sodium lactate is obtained after clarification. This solution is put in contact with a tertiary amine contained in and organic phase (Alamine 336, n-octanol, paraffin) under CO_2 pressure, which permits lactate extraction without an acidification step with H_2SO_4. The organic phase charged in acid is subsequently put in contact with water at a temperature between 100 – 150°C, in order to recover lactic acid. Performing like this, the organic phase is regenerated and can be recycled. This reduces considerably the solid and liquid wastes of the process compared to precipitation (Baniel *et al.*, 2002). Later, Hyflux (Canada) patented a process that also includes reactive extraction with tertiary amine. The difference is that contact between the lactic acid solution and the organic phase is carried out in a membrane contactor, reducing the organic phase volume needed for an efficient lactic acid extraction. However, the acidification step with H_2SO_4 before reactive extraction is needed (Lum *et al.*, 2014). Currently, there are many studies on these technologies for carboxylic acid recovery, however its application in the industry is very limited.

1.1.4. Carboxylic acids production from waste streams and their recovery

The seeking of alternative methods for chemical compounds production has generated new trends for renewable resources exploitation. One of them is the use of organic-rich wastes from different sources (food, agricultural, chemical and municipal water treatment industries) as feedstocks for organic acids production. The main type of acids that can be produced from these resources are classified as volatile fatty acids (VFAs). They are short-chain carboxylic acids (usually from 2 to 6 carbon atoms), able of being distilled at atmospheric pressure, *e.g.*, formic, acetic, propionic, valeric, butyric and caproic acid (Raposo *et al.*, 2013; Singhania *et al.*, 2013).

VFAs production from waste resources is performed mainly by anaerobic bioconversion, because it requires lower investment and operating costs than aerobic processes (Angenent

et al., 2016). At present, the preferred method is anaerobic digestion, which generates biogas and other high value-added products. Microorganisms able to produce VFAs in this conditions are very diverse, but they are also subjected to product inhibition, limiting VFAs production and shifting the metabolic pathways to other products (Pind *et al.*, 2003). Therefore, continuous recovery of the produced VFAs is critical to achieve an efficient and stable production.

In theory, all the conventional techniques for carboxylic acid could be applied to VFAs. However, obtaining a mixture of different VFAs with very similar properties is characteristic of these processes. In addition, they are normally produced at concentrations much lower than in bioconversion of refined feedstocks. Thus, the choice of the right recovery methods becomes more complicated. Application of traditional techniques such as precipitation, crystallisation and conventional distillation is not completely adapted, having low yields and high costs (Wang *et al.*, 2006). Therefore, alternative methods have been studied, among them membrane-based separation is one of the most promising alternatives, since they can be integrated into different processes configurations. The typical first step of clarification of the fermented effluents can be performed by microfiltration and/or ultrafiltration. Further VFAs separation can be obtained by nanofiltration (NF) or reverse osmosis (RO), that use a combination of size and charge effects. In general, these methods can provide permeate streams of high purity, but there are still challenges related to the overall recovery rate (Aghapour Aktij *et al.*, 2020).

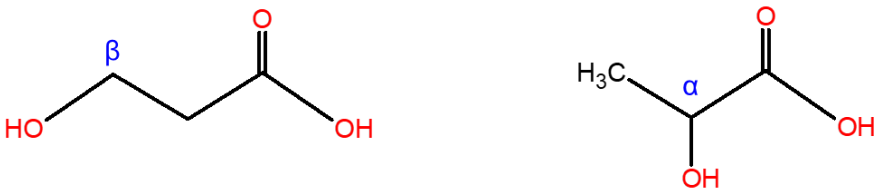
The development of hydrophobic membranes with suitable thermal stability and chemical resistance, enables their application on reactive liquid-liquid extraction. This process could be adapted to obtain high recovery yields of a wide range of carboxylic acids. Moreover, it works well on solutions at low concentrations (Reyhanitash *et al.*, 2018). This process was tested on a fermenter where ethanol-producing biorefinery wastewater was used to produce VFAs, with promising results: 53% removal of the overall VFAs was obtained, with further optimization potential (De Sitter *et al.*, 2018).

All the previous examples indicate that the proper method for organic acids recovery strongly depends on the nature of the acid, but also on the characteristics of the production process, especially the pH and the presence of other metabolites produced. Also, the use of microorganisms (especially those genetically modified) able to tolerate low pH represent a key aspect to obtain high productivities and for reducing costs of the subsequent acid recovery processes. All these aspects will be taken into account for the design of an integrated process for the production of our molecule of interest, 3-hydroxypropionic acid (3-HP), introduced in the following section.

1.2. 3-hydroxypropionic acid – A promising bio-based building-block

1.2.1. Potential applications of 3-hydroxypropionic acid

3-hydroxypropionic acid (3-HP) is a carboxylic acid of 3 carbons and one hydroxyl (–OH) group additional to the carboxylic (–COOH) group. It is a structural isomer of lactic acid (2-hydroxypropionic acid), which has the –OH group in α position (Figure 1.1). The difference in the –OH group position makes 3-HP a weaker acid (pKa = 4.51) than lactic acid (pKa = 3.86). This bifunctional organic acid presents a high reactivity and has tendency to dimerise at high concentrations. Therefore, it can be used as an intermediate for the synthesis of a rich variety of compounds and materials such as acrylic acid, 1,3-propanediol, methyl acrylate, acrylamide, ethyl 3-HP, malonic acid, propiolactone and acrylonitrile (Figure 1.2). This potential justifies a position in the DoE list as one of the most promising building-blocks for the chemical industry (Bozell and Petersen, 2010; Werpy and Petersen, 2004). These compounds are used for making adhesives, coatings, fibres, plastic packing, resins, detergents and biodegradable polymers. In the case of 3-HP, the most attractive valorisation line is the production of bio-based acrylic acid.



3-hydroxypropionic acid (3-HP)

2-hydroxypropionic acid (lactic acid)

Figure 1.1 Chemical structures of 3-hydroxypropionic acid (3-HP) and 2-hydroxypropionic acid (lactic acid)

Acrylic acid is used for making polymers and esters that possess super-absorbent properties and are attractive additives for co-polymerisation. These compounds are mainly used in the fabrication of product like diapers, synthetic rubbers, adhesives, plastics and paintings (Figure 1.2). Acrylic acid production is currently dominated by chemical synthesis from petrochemical resources. However, the recent inclination for a bio-based economy has driven the development of biotechnological alternatives for its production from renewable resources. Several routes for acrylic acid production from bio-based intermediates have been explored. Among them, the use of lactic acid as intermediate gained special attention. This is because its production from cheap renewable resources is well established in industry and it can be directly converted into acrylic acid by dehydration. Nevertheless, this chemical reaction presents selectivity issues, resulting in low conversion yields. The highest reported yield is 78%, from a lactic acid feed solution of 25% (w/w) (Ghantani *et al.*, 2014) and it has been observed that the acrylic acid yield is lower with feeds containing high concentrations of lactic acid (Beerthuis *et al.*, 2015). After the DoE list publication

(Werpy and Petersen, 2004), studies dedicated to 3-HP increased significantly. Soon after, 3-HP was proven to be more easily dehydrated into acrylic acid than lactic acid, presenting yields in the range of 90 – 100% (Holladay *et al.*, 2007). These results triggered the interest of several companies to bring the production of acrylic acid, using 3-HP as intermediate, to an industrial scale. Consortia of important companies were created in order to pursuit this goal, such as Cargill and Novozymes in 2008. They were joined by BASF in 2012, and in 2014 they announced the successful conversion of bio-based 3-HP into glacial acrylic acid and superabsorbent polymers (BASF, 2014). In 2011, Dow chemical and the start-up OPX Biotechnologies Inc. signed a joint development agreement for 3-HP production from sugar feedstocks and further dehydration to acrylic acid. They claimed to have a lower-cost route than Cargill and also competitive to petroleum-based route (Runge, 2014). In 2015, Cargill acquired OPX Biotechnologies (Biofuels Digest, 2015) and BASF left the partnership with Cargill and Novozymes (Bioplastics Magazine, 2015). This happened in a period when oil prices were falling, rendering petrochemicals cheaper (European Central Bank, 2016). The variability of the market around these chemicals encourages to develop more efficient bio-based routes and alternative applications for 3-HP, to make this platform more robust.

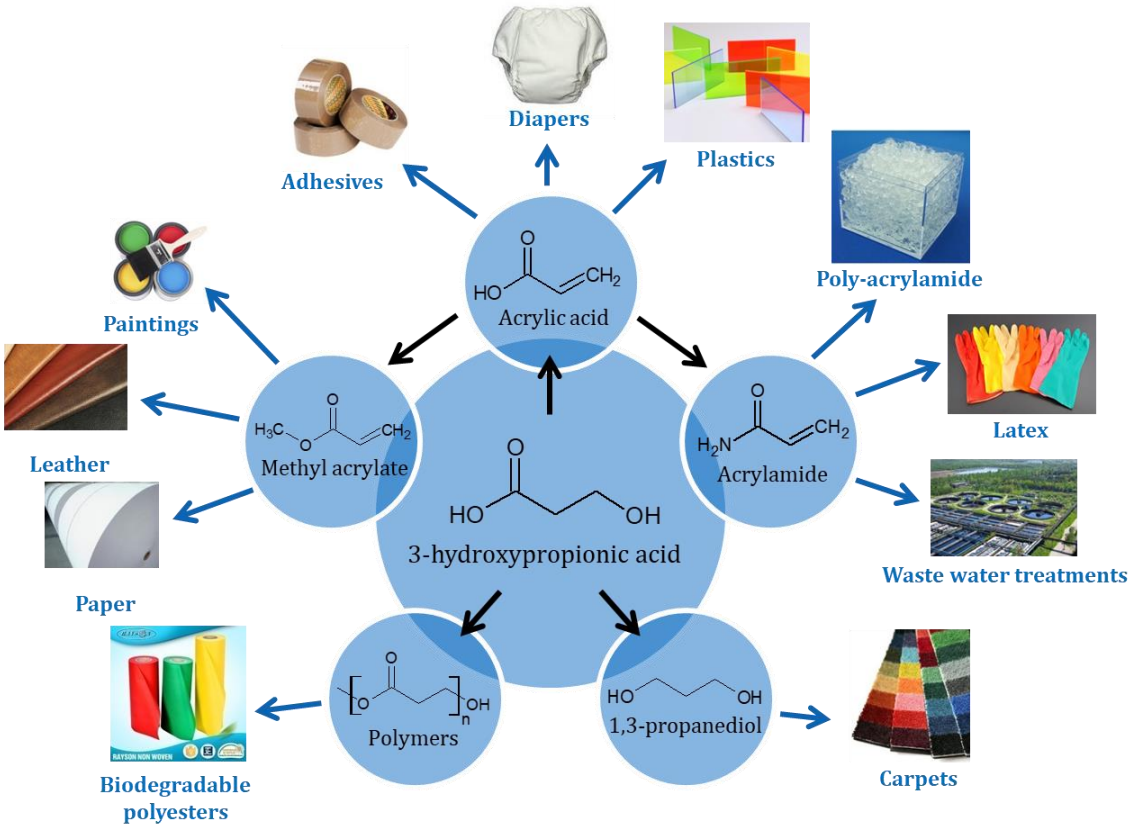


Figure 1.2 Examples of chemicals and corresponding materials produced from 3-HP

3-HP chemical synthesis from petrochemical resources at large scale is not viable due to expensive substrates and the toxicity of substrates and intermediates (Vidra and Németh, 2017). However, the seeking of bio-based routes with competitive costs and feasibility for 3-HP production has increased the interest for alternative applications. For example, 3-HP

polymerisation to poly(3-HP) is of great interest due to its particular properties: in addition to being biodegradable, it has a low melting point (77°C), low glass transition temperature (-22°C) and a high elongation-to-break factor (634%). This results in a flexible and stable biomaterial that could be used for the fabrication of thermoplastics. In addition, the incorporation of 3-HP monomers can improve the quality of other bio-based polymers. For example, poly(3-hydroxyburtyrate) presents limitations on its physical properties (notably a low elongation-to-break factor), resulting in a very crystalline and fragile biopolymer. Interestingly, 3-HP incorporation as a monomer improves flexibility and resistance of the resulting biopolymer (Andreeßen and Steinbüchel, 2010). The obtained materials have potential application as a surgical bio-composite materials and drug release (Pandey *et al.*, 2015).

1.2.2. Methods for 3-HP production

1.2.2.1. Chemical synthesis from petrochemical resources

As mentioned before, chemical synthesis using petrochemical resources is currently not a viable method for 3-HP production at large scale. For biopolymers fabrication, one of the considered methods is acrylic acid hydration. Moreover, economical acrylic acid production from petrochemical resources has to be implemented. The hydration process uses temperatures of 170 – 200°C with a CO_2 pressure between 7 – 35 bar. The reaction yields 3-HP together with the ether dimer of 3-HP (3-3'oxy-dipropionic acid) as impurity. This process is the one used to produce 3-HP commercial solution by chemical reagent suppliers such as TCI chemicals and Sigma-Aldrich. The selling price of the obtained 3-HP reflects the high costs of the process. It is commercialised in form of a 30% w/v aqueous solution with 3-3'oxy-dipropionic acid reported as the main impurity by TCI chemicals. The price of a 25 g solution is currently 194 euros (USD $216) from TCI chemicals (TCI Chemicals Europe, 2019), while Sigma-Aldrich sells a solution of 1 g at 50 euros (USD $55) (Sigma-Aldrich, 2019).

There are other methods reported in literature such as production from ethylene chlorohydrine *via* 3-hydroxypropionitrile hydrolysis. However, the complex route involves the use of harmful and noxious reagents such as sodium cyanide. Finally, 3-HP can be obtained from 3-propiolactone produced from ketene and formaldehyde. The alkali and acid-catalyzed hydrolysis produces 3-HP. The disadvantage of this method is the handling of the noxious ketene and formaldehyde and the carcinogen 3-propiolactone (Della Pina *et al.*, 2011).

1.2.2.2. Biotechnological production of 3-HP

Contrary to chemical synthesis, 3-HP production through biotechnological processes does not require handling of toxic substances. In addition, processes can be performed at mild conditions (temperature and pressure), making them more energy-efficient than chemical synthesis (Gavrilescu and Chisti, 2005; OECD, 2001). Figure 1.3 gives an overview of

research evolution related to 3-HP. In the early 2000s, research started focusing in 3-HP microbial production using recombinant bacterial strains. Few years after, publication of the "Top value added chemicals from biomass" report by the US Department of Energy (2004) seems to have triggered the investigation of 3-HP microbial production. The number of studies and patents related to this molecule increased significantly and several microbial routes has been investigated and developed for 3-HP production since then.

The overall approaches to improve 3-HP microbial production involve testing new microorganisms and metabolic pathways, the implementation of different process modes, and the implementation of new and emerging technologies. The selection of a relevant microbial agent with an efficient metabolic pathway, is one of the most important choices to make when designing a new biotechnological process. Several microorganisms capable of producing 3-HP have been discovered and studied. An exhaustive review of the process engineering strategies investigated for 3-HP microbial production was made by de Fouchécour *et al.* (2018). Understanding of the metabolic pathways involved in 3-HP production have given valuable insights for process design and the development of more efficient microbial agents through genetic engineering. Examples of the best 3-HP production performances are given in Table 1.1. Description of the involved metabolic pathways and the strategies used in order to achieve those performances are given below.

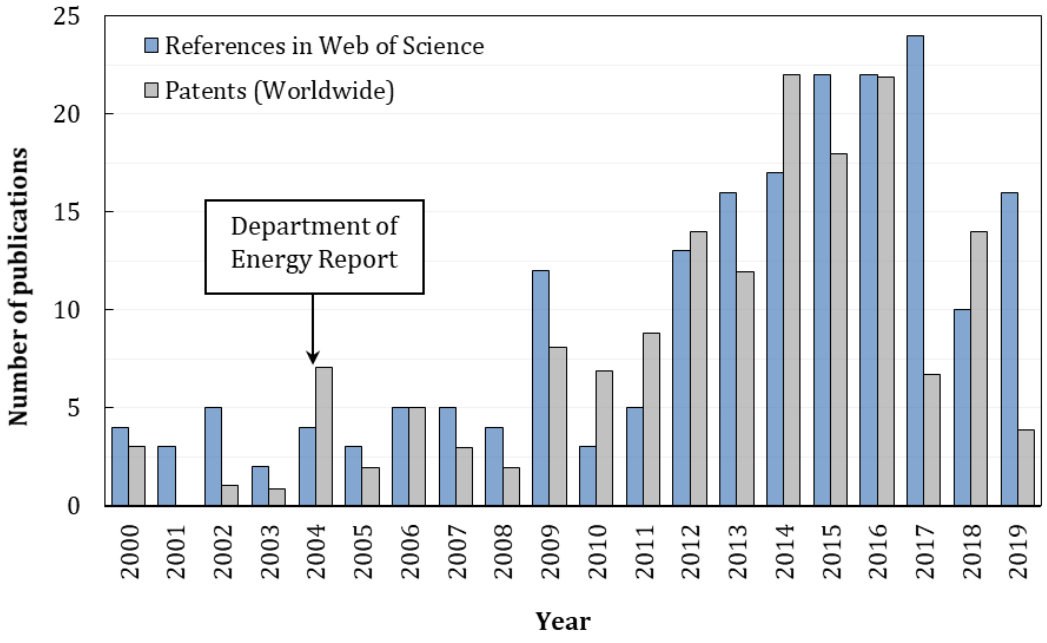


Figure 1.3 Evolution of the numbers of articles and patents related to 3-HP on the last 19 years. Results obtained by searching "3-hydroxypropionic acid" or "3-hydroxypropionate" in the title of articles (Web of Science) and patents (European Patent Office). Accessed December 21th 2019. Adapted from de Fouchécour *et al*. (2018).

Table 1.1 Best 3-HP microbial production performances listed by type of metabolic pathway (de Fouchécour *et al.*, 2018)

Microorganism	Carbon source	Operational technique	Titre (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield (mol_C mol_C^{-1})	Reference
Glycerol oxidation (CoA – dependent) pathway						
Lactobacillus reuteri	Glycerol	Fed-batch 3 L bioreactor	14	0.25	0.49	(Dishisha *et al.*, 2015)
Glycerol oxidation (CoA – independent) pathway						
Klebsiella pneumoneae	Glycerol	Fed-batch 5 L bioreactor	102.61	1.07	0.86	(Zhao *et al.*, 2019)
Escherichia coli	Glycerol + glucose	Fed-batch 5 L bioreactor	71.9	1.8	-	(Chu *et al.*, 2015)
Corinebacterium glutamicum	Glycerol	Fed-batch 5 L bioreactor	62.6	0.87	0.51	(Chen *et al.*, 2017)
Malonyl-CoA pathway						
Escherichia coli	Glucose	Fed-batch 1.8 L bioreactor	48.4	-	0.53	(Lynch *et al.*, 2014)
Escherichia coli	Glucose	Fed-batch 5 L bioreactor	40.6	0.56	0.19	(Liu *et al.*, 2016)
β-Alanine pathway						
Escherichia coli	Glucose	Fed-batch 6.6 L bioreactor	31.1	0.63	0.423	(Song *et al.*, 2016)
Saccharomyces cerevisiae	Glucose	Fed-batch 1 L bioreactor	13.7	0.17	0.14	(Borodina *et al.*, 2015)
Aerobic 1,3-PDO oxidation						
Gluconobacter oxydants	1,3-PDO	Batch 7 L bioreactor	60.5	2.52	0.94	(Zhao *et al.*, 2015)
Acetobacter sp.	1,3-PDO	Batch 10 mL reaction volume	67.0	0.90	0.93	(Li *et al.*, 2016a)
Acetobacter sp.	1,3-PDO	Fed-batch 1 L bioreactor	69.8	2.79	1	(de Fouchécour, 2019)

1.2.2.2.1. Glycerol oxidation through a coenzyme A-dependent pathway

3-HP production through glycerol oxidation has been investigated in *Lactobacillus reuteri* strains, a hetero-fermentative, gram-positive bacterium. Although this species is not able to grow on glycerol as the only carbon or energy source (Sobolov and Smiley, 1960), cells growing on other substrates (*e.g.* glucose or lactose), as well as resting (*i.e.* non-growing) cells are able to consume glycerol. *L. reuteri* contains a vitamin B_{12}-dependent diol dehydratase, that converts glycerol into 3-hydroxypropionaldehyde (3-HPA). In the case of growing cells, 3-HPA is used as electron acceptor and reduced into 1,3-propanediol (1,3-PDO). On the other hand, when glycerol is supplied as the only carbon source to resting cells, 3-HPA is mainly accumulated in the medium (Krauter *et al.*, 2012). Interestingly, 3-HP is also found together with 1,3-PDO as by-products (Talarico *et al.*, 1988). In this case, 3-HPA is converted into 3-HP due to the activation of an additional oxidative pathway, which is coenzyme A (CoA)-dependent. This step is necessary to maintain the redox balance of the metabolic pathway and results in an equimolar production of 3-HP and 1,3-PDO, with the last being an obligated by-product (Dishisha *et al.*, 2014). Therefore, the maximal 3-HP yield is limited to 0.5 $mol_{3\text{-}HP}$ $mol_{glycerol}^{-1}$. In practice, yields are even lower due to accumulation of 3-HPA. Moreover, this aldehyde shows a high toxicity towards cells (Schaefer *et al.*, 2010).

Accumulation of 3-HPA in the medium can be avoided by progressively supplying glycerol (*i.e.* fed-batch bioconversion). Dishisha *et al*. (2015) achieved glycerol bioconversion into 3-HP and 1,3-PDO by supplying glycerol at a feeding rate equivalent to half of the maximum metabolic flux of glycerol determined for the used strain. No 3-HPA accumulation was detected with the used conditions. They achieved a final titre of 14 g L^{-1}, with a productivity of 0.25 g L^{-1} h^{-1} and a yield of 0.49 $mol_{3\text{-}HP}$ $mol_{glycerol}^{-1}$ (Table 1.1) (Dishisha *et al.*, 2015). Interestingly, no other by-products were found in the medium, which is advantageous for downstream processes. Additional advantages of using *L. reuteri* cells are their capacity to produce vitamin B_{12} (Dishisha *et al.*, 2014) which represents an expensive substrate, and that this specie has the Generally Recognised As Safe (GRAS) status.

1.2.2.2.2. Glycerol oxidation through a coenzyme A-independent pathway

This metabolic pathway is present in several bacterial strains able to grow on glycerol during anaerobic fermentation. Glycerol has a high degree of reduction (4.67 e^-/C), so its fermentation requires efficient routes for the dissipation of the reducing equivalents. While part of the glycerol is converted into end-products like organic acids (such as acetic, lactic and succinic acids) and alcohols (such as ethanol and butanol), another part is converted into 3-HPA by a B_{12}-dependent glycerol dehydratase. In order to maintain the redox balance, 3-HPA is converted to other end-products, mainly 1,3-PDO (Biebl *et al.*, 1999). It has been observed that *Klebsiella pneumoneae* can metabolise glycerol, with acetic acid and 1,3-PDO as the main products (Clomburg and Gonzalez, 2013). 3-HP can also be produced naturally by this species because it possesses a NAD^+-dependent aldehyde

dehydrogenase able to convert 3-HPA into 3-HP, but not in a significant amount (Raj *et al.*, 2010). However, overexpression of this enzyme permits to obtain high amounts of 3-HP from glycerol. For example, the highest titre reported so far in literature (102.61 g L^{-1}, with a productivity of 1.07 g L^{-1} h^{-1} and a yield of 0.86 $mol_{3\text{-}HP}$ $mol_{glycerol}^{-1}$, Table 1.1) was achieved by engineering a *K. pneumoneae* strain using three tandem repetitive *tac* promoters induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), in order to overexpress the endogenous aldehyde dehydrogenase (Zhao *et al.*, 2019). An interesting outcome is that lactic acid, a usual by-product from glycerol fermentation by this strain, was inhibited, which is advantageous for downstream processes.

This metabolic pathway has been intensively studied, resulting in the highest titres achieved in literature. It has even been implemented in other microbial hosts such as *Escherichia coli* and *Corynebacterium glutamicum*, to name a few, with very promising results (Table 1.1).

1.2.2.2.3. Malonyl-coenzyme A pathway

3-HP can also be produced from sugars, such as glucose, through the malonyl-CoA pathway. Glucose is first converted into two acetyl-CoA molecules, passing through glycolysis. Then, malonyl-CoA is obtained by decarboxylation of acetyl-CoA using an acetyl-CoA carboxylase. Malonyl-CoA conversion into 3-HP is achieved by the induction of a malonyl-CoA reductase (MCR) from the cyanobacterium *Chloroflexus aurantiacus*. This enzyme has two distinct functional domains: i) a C-terminal fraction that catalyses the reduction of malonyl-CoA into malonate semialdehyde (MSA), and ii) a N-terminal fraction that catalyses the reduction of (MSA) into 3-HP. One of the best yields in literature was obtained by separating MCR in two fragments, MCR-C and MCR-N, and balancing both enzyme activities, resulting in a final titre of 40.6 g L^{-1} (Table 1.1) (Liu *et al.*, 2017).

This pathway is redox neutral, which means that is does not need an effective dissipation of reducing equivalents like the glycerol oxidation pathways described previously. Furthermore, it is independent of vitamin B_{12}, which is an economical advantage in 3-HP production (Suyama *et al.*, 2017). Also, since this pathway uses common metabolic intermediates, a great variety of feedstocks could be used for 3-HP production. On the other side, this leads to the production of a wide range of by-products, limiting the maximal 3-HP yield. In this case, strategies to limit the diversion of carbon flux towards other products need to be implemented. For example, OPX Biotechnologies process (Lynch *et al.*, 2014) aimed to increase MCR activity in *E. coli* while decreasing the fatty acids synthesis as by-products. This resulted in the best 3-HP performance so far using this metabolic pathway (48.8 g L^{-1}, Table 1.1).

E. coli has been the most investigated host for 3-HP production through this pathway. Unfortunately, it exhibits low tolerance to 3-HP (Warnecke *et al.*, 2010) and low extracellular pH, as most bacterial strains. In contrast, yeast cells are known to have high tolerance to low pH. Therefore, this pathway has been introduced in *Saccharomyces cerevisiae* (Liu *et al.*, 2017) and *Schizosaccharomyces pombe* (Suyama *et al.*, 2017) as an attractive approach to tackle the inhibition by 3-HP accumulation in the medium. Also, it

represents a great advantage for downstream processes, as it will be discussed in Section 1.3. However, the performances obtained so far has been much lower than those achieved with *E. coli*, with the highest final yield being 11.4 g L^{-1}, using *Schizosaccharomyces pombe* (Takayama *et al.*, 2018).

1.2.2.2.4. β-alanine pathway

Another metabolic pathway proposed for 3-HP production involves glucose conversion into β-alanine as intermediate. The first step is glucose oxidation into pyruvate, which is subsequently converted into β-alanine through the tri-carboxylic acids (TCA) cycle. Then, β-alanine is converted to malonate semialdehyde (MSA) by a β-alanine pyruvate transaminase and finally, 3-HP is obtained by MSA reduction (Song *et al.*, 2016). This metabolic pathway has been patented using model strains like *E. coli* by Cargill (Liao *et al.*, 2005), and *S. cerevisiae* by Novozymes (Jessen *et al.*, 2012).

This pathway has a higher maximum theoretical 3-HP yield than the malonyl-CoA pathway and has been identified as the most economically attractive according to metabolic modelling (Borodina *et al.*, 2015). This is true at least for *Saccharomyces cerevisiae*, which has been observed to produce a higher concentration of 3-HP through the β-alanine pathway (6.1 g L^{-1}) than with the malonyl-CoA pathway (2.3 g L^{-1}) (Kildegaard *et al.*, 2015). Indeed, the maximal performance in *Saccharomyces cerevisiae* has been achieved using this metabolic pathway (13.7 g L^{-1}, Table 1.1). Although better 3-HP production performances has been achieved with the malonyl-CoA pathway with *E. coli* (Table 1.1), the use of yeast strains is a more attractive approach for industrial processes. The best performance achieved by a yeast strain was reported in a patent from Cargill: a titre of 80 g L^{-1}, a productivity of 2 g L^{-1} h^{-1}, and a yield of 0.75 $g_{3\text{-}HP}$ $g_{glucose}^{-1}$. This was obtained in a batch fermentation of 107 g L^{-1} of glucose at a pH of 4 (Abraham *et al.*, 2016). They even mention that with an appropriate organism and fermentation parameters, the final 3-HP titre can be up to 100 g L^{-1}, with a productivity of 2.5 g L^{-1} h^{-1}. Taking into account the high resistance of yeast, these are probably the best conditions for industrial production of 3-HP. However, the name of the microorganism used is not indicated.

1.2.2.2.5. Aerobic 1,3-propanediol oxidation

One of the most recent strategies explored for 3-HP production is the use of acetic acid bacteria (AAB) for 1,3-propanediol oxidation. The general idea is to do a two-step process with an initial production of 1,3-PDO, followed by its further oxidation into 3-HP, taking advantage of the capacity of AAB to selectively oxidise alcohols into their corresponding organic acids (Rogers *et al.*, 2013). These bacteria are obligated aerobes and gram-negative. This approach was reported for the first time by two different studies, almost at the same time: i) the use of a *Klebsiella pneumoneae* strain for an initial bioconversion of glycerol into 1,3-PDO, which is further oxidised into 3-HP (Zhao *et al.*, 2015), and ii) the initial production of 1,3-PDO and 3-HP from glycerol, using a *Lactobacillus reuteri* strain, with further oxidation of 1,3-PDO into 3-HP (Dishisha *et al.*, 2015). The strain used to oxidise

1,3-PDO of 3-HP in both studies was *Gluconobacter oxydans*. The proposed metabolic pathway is similar to the conversion of ethanol in acetic acid: first, an alcohol dehydrogenase oxidises 1,3-PDO into 3-HPA, which is further oxidised into 3-HP by an aldehyde dehydrogenase (De Muynck *et al.*, 2007; Dishisha *et al.*, 2015).

It was previously mentioned that the maximal theoretical yield of glycerol bioconversion into 3-HP by *L. reuteri* is 0.5 mol_C mol_C^{-1} because of the obligated production of 1,3-PDO (Section 1.2.2.2.1). In the study of Dishisha *et al.* (2015), almost all the 1,3-PDO was converted into 3-HP, yielding a final concentration of 23.6 g L^{-1}, meaning that the yield of 3-HP from glycerol using this strategy was approx. 1 mol_C mol_C^{-1}. The advantage of using *L. reuteri* resting cells is that no other by-products are found, resulting in a final 3-HP solution with a purity of 95%. While in the study of Zhao *et al.* (2015), the molar yield of 3-HP from 1,3-PDO was also high (0.94 mol_C mol_C^{-1}). Although 1,3-PDO yield from glycerol was 0.55 mol_C mol_C^{-1} due to the production of other by-products such as acetic acid and 3-hydroxybutanone, the final titre was much higher than the one obtained with *L. reuteri* (60.5 g L^{-1}, Table 1.1).

Another explored microorganism is *Acetobacter* sp., which has been found to be an interesting candidate for 1,3-PDO oxidation into 3-HP, achieving a titre of 69.95 g L^{-1} using cells immobilisation (Table 1.1) (Li *et al.*, 2016a). The 3-HP production capacity of *Acetobacter* sp. in a fed-batch bioreactor has been recently evaluated in our laboratory. The maximal titre obtained from pure 1,3-PDO was 69.8 g L^{-1}, with a productivity of 2.79 g L^{-1} h^{-1} (Table 1.1) (de Fouchécour, 2019). These results are highly encouraging, indicating that this strain is a very good candidate to be used in an integrated process for 3-HP production.

Remarkable progress on 3-HP microbial production has been accomplished. However, most of the aforementioned examples still present limited performances and cannot ensure an economically viable production at industrial scale. According to the DoE, a productivity of at least 2.5 g L^{-1} h^{-1} is required for an economically competitive process (Werpy and Petersen, 2004). Also, industrial production generally requires a high final titre to reduce recovery costs. For example, it has been determined that titres higher than 100 g L^{-1} are required for economic production (Litchfield, 2009).

1.2.3. Downstream processes for 3-HP recovery

3-HP recovery from bioconversion broth can be particularly challenging, due to its physicochemical properties. For example, its high solubility in water and low affinity for organic solvents (log $P_{O/W}$ = -0.89) make 3-HP separation from aqueous phases more difficult than other organic acids such as lactic acid, its positional isomer (Table 1.2). In addition, some methods such as traditional distillation and evaporation are not effective because 3-HP easily dimerises at high concentrations and decomposes at high temperatures (Hoppe *et al.*, 2015). However, efficient recovery of 3-HP at high concentration and purity for the corresponding applications, is a crucial point for its industrial production. To this end, several methods have been explored.

Table 1.2 Physicochemical properties of 3-HP and lactic acid

	3-HP	**2-HP (lactic acid)**
Formula	$C_3H_6O_3$	$C_3H_6O_3$
CAS number	503-66-2	50-21-5
Molecular weight (g mol^{-1})	90.08	90.08
Henry's constant (atm m^3 mol^{-1})	$2.66 \cdot 10^{-11}$	$1.13 \cdot 10^{-7}$
log $P_{O/W}$	-0.89	-0.65
pKa	4.51	3.86
Boiling point (°C)	Decomposes before evaporation	122
Solubility in water	Miscible	Miscible

1.2.3.1. 3-HP recovery from clarified bioconversion medium

The processes currently used for recovery and purification of commercial 3-HP begin with a common protocol followed for most organic acids produced at industrial scale (Section 1.1):

i) Cells removal after the end of bioconversion, which can be performed by centrifugation or appropriate filtration technique.

ii) Medium concentration: when needed, it can be performed by evaporation at a temperature between 40 – 60 °C and a pressure of 330 – 400 kPa. This permits to obtain a concentration of 20 – 30 % w/w of 3-HP (Abraham *et al.*, 2016). Another applied method is electrodialysis that permits a concentration from 10 % w/w to a final concentration of about 30 % w/w (Jump, 2012).

iii) Conversion to free acidic form and demineralisation: since 3-HP microbial production is commonly performed at controlled pH by addition of a neutralising base solution, 3-HP is normally present in the form of the corresponding salt. In addition, there are other minerals present in the complex bioconversion medium. In order to recover the acidic form of the molecule, the acidification of the medium is applied (with gypsum production as waste), followed by ion exchange for demineralisation (Abraham *et al.*, 2016). Other method applies electrodialysis using a bipolar membrane, which does not need a previous acidification step. This method also removes neutral components such as glucose and other nutrients present in the bioconversion medium. Also, the regenerated base solution in the retentate can be recycled for pH control of bioconversion (Jump, 2012).

Further 3-HP purification steps can be applied depending on the desired purity and concentration of the 3-HP solution:

Distillation. The 3-HP separation process described by Cargill (Abraham *et al.*, 2016) permits to have a final 3-HP solution at high concentration and purity. After cells removal, concentration, conversion into its free acidic form and demineralisation, the 3-HP solution is subjected to further evaporation at a temperature of 80 – 85 °C and a pressure of 0.39 bar, to a final concentration of about 60 % w/w. The obtained solution is then fed to a two-

step distillation. The first column operates at 135 °C and 35 mmHg, the distillate consists of 3-HP and water, while the bottoms containing impurities such as sugars and oligomers of 3-HP are fed into a second distillation column that operates at 125 °C and 3 mmHg. The distillates from both columns are condensed, resulting in a final product of 60% w/w of 3-HP. This two-step distillation approach is to avoid extreme conditions that could increase 3-HP decomposition into acrylic acid, also the residence times are very short (30 – 60 s).

Heated reactive liquid-liquid extraction. Another process used for 3-HP recovery from a bioconversion medium in its salt form was presented by Cargill (Meng *et al.*, 2007). The 3-HP solution has been neutralised during bioconversion with ammoniac, giving a solution rich in ammonium 3-hydroxypropionate. This solution is put in contact with an organic phase containing an amine, a long-chain alcohol, or a mixture of both. The extraction system is heated (100 – 140 °C) under reduced pressure (1.1 – 1.7 mmHg) during 1 – 1.5 h, in order to evaporate the ammoniac, and the 3-HP liberated goes to the organic phase. The evaporated ammoniac can be recycled for bioconversion neutralisation. Conversion of 84 – 98% of the ammonium 3-hydroxypropionate into 3-HP was reported by this method.

1.2.3.2. Extractive bioconversion

Traditional recovery and purification methods can be highly costly if the final 3-HP solution is desired at high purity and concentration. The 3-HP produced by microbial processes is normally present at relatively low concentrations in a complex bioconversion medium. This makes downstream processes more challenging and costly, compared with other carboxylic acids produced at higher concentrations. In addition, 3-HP exhibits toxicity towards producing cells, so the continuous removal from the bioconversion medium can help to improve the process performance, alleviating end-product inhibition (Yang *et al.*, 2007).

In-situ or in stream product recovery (ISPR) is a promising strategy for the intensification of processes affected by end-product inhibition, such as carboxylic acids microbial production (Van Hecke *et al.*, 2014; Yang *et al.*, 2007). The principle of ISPR is the continuous removal of the desired product from the bioconversion medium as it is being produced. The advantages of this approach are:

i) Alleviation of end-product inhibition, which improves the production performance.

ii) Yield improvement by removing the target product from the bioconversion medium, making it unavailable for side reactions.

iii) Recovery of the product in a more concentrated and pure solution, which decreases the subsequent downstream processes costs.

iv) Decrease of the overall process steps and duration.

v) Reduction of the amount of waste generated.

There are numerous configuration for ISPR, and the selection of the best techniques depends on the properties of the targeted product (Figure 1.4). In the case of carboxylic

acids, ISPR has been already implemented and proved to enhance the overall productivity of the processes. To date, there are no studies reporting a successful ISPR process for 3-HP bioconversion; nevertheless, taking lactic acid (positional isomer of 3-HP) as an example, the techniques used to implement an extractive bioconversion of this molecule follows the decision diagram in Figure 1.4. Most of the microbial processes studied for ISPR has been performed at a pH higher than the pKa of lactic acid (3.86, Table 1.2). Therefore, ion exchange and electrodialysis were the most often selected techniques (Yang *et al.*, 2007). This is because when the pH is above the pKa of the corresponding acid, the carboxylic group is in a deprotonated or dissociated form with negative charge (A^-). This anion can easily interact in reactions like ion exchange or reprotonation using the adequate ion-exchange resins (Ataei and Vasheghani-Farahani, 2008; Boonmee *et al.*, 2016) or electro-membrane processes (Boontawan *et al.*, 2011; Wang *et al.*, 2013b). On the other side, when bioconversion pH is below the pKa of the acid the carboxylic group is in its protonated or non-dissociated form (AH), which makes it available for other interactions such as liquid-liquid extraction, particularly reactive extraction (Datta *et al.*, 2015).

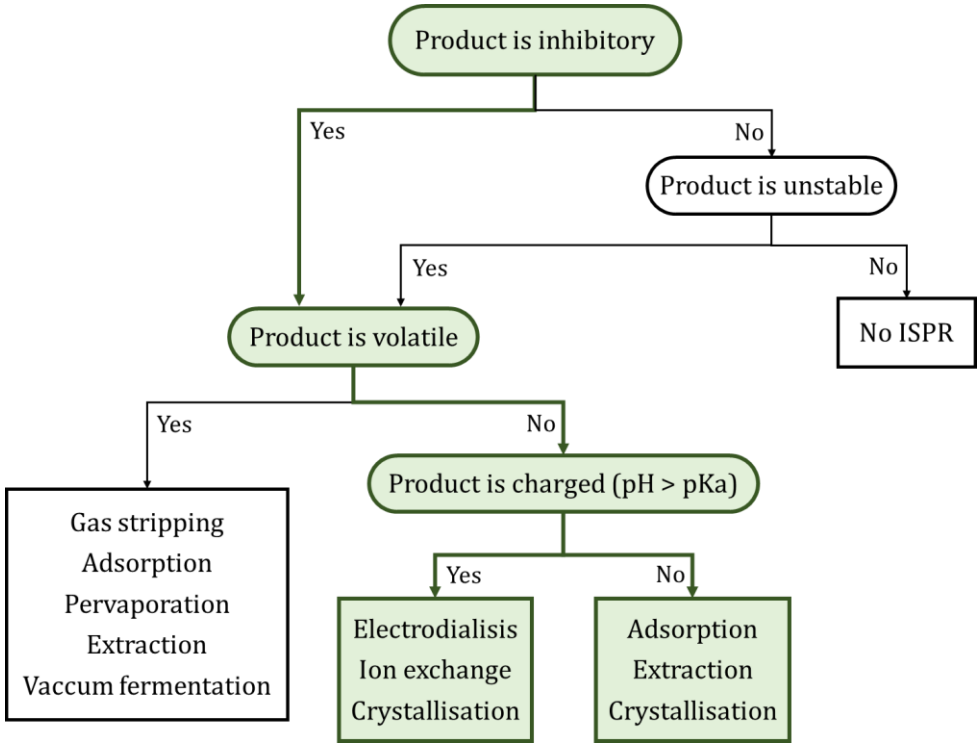


Figure 1.4 Decision diagram for the selection of an appropriate ISPR technique for a fermentation or bioconversion product. Pathway adapted to 3-HP recovery is highlighted in green. Last decision choice depends of the fermentation pH (pKa = 4.51). Adapted from Van Hecke *et al.* (2014).

Table 1.3 summarises some examples of ISPR configurations applied to acid lactic extractive bioconversion, an increase in the overall productivity of the processes can be attained by continuous removal of lactic acid from bioconversion medium. Ion-exchange resins have been widely studied for the recovery of other carboxylic acids such as citric, fumaric and acetic acids (Anasthas and Gaikar, 2001; Cao *et al.*, 1996; Jianlong *et al.*, 2000). In general, resins are not toxic to cells and the removal of the acid product can provide good

pH control and increase reactor productivity significantly (up to 3 to 4 fold increase). However, the bioconversion is limited by the adsorption capacity of the resins, which also are more expensive than other methods like solvent extraction. Therefore it has not be used for industrial application (Yang *et al.*, 2007). In general, electrodialysis fermentation can provide good pH control without base addition, reducing chemical use and waste generation. It can also improve dramatically the productivity of the bioconversion process (Table 1.3). However, it also suffers process limitation such as membrane fouling and lower final product concentration than other extractive fermentation processes (Yang *et al.*, 2007). Finally, liquid-liquid extraction using an organic phase for acid removal is probably the most promising method to be adapted at industrial scale. The principle and more detailed information about this recovery technique are given in the next section.

Table 1.3 ISPR techniques applied for lactic acid extractive bioconversion

Extraction agent	Microorganism	pH	Productivity increase (compared w/control)	Reference
Ion exchange resins				
Resin Amberlite IRA 400	*Lactobacillus casei*	6.1	5.4-fold	(Ataei and Vasheghani-Farahani, 2008)
Resin Amberlite IRA 67	*Lactococcus lactis*	6.5	5.9-fold	(Boonmee *et al.*, 2016)
Electrodialysis				
Electrodialyzor (Micro Acylizer G3)	*Lactobacillus rhamnosus*	6	19.5-fold	(Min-Tian *et al.*, 2005)
Bipolar membrane	*Lactobacillus plantarum*	6.8	1.4-fold	(Wang *et al.*, 2013b)
Reactive liquid-liquid extraction				
15% v/v Alamine 336/oleyl alcohol	*Lactobacillus delbrueckii*	4.3	1.7-fold	(Yabannavar and Wang, 1991b)
33% v/v Tridecylamine/oleyl alcohol	*Saccharomyces cerevisiae*	2.2	1.8-fold	(Gao *et al.*, 2009a)

1.3. Reactive liquid-liquid extraction

Liquid-liquid extraction consists of the transfer of a solute from one liquid phase to another, both phases being immiscible or partially miscible with each other (Berk, 2013). One of the advantages of this technique is its versatility, since it can be adapted for a wide variety of compounds, just by the adequate selection of the extraction phase. Therefore, this separation process is widely used for the downstream recovery of bioconversion products for the chemical, pharmaceutical and food industries (Yang *et al.*, 2007).

Depending on the characteristics and needs of the bio-production process there are several configurations for putting the two liquid phases in contact to perform extraction (Figure 1.5). These could be classified according to the location of the separation unit (Van Hecke *et al.*, 2014):

i) Internal-direct contact. When the extraction phase is inside the bioconversion unit (also known as *in-situ* extraction) and in direct contact with the producing cells. This configuration correspond to a two-phase partitioning bioreactor (Figure 1.5a) (Malinowski, 2001; Matsumoto, 2018; Rosinha-Grundtvig *et al.*, 2018) or to solvent-impregnated particles dispersed in the bioconversion medium (Figure 1.5b) (Kabay *et al.*, 2010; Van Den Berg *et al.*, 2008)

ii) Internal-indirect contact. In this configuration, the extraction phase remains inside the bioconversion unit but there is a barrier to avoid direct contact with the producing cells. For example, using immobilized cells in the bioconversion medium (Figure 1.5c) (Wu and Yang, 2003a; Zaushitsyna *et al.*, 2017) or using membrane-based extraction inside the bioreactor (Figure 1.5d) (Chen and Lee, 1997; Heerema *et al.*, 2011).

iii) External-direct contact. This configuration consists of transferring the bioconversion medium to a separation unit located externally, this is also known as in stream extraction. An example of this configuration involving direct contact with the producing cells is to pump the bioconversion broth to a column packed with solvent-impregnated particles (Figure 1.5e) (Blahušiak *et al.*, 2015).

iv) External-indirect contact. The bioconversion broth is transferred to an external separation unit equipped with a barrier that avoids direct contact of the extraction phase with the producing cells. For example, membrane-based extraction outside the bioreactor (Figure 1.5f) (Cai *et al.*, 2017; Schlosser *et al.*, 2005), and introducing a microfiltration unit to separate cells from the medium before contact with the extraction phase (Figure 1.5g) (Eggert *et al.*, 2019; Yang and Tsao, 1995).

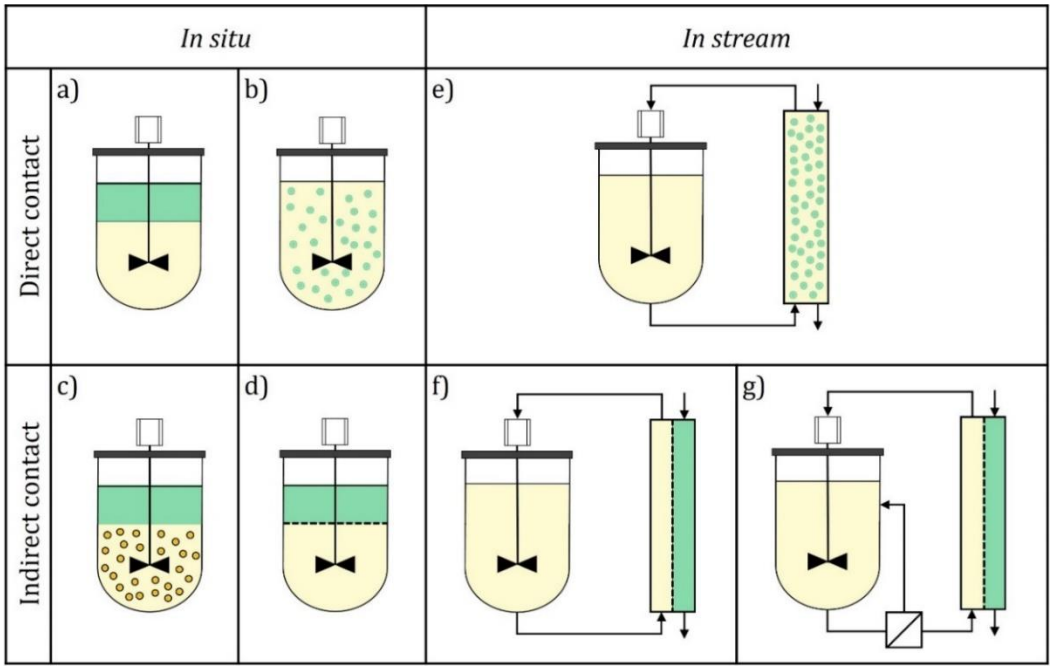


Figure 1.5 Different configurations for liquid-liquid extraction of compounds produced in bioreactors. In situ extraction with a direct contact between the cells and the extraction phase: a) two-phase partitioning bioreactor; b) solvent-impregnated particles dispersed in bioconversion medium. In situ extraction with indirect contact: c) immobilised cells in the bioconversion medium; and d) using membrane-based extraction inside the bioreactor. In stream extraction with direct contact: e) pumping bioconversion broth to a column packed with solvent-impregnated particles. In stream extraction with indirect contact; f) membrane-based extraction outside the bioreactor; and g) introducing a microfiltration unit to separate the biocatalyst from broth before contact.

Because of the hydrophilic nature of short-chain carboxylic acids, and especially the hydroxylated acids like 3-HP, traditional liquid-liquid extraction suffers from poor extraction yield. This property can be related to the value of the logarithm of the partition coefficient of the corresponding acid in an octanol/water system (known as log $P_{o/w}$). If this value is negative, as is the case of 3-HP (-0.89) and lactic (-0.65) for example (Table 1.2), it means that the compound is more compatible with the aqueous phase and will have a low partition coefficient with an organic phase. For this reason, reactive extraction has been extensively studied for removal of organic acids from aqueous media (Datta *et al.*, 2015; Djas and Henczka, 2018) where the organic phase contains an *extractant* molecule able to react with the acid that improves significantly the extraction yield of the organic phase. Moreover, additional advantages can be attained when using this technique as an ISPR approach (Datta *et al.*, 2015):

i) the selectivity of the organic phase for the acids in the bioconversion medium
ii) a purified product solution is obtained
iii) pH control of the bioreactor without base solution addition
iv) reactive extraction is effective even at low acid concentrations
v) reaction equilibrium can be positively influenced
vi) reduction of downstream processing load and recovery costs

1.3.1. Organic phase composition for carboxylic acids reactive extraction

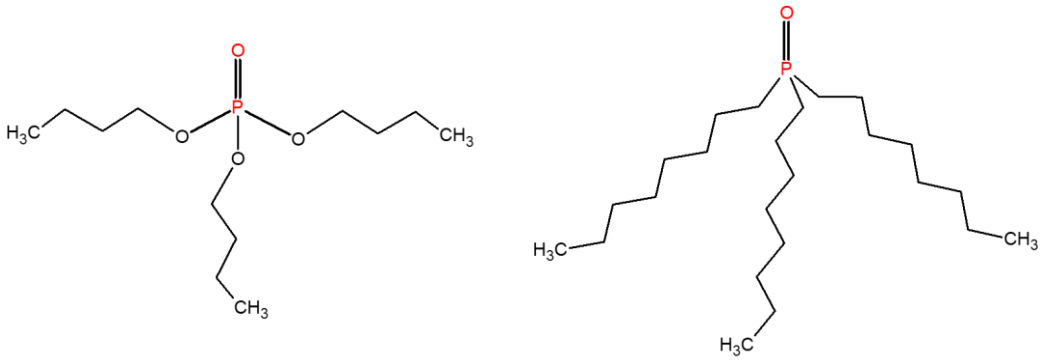
Numerous studies have shown that an adequate composition of the organic phase used for carboxylic acids extraction consists of a molecule that reacts with the acid (*extractant*) and a *diluent* able to stabilise the acid-*extractant* complex in the organic phase, and control at the same time the physical properties of the organic phase such as density, viscosity and surface tension (Datta *et al.*, 2015).

1.3.1.1. Extractants

Extractant is the name assigned to a molecule able to interact with the compound of interest in reactive extraction. According to Kertes and King (1986), *extractants* can be classified as three major types: i) carbon bonded oxygen bearing extractants, ii) phosphorus bonded oxygen bearing extractants, and iii) high molecular weight aliphatic amines. However, in the case of carboxylic acids extraction, molecules of the first category (such as long-chain alcohols), normally show a low extraction yield when used alone. Therefore another classification put these type of molecules in the category of *active diluents* or modifiers (Marinova and Yankov, 2009; Marták *et al.*, 1997).

1.3.1.1.1. Phosphorus bonded oxygen bearing extractants

The oxygen atom bonded to a phosphorus atom (P = O) in these molecules gives them the properties of a Lewis base. In fact, there is no chemical reaction in contact with carboxylic acids, however there is a strong and selective solvation process, especially when there is an interaction with weak carboxylic acids (Kertes and King, 1986). The two molecules most frequently used from this group are tri-butyl phosphate (TBP) and tri-octyl-phosphine oxide (TOPO) (Figure 1.6). An interesting characteristic of these molecules is that they have very good solvation properties, thanks to the high polarity of the P = O group. For example, TBF has a very high dipole moment (3.07 D) compared to water (1.85 D), which makes it a better solvation agent (Marcus, 1999). Because of this, phosphorus *extractants* are not sensitive to the type of diluents used, which permits to choose freely an adequate diluent focusing only in the physical properties of the organic phase.



Tri-butyl phosphate (TBP) Tri-octyl-phosphine oxide (TOPO)

Figure 1.6 Chemical structures of tri-butyl phosphate (TBP) and tri-octyl phosphine oxyde (TOPO)

The solvation interaction between an organic acid and the phosphorus extractant can be written as (Kertes and King, 1986):

$$[AH]_{aq} + p[S]_{org} \rightleftharpoons [AH\ S_p]_{org} \qquad \text{(Reaction 1.1)}$$

where $[AH]_{aq}$ is the organic acid in the aqueous phase, $[S]_{org}$ is the phosphorus extractant, p is the stoichiometric coefficient and $[AH\ S_p]_{org}$ is the complex solvated in the organic phase.

The extraction performance of an organic phase can be quantified by the distribution coefficient K_D of the corresponding acid in the organic phase and the aqueous phase. As mentioned before, these type of extractant are more effective with weak carboxylic acids. This was observed by Pagel and Schwab (1950), where propionic acid was the best extracted (Table 1.4). From this study, it could also be observed that monobasic acids are more easily extracted than dibasic ones. Interestingly, the presence of a hydroxyl group (as in lactic acid) decreases its extractability. In general, they are less efficient with hydrophilic organic acids. It has been also observed that these extractants need relatively high acid concentrations (around 1 mol L^{-1} or higher) in order to have an adequate efficiency, and also a high concentration of the extractant in the organic phase (Kertes and King, 1986). In general TOPO is more efficient than TBP, but it is also much more expensive.

Table 1.4 Distribution coefficient of organic acids extracted with tri-butyl phosphate (TBP)[a]

Organic acid	pKa	Log $P_{o/w}$[b]	Distribution coefficient K_D
Tartaric acid	3.01, 4.38	-1.91	0.6
Malic acid	3.22, 4.70	-1.26	1.3
Lactic acid	3.86	-0.72	1.4
Citric acid	3.13, 4.76, 6.40	-1.64	2
Maleic acid	1.93, 6.14	-0.48	3.6
Succinic acid	4.21, 5.64	-0.59	5.7
Propionic acid	4.85	0.33	8.4

[a] From Pagel and Schwab (1950), [b] from the EPI Suite experimental database (Syracuse Research Corporation and US Environmental Protection Agency, 2000).

1.3.1.1.2. High molecular weight aliphatic amines

This type of *extractants* has proven to be very effective for separation of carboxylic acids. Their extraction mechanism is different from that of phosphorus extractants, since there is a chemical reaction with the organic acid instead of solvation. Among aliphatic amines, primary amines are excessively soluble in water and therefore not suitable for the extraction of acids from an aqueous phase (Kertes and King, 1986). Secondary amines show a very good performance, with the highest reported distribution coefficients, but tend to form amides during the organic phase regeneration by distillation and form a gel-like third phase at the interface, which makes them potentially problematic for downstream processes

(Datta *et al.*, 2015; Kumar and Babu, 2008). Tertiary amines are the most effective thanks to their high extraction capacity, high selectivity and low water solubility (Kertes and King, 1986; Krzyzaniak *et al.*, 2011; Li *et al.*, 2016b; Tamada *et al.*, 1990). However, this is true only for tertiary amines having more than 6 carbons per chain (Kertes and King, 1986). The most frequently used tertiary amines have chain lengths between 8 – 10 C, such as trioctylamine (TOA, Figure 1.7) and Alamine 336 which is a commercial mixture of trialkylamines with C_8 – C_{10} chains (Sprakel and Schuur, 2019).

Figure 1.7 Comparison between the chemical structure of N-methyldioctylamine with tri-octylamine

The efficiency of a given tertiary amine depends on its structure, the nature of the acid, and contrary to phosphorus extractants, on the type of diluent used. Kaur and Elst (2014) evaluated the distribution coefficient K_D of itaconic acid with different amines and various diluents. They observed that for tertiary amines diluted in 1-octanol (*active diluent*), K_D increased with the length of their carbon chain, but when the chain was too long (12 C per chain), K_D was lower due to steric hindrance (Table 1.5). When the diluent was n-octane (*inert diluent*) K_D decreased with the chain length. Interestingly, N-methyldioctylamine has the highest K_D with the inert diluent, probably because the N group was more available to react with the acid (Figure 1.7). Kyuchoukov and Yankov (2012) observed the same behaviour with different amines diluted in oleyl alcohol for the extraction of lactic acid, where K_D decreased with the steric hindrance of the amines. This suggests that the use of tertiary amines with two long carbon chains and one short chain, could give better distribution coefficients. Evaluation of the extraction performances of other amines with these characteristics could contribute to discover better options for reactive extraction, for example N,N-didodecylmethylamine (DDMA) is a tertiary amine with two long chains (12 C) and one methyl chain which have a promising structure for high distribution coefficients. However, its use for reactive extraction has not been reported in the literature.

When amines are used alone, the acid-amine complex is not well solvated in the organic phase. For example, trioctylamine (TOA) has a dipole moment of only 0.8 D. In this case, the complex forms a third phase, leading to separation problems and low extraction yields (López-Garzón and Straathof, 2014). In addition, their high viscosity make mass transfer more difficult. In order to stabilise the complex in the organic phase and enhance the extraction yield, an *active diluent* with a functional group able to interact with the acid-base

complex is needed (Sprakel and Schuur, 2019).

Table 1.5 Distribution coefficient of an itaconic acid solution at 65 g L^{-1} for different amines diluted in two different diluents[a]

Tertiary amine (1 M)		1-Octanol	n-Octane
Name	**Carbon-chain length**		
Tributylamine	3 chains of 4 C	1.5	0.3
Trihexylamine	3 chains of 6 C	57	9
Trioctylamine	3 chains of 8 C	218	7
Tridodecylamine	3 chains of 12 C	132	3
N-methyldioctylamine	1 chains of 1 C, and 2 chains of 8 C	84	20
N,N-dimethyloctylamine	2 chains of 1 C, 1 chain of 8 C	5	0.2

[a] From Kaur and Elst (2014)

1.3.1.2. Diluents

A diluent in reactive extraction is an organic solvent used to control de physical properties such as viscosity, density and interfacial tension, which will impact the mass transfer and phase separation (López-Garzón and Straathof, 2014). The *extractant* molecule is preferably used together with the diluent in order to reduce the viscosity and corrosive nature of the organic phase. Moreover, in the case of extraction with amines, the diluent plays a very important role in the stability of the formed acid-amine complex (Datta *et al.*, 2015). Diluents can be divided into two types: active and inert.

1.3.1.2.1. Active diluents

Active diluents are organic solvents with a functional group, such as hydroxyl or carbonyl groups, that is able to interact with the organic acid (in the form of a complex and to a lesser extent in its free form) through hydrogen bonds and dipole-dipole interactions (Kertes and King, 1986; Tamada and King, 1990a). They are also known as modifiers, since their addition to an organic phase modifies its solvation properties, improving the extraction yield (López-Garzón and Straathof, 2014). This category includes chlorinated hydrocarbon, ketone, alcohol, and halogenated aromatic solvents. Nevertheless, for reactive extraction with amines, long-chain alcohols are among the best active diluents due to their polarity and specific H-bond donor character that favours complex formation and solvation (Tamada *et al.*, 1990; Tamada and King, 1990a; Uslu *et al.*, 2009).

1.3.1.2.2. Inert diluents

Inert diluents, also known as inactive diluents, are non-polar solvents such as alkanes, benzene and alkyl substituted aromatic compounds. In extraction with a solvating

extractant, these solvents can be used to decrease the viscosity and increase the interfacial tension of the organic phase, in order to facilitate mass transfer and phase separation during reactive extraction (López-Garzón and Straathof, 2014). In the case of extraction with amines, inert diluent addition results in lower extraction yields because it cannot interact with the amine-complex formed, thus it is not able to stabilise the complex in the organic phase. However, it could be used in combination with an extractant and an active diluent, to decrease viscosity and increase the interfacial tension, despite the decrease in the reaction yield. It has also been observed that it decreases the toxicity of the organic phase (Kumar *et al.*, 2012; Pérez-Ávila *et al.*, 2018; Wasewar *et al.*, 2011; Yabannavar and Wang, 1991a; Yamamoto *et al.*, 2011).

In fact, the reduction of the extraction capacity of the organic phase can be exploited for the back-extraction of the acid. The equilibrium curve can be shifted towards the aqueous phase by increasing the concentration of the inert diluent in the organic phase. With this method the organic phase can be regenerated for ulterior extraction (Chemarin *et al.*, 2019b; Han and Hong, 1996).

1.3.2. Reaction mechanisms of the extraction with amines

As previously mentioned, reactive extraction with amines presents interesting extraction performance for a wide range of carboxylic acids. They have been found to be more effective and less expensive than phosphorus extractants (Hong *et al.*, 2001; Wardell and King, 1978; Wasewar, 2012). Therefore, focus will be made on amines throughout the following sections. The reaction mechanism can vary depending on several other factors besides the structure of the amine used, such as the nature of the diluent and the type and concentration of the carboxylic acid to be extracted. The pioneer works of King and co-workers have elucidated many aspects of the reaction mechanism with amines using different carboxylic acids and diluents (Tamada *et al.*, 1990; Tamada and King, 1990a, 1990b), showing that there is a difference between complex formation with mono-carboxylic acids and di- or tri-carboxylic acids. In this section, focus will be made on monocarboxylic acids since the molecule of interest is the 3-HP.

Tertiary amines react with the non-dissociated form of the acid and create an acid-base complex that is insoluble in the aqueous media (Equation 1.2).

$$p[AH]_{aq} + q[R_3N]_{org} \rightleftharpoons [AH_p\ R_3N_q]_{org} \qquad \text{(Reaction 1.2)}$$

where $[AH]_{aq}$ is the organic acid in the aqueous phase, $[R_3N]_{org}$ is the tertiary amine in the organic phase, p and q are the stoichiometric coefficients, and $[AH_p\ R_3N_q]_{org}$ the complex formed, which is stable in the organic phase.

The nature of the acid-amine bond can be quite complex and vary depending on the nature of the phase. The interaction between the non-bonding pair of the amine and the proton of the acid can generate two main boundary forms: i) a hydrogen bond (Figure 1.8A) and ii) an ion pair related to the protonation of the amine (Figure 1.8C).

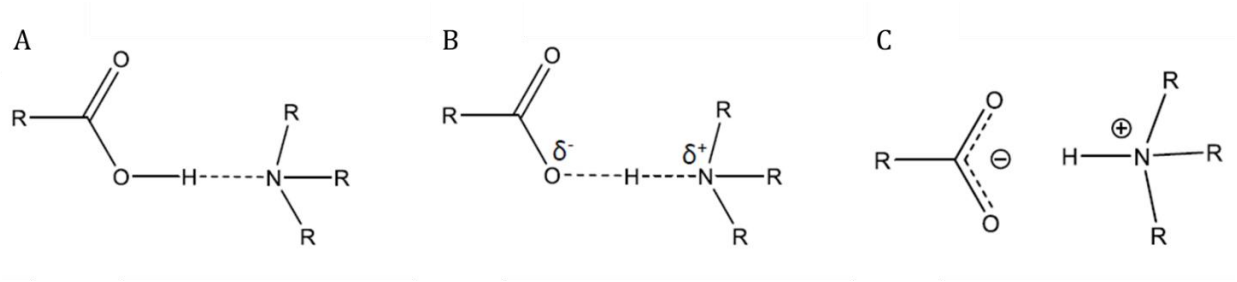


Figure 1.8 Representation of the different boundary forms of the acid-amine bond: A) hydrogen bond, B) intermediate bond with partial charges and C) ionic bond.

All these forms are present in equilibrium and the privileged form depends on the nature of all the chemical species involved. The type of bond depends on the acid strength, for example if the acid is strong it will have more tendency to form an ionic bond with the amine. Also, the type of diluent plays a very important role in the formation of the complex. An active diluent with a H-bond donor ability will promote the separation of the charges and the formation of the ionic bond, while an inert diluent will have the opposite effect (Tamada and King, 1990a). Long-chain alcohols have a very good H-bond donor ability, this is why they are preferred for acids reactive extraction with amines, since they promote a strong ionic bond, facilitating the acid-base complex formation. It has been observed that complexes formed with monocarboxylic acids may present a 1:1 acid-amine composition, but also have tendency to form complexes with a 2:1 stoichiometry. In the 2:1 complex formation, the first acid forms an ionic bond with the amine and the second acid forms a H-bond with the conjugated C=O of the complex (Figure 1.9B). This behaviour is diluent-dependent and it has been found that H-bond donor diluents inhibit the formation of 2:1 complexes, favouring 1:1 configuration (Figure 1.9A), because the H-bond of the active diluent compete with the interaction between the two acids. On the contrary, when the diluent is inert, interactions between the acid molecules are favoured (Tamada *et al.*, 1990).

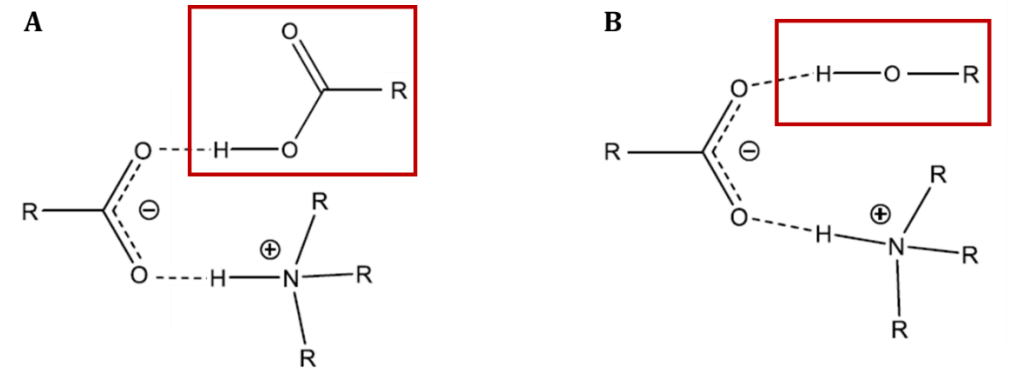


Figure 1.9 Acid-amine complex formation: A) when there is no active diluent in the organic phase, acid molecules (encased in red) are favoured to form H-bonds with the complex. B) When there is an active diluent (encased in red), it competes with the acid to form H-bonds with the complex.

The type of diluent also affects the stability of the complex in the organic phase. When an active diluent is used, in addition to the polarity that provides a good solvating medium for

the ion pair (as in the case of ketones, nitrobenzene, and halogenated aromatic solvents), specific H-bonding between the diluent and the acid-amine complex explains the strong solvation ability of molecules like long-chain alcohols. In contrast, when an inert diluent is used, the organic phase does not have the appropriate solvation properties, leading to low extraction yields and the formation of a third layer between the aqueous phase and the bulk organic phase. The third layer contains most of the extracted acid, amine and water. This phenomenon is also observed when pure amine or a high concentration of amine is used with an active diluent (Tamada and King, 1990a).

1.3.2.1. Reactive extraction of 3-hydroxypropionic acid with trioctylamine

The mechanism of the reactive extraction of 3-HP with tri-octylamine (TOA) diluted in long-chain alcohols has been previously studied in detail in our laboratory (Chemarin *et al.*, 2017b, 2017a). In this system, the mechanism for the acid-amine complex formation is as follows:

$$AH_{aq} \rightleftharpoons A^-{}_{aq} + H^+{}_{aq} \qquad \text{Reaction 1.3}$$

$$R_3N_{org} + H^+{}_{aq} \rightleftharpoons R_3NH^+{}_{org} \qquad \text{Reaction 1.4}$$

$$R_3NH^+{}_{org} + A^-{}_{aq} \rightleftharpoons R_3NH^+A^-{}_{org} \qquad \text{Reaction 1.5}$$

It should be noted that the stoichiometry of the acid-amine complex is 1:1 with this combination of solvents (Chemarin *et al.*, 2017a). A schematic representation of 3-HP reactive extraction with TOA is given in Figure 1.10. The acid is initially in the aqueous phase and the organic phase is composed of TOA and a long-chain alcohol (Figure 1.10A). First, the non-dissociated form of the acid AH_{aq} reacts with TOA at the interface, protonating the amine and creating an ionic bond (Figure 1.10B). The acid-amine complex is then formed, which is soluble in the organic phase. In addition, the long-chain alcohol form H-bonds with the complex, facilitating its stabilisation in the organic phase (Figure 1.10C). Solvation of the free acid by the long-chain alcohol also occurs, the physical extraction of 3-HP contributes to the overall extraction yield (Figure 1.10C). When polar solvents such as long-chain alcohols are used alone, acid partitioning is independent of its concentration (Reaction 1.6) (Kertes and King, 1986).

$$AH_{aq} \rightleftharpoons AH_{org} \qquad \text{Reaction 1.6}$$

The contribution of each chemical species involved in this reaction system has been elucidated (Chemarin *et al.*, 2017b, 2017a). The most remarkable findings are summarised below.

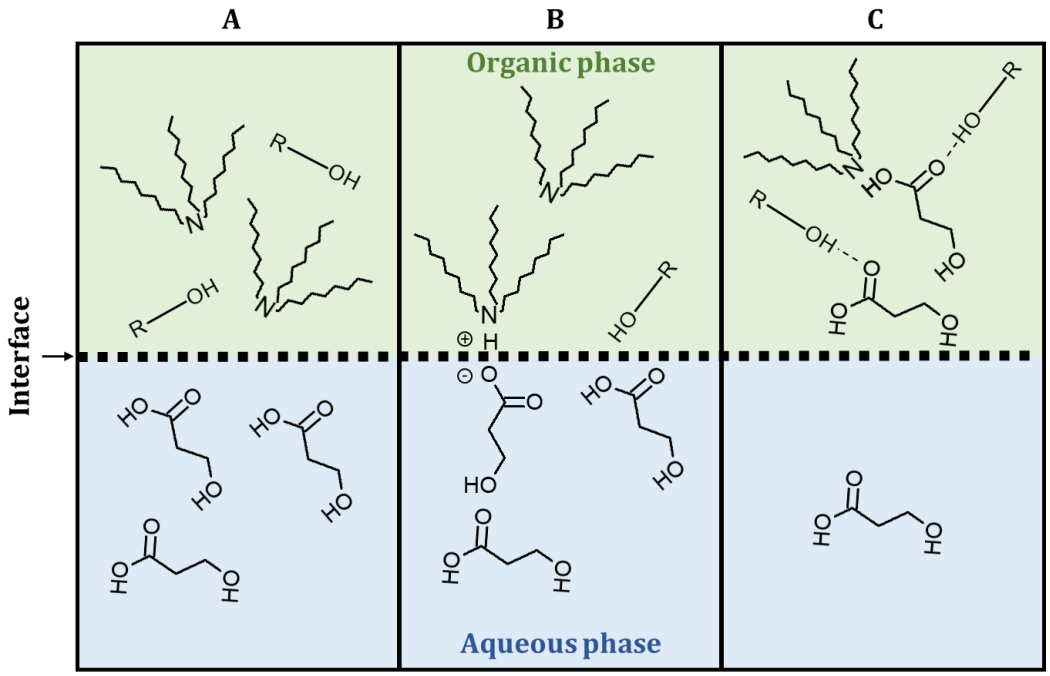


Figure 1.10 Schematic representation of 3-HP reactive extraction with TOA diluted in a long-chain alcohol. A) 3-HP is dissolved in the aqueous phase and the organic phase contains TOA and a long-chain alcohol. B) Ion-pair formation between 3-HP and TOA. C) The acid-amine complex is solvated by the long-chain alcohol in the organic phase. Physical 3-HP extraction by the long-chain alcohol also occurs with limited extent.

1.3.2.1.1. Organic phase composition

The organic phase composition is a key aspect on reactive extraction, as previously mentioned, since extraction with amines is extremely dependent on the diluent used. The structure of the amine is also very important, however, most of the studies of 3-HP reactive extraction with amines have been performed with TOA (Burgé *et al.*, 2016, 2017; Chemarin *et al.*, 2017b, 2017a, 2019a, 2019b; Matsumoto *et al.*, 2017; Moussa *et al.*, 2016). Although some studies revealed the synergistic effect of using Aliquat 336, a quaternary ammonium salt, together with TOA diluted in decanol (Burgé *et al.*, 2016; Moussa *et al.*, 2016). However, due to the high toxicity (Seevaratnam *et al.*, 1991) and elevated price of Aliquat 336, its utilisation for ISPR is not recommended.

The impact of the type and concentration of the diluent used has been elucidated. In the study made by Chemarin *et al.* (2017b) a set of 11 solvents comprising the categories of long-chain alcohols, esters and alkanes were tested in a mixture with 20% v/v of TOA. Among them, long-chain alcohols gave the best extraction performances and the extraction yields decreased with the carbon-chain length: 91% with hexane (6 C) and 53% with oleyl alcohol (10 C). Hexane and octanol (8 C) gave the best extraction yields but their excessive solubility in water (5 900 and 540 mg L^{-1} at 25 °C, respectively) represent a risk of solvent toxicity towards the 3-HP producing microorganisms. Therefore, decanol was considered to display a good compromise between extraction performance and solubility in water (37

mg L^{-1}).

TOA concentration in the organic phase was also evaluated using decanol as diluent. Results showed a bell-shape behaviour (Figure 1.11): the extraction yield increased with TOA concentration at lower concentrations, until an optimum value at 20% v/v TOA (extraction yield = 70%), and then it decreased with further increase in TOA concentration. Extraction with n-decanol at 100% corresponded to a partition coefficient of 0.02, which correspond to the physical extraction (Reaction 1.6). This low value confirms that 3-HP extraction needs to be driven by a chemical reaction (Chemarin *et al.*, 2017b). Oleyl alcohol (was also used at different concentration with TOA, showing the same bell-shape behaviour but with a lower optimum extraction yield (53% at a TOA concentration of 20% v/v, Figure 1.11).

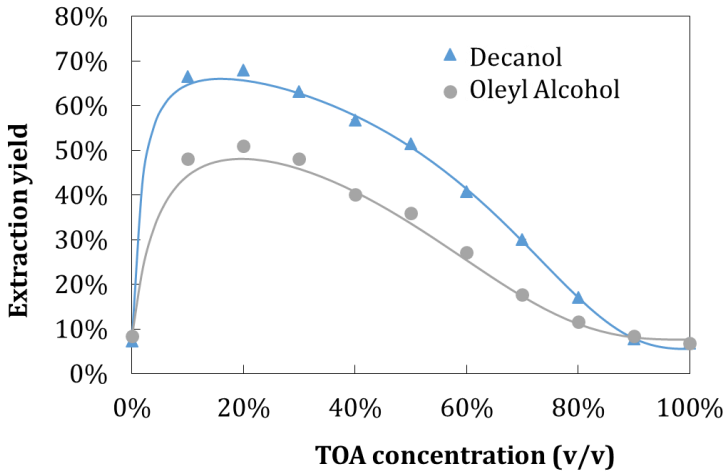


Figure 1.11 3-HP extraction yields for different TOA concentrations in n-decanol and oleyl alcohol. From Chemarin *et al*. (2017b).

The organic phase composed of TOA and decanol was then used to study the acid-amine complexation mechanism during 3-HP reactive extraction (Chemarin *et al.*, 2017a). It was shown that the stoichiometry (1:1) is privileged in this organic phase composition when the amine is in excess compared to 3-HP concentration. Two different mechanistic models were proposed to account for the experimental results using different TOA concentrations in the organic phase and 3-HP concentrations in the aqueous phase. The first model considers the diluent (decanol) as a reagent that is involved in the complexation as a synergistic extractant, but with competition for solvation via H-bond interactions with TOA; the second model considers the diluent as a phase modifier that improves the physicochemical properties of the extractant and that changes the complexation equilibrium. Both models described the experimental results with high accuracy, but the second model the second model was preferred because solvating properties of the diluent were better described as non-stoichiometric interactions.

1.3.2.1.2. Aqueous phase composition

The composition of the aqueous phase also affects the reactive extraction performance. Several aspects have been evaluated in our laboratory with the aim of elucidating the effect

of a real bioconversion medium during 3-HP extraction. Comparison with its positional isomer (lactic acid, 2-HP), as well as the effect of initial acid concentration in the aqueous phase, pH of the medium, and the presence of bioconversion medium components such as dissolved salts and proteins have been studied. This has given valuable information for the design of an integrated process for 3-HP extractive bioconversion.

Nature of the acid. As mentioned before, the nature of the acid influences its extractability from aqueous media. Hydroxy-acids such as 3-HP and lactic acid (2-HP), are more difficult to extract than their equivalents without OH, because they are more hydrophilic. It has been observed that even the change in the position of the OH group dramatically changes the extraction yield at the same conditions. In fact, lactic acid is better extracted than 3-HP which was attributed to the stronger acidity of lactic acid ($pK_{A2\text{-}HP}$ = 3.86 *vs*. $pK_{A3\text{-}HP}$ = 4.51), which has a greater tendency to dissociate (Reaction 1.3) and favours the ion pair formation with the amine (Reactions 1.4-1.5). In addition, lactic acid ($\log K_{o/w}$ = -0.65) is less hydrophilic that 3-HP ($\log K_{o/w}$ = -0.89), which means that it will be better solvated in the organic phase. Indeed, lactic acid was better extracted (K_D= 8.8) than 3-HP (K_D= 2.5) with an organic phase composed of 20% v/v TOA in decanol, at the same conditions (Moussa *et al.*, 2016).

Initial acid concentration. Normally, at low initial acid concentrations the extraction yield is higher and it decreases with the increase of acid concentration. This is because the amine is more in excess at lower acid concentrations. This was observed for 3-HP in the concentration range of 10 – 50 g L^{-1} (Chemarin *et al.*, 2017b). However, for the lowest concentrations between 0.4 – 2 g L^{-1}, the extraction yield decreased. This was explained by the presence of water soluble impurities from the TOA, such as octylamine and dioctylamine. These molecules pass to the aqueous phase and neutralise the acid that can be no longer extracted, which decreases the extraction yield at equilibrium. Once the TOA was further purified, the extraction yield values were more or less constant in the concentration range of 0.4 – 7 g L^{-1} and they decreased at higher acid concentrations. Results at higher acid concentrations were very similar with the TOA at different purities, because there was much more acid than impurities and their effect was not significant.

Initial pH of the aqueous phase. Reactive extraction with amines is strongly affected by the pH of the solution because only the non-dissociated form of the acid can be extracted (Kertes and King, 1986). Reactive extraction of a 3-HP solution at 1 gL^{-1}, with an organic phase composed of 20% v/v TOA and n-decanol was performed, at different initial pH values (Chemarin *et al.*, 2019a). As expected, higher pH values lead to lower extraction yields, the obtained yields were 74% (pH =3.2), 43% (pH = 4) and a very limited extraction at pH 5 (extraction yield = 5%). Indeed, the non-dissociated form at this pH is only of 24% (Chemarin *et al.*, 2019a). This highlights the importance of using microorganisms that are tolerant to low pH, in order to have a successful integration of reactive extraction with bioconversion. In the case of 3-HP production, a pH lower than its pK_A (4.51) is preferred during bioconversion for a good extraction performance.

Components found in a real bioconversion medium. The ions Cl^- and $H_2PO_4^-$, are frequently found in bioconversion medium compositions because of the utilization of salts

to regulate the osmotic pressure. It has been observed that the presence of these ions in the aqueous phase hinders the extraction of 3-HP (Chemarin *et al.*, 2019a). They have been shown to induce an ion exchange with the organic acid in the complex, as described in Reactions 1.7 and 1.8.

$$R_3NH^+A^-{}_{org} + Cl^-{}_{aq} \rightleftharpoons R_3NH^+Cl^-{}_{org} + A^-{}_{aq} \qquad \text{Reaction 1.7}$$

$$R_3NH^+A^-{}_{org} + H_2PO_4^-{}_{aq} \rightleftharpoons R_3NH^+H_2PO_4^-{}_{org} + A^-{}_{aq} \qquad \text{Reaction 1.8}$$

The subsequent release of the carboxylate conjugate of 3-HP in the aqueous phase increases the pH of the aqueous medium, lowering the final extraction yield. It was also observed that the extraction yield values are less affected by $H_2PO_4^-$ than with Cl^-.

In the case of proteins contained in the bioconversion medium, no significant effect was observed in the extraction yield between the presence or absence or proteins (albumin from bovine serum) at a concentration of 8 mg L^{-1} (Chemarin *et al.*, 2019a). Glycerol and other components frequently used in 3-HP bioconversion mediums did not have a significant effect either. In fact, reactive extraction showed to have a great selectivity for 3-HP against the metabolites found in glycerol bioconversion with *L. reuteri* such as 1,3-propanediol and 3-hydroxypropionaldehyde (Burgé *et al.*, 2016; Moussa *et al.*, 2016).

1.3.2.2. Back-extraction

As mentioned before, the acid-amine complex formed during reactive extraction is reversible and there are several factors that play against this complex stabilisation in the organic phase, decreasing extraction yields. These factors can be used for the regeneration of the organic phase and simultaneous recovery of the acid in another aqueous phase. This is frequently called back-extraction in the literature (López-Garzón and Straathof, 2014; Malmary *et al.*, 2001; Tamada and King, 1990a). There are different strategies proposed in the literature to recover organic acids from amines extracting systems. They can be separated into three categories:

Chemical swing. A chemical compound with more affinity for the tertiary amine can be used to recover the carboxylate ion from the acid-amine complex. To this end, the use of salts for ion-exchange can be applied, especially when the amine is a quaternary salt such as Aliquat 336 (Coelhoso *et al.*, 1997). But they can also be applied for tertiary amines. An original approach is to use an acid with better affinity for the amine such as HCl to displace the organic acid. The desired acid is released in the aqueous phase in its acidic form. Then, the organic phase can be regenerated by distillation (Ricker and King, 1980). Another widely reported method is the use of a basic water soluble compound like NaOH, a carbonate or tri-methylamine (TMA) (Ahsan *et al.*, 2013; Harington and Hossain, 2008; Jarvinen *et al.*, 2000; Keshav *et al.*, 2009). In aqueous phase, these compounds impose a high pH that induces the release of the acid under its dissociated form. The advantage of this method is that the organic phase is directly regenerated, which permits continuous extraction and back-extraction. The disadvantage is that the acid is recovered in the conjugated salt of the used base. The use of TMA is attractive because it could be easily

distilled from the aqueous phase in order to obtain the pure acid.

Diluent swing. This method consist in adding a diluent, frequently called "anti-solvent", to decrease the affinity of the organic phase for the acid (Han *et al.*, 2000; Tamada and King, 1990b; Yankov *et al.*, 2004). With this, the organic phase will reject the acid in the aqueous phase (Keshav and Wasewar, 2010). The organic phase can then be regenerated by distillation of the anti-solvent (if it is more volatile) which is then recycled.

Temperature swing. This method relies on the thermal reversibility of the acid-amine complexation reaction (Tamada and King, 1990b). Increasing the temperature of the organic phase in the presence of an aqueous phase can lead to the dissociation of the complex into its two components: the acid in the aqueous phase and the amine in the organic phase. Therefore, heating a loaded organic phase in contact with an aqueous phase can lead to some back-extraction. This method is attractive as it does not need the addition of any chemicals and allows the recovery of the protonated acid.

A quite complete back-extraction study of 3-HP from the organic phase was previously made in our laboratory (Chemarin *et al.*, 2019b). Strategies from the three different types of back-extraction were explored to recover 3-HP from an organic phase composed of 20% v/v TOA in decanol: base and salts addition, diluent-swing with hexane, and temperature swing was explored using temperatures from 4 to 140 °C. All of the strategies tested turned out to be fairly efficient for 3-HP. This was attributed to the high hydrophilic character of this molecule. Mineral base addition such as NaOH and Na_2CO_3 permitted to recover nearly all the 3-HP present initially (≥ 98%). Hexane addition at 60% gave a recovery yield of 83%, while the temperature swing approach was the less efficient with a 3-HP recovery of 78% at 140°C. However, the three strategies gave promising results and could be applied depending of the envisaged integrated process.

1.3.3. Toxicity of the organic phase

Although reactive extraction can provide high extraction performance and selectivity, the solvents toxicity towards microbial strains is the main drawback for its utilisation for ISPR. The presence of an organic solvent in the medium can generate cell membrane alteration affecting the functionality of the membrane-bounded proteins or causing membrane rupture and metabolite leakage. These effects are often followed by cell lysis and death (Gu *et al.*, 1998; Marinova and Yankov, 2009; Ramos *et al.*, 2002). Solvent toxicity is considered to affect cells in two different levels: by direct contact between the cells and the solvent at the aqueous- organic interface (phase level toxicity); and the organic solvent molecules that are soluble in the aqueous medium (molecular level toxicity). Some solvents can be toxic at a phase-level, but non-toxic at the molecular level (Bar and Gainer, 1987; Marinova and Yankov, 2009).

A wide number of studies have evaluated the toxicity of different extractants and diluents used for reactive extraction towards carboxylic acid producing strains (Burgé *et al.*, 2017; Choudhury *et al.*, 1998; Marinova and Yankov, 2009; Matsumoto *et al.*, 2004; Pérez-Ávila *et al.*, 2018; Seevaratnam *et al.*, 1991; Solichien *et al.*, 1995; Yabannavar and Wang, 1991a).

Toxicity evaluation is performed by different methods in these studies, for example: i) by measuring the growth ability (Solichien *et al.*, 1995; Yabannavar and Wang, 1991a), ii) the ability to consume a substrate or produce the metabolite of interest (Bar and Gainer, 1987; Matsumoto *et al.*, 2004), and more recently iii) the assessment of the physiological state of the cell by evaluation of its enzymatic activity and membrane integrity (Burgé *et al.*, 2017) when cells are in contact with the organic solvents.

Toxicity depends on the type of organic solvent. In general, the molecules used as extractants such as amines and phosphorus molecules have presented high toxicity towards acid producing cells (Marinova and Yankov, 2009; Roffler *et al.*, 1984). Therefore, the use of an adequate diluent can contribute not only to improve the physical properties and extraction performance of the organic phase, but also to decrease its toxicity. However, active diluents such as alcohols have been reported to be highly toxic at phase and molecular levels (Marinova and Yankov, 2009; Ramos *et al.*, 2002). For example, solvents with the best extraction performances were also the most toxic towards the acid lactic producing strain *Lactobacillus delbrueckii* (Roffler *et al.*, 1984). On the other side, inert diluents such as alkanes, paraffin (or kerosene) and vegetable oils are generally less toxic or show no toxicity towards cells (Choudhury *et al.*, 1998; Gu *et al.*, 1998; Marinova and Yankov, 2009; Roffler *et al.*, 1984). In fact, it has been observed that solvents with poor extraction ability but good biocompatibility can be added to a toxic solvent with good extraction performance to reduce the overall toxicity (Kumar *et al.*, 2012; Pérez-Ávila *et al.*, 2018; Wasewar *et al.*, 2011; Yabannavar and Wang, 1991a; Yamamoto *et al.*, 2011). A compromise between extraction performance and toxicity should thus be found.

Solvent toxicity strongly depends on the intrinsic tolerance of the microbial species and strains. In fact, a solvent can affect differently cells even of the same specie but from two different strains. For example, dodecanol resulted to be highly toxic for the strain *Lactobacillus fructivorans* NRIC 0224 but resulted to be non-toxic to *Lactobacillus fructivorans* NRIC 1814 (Matsumoto *et al.*, 2004). Therefore, generalities cannot be made regarding solvent toxicity. However, a parameter that can be used as a guide for the organic phase selection is the polarity of the solvent. In fact, toxicity have been related to the log $P_{o/w}$ of the solvent (Fernandes, 2003; Isken and de Bont, 1998; Laane *et al.*, 1985; Ramos *et al.*, 2002; Sardessai and Bhosle, 2002). In general, solvents with a log $P_{o/w}$ below 4 such as octanol (log $P_{o/w}$ = 2.68), are considered extremely toxic towards cells; while solvents having values higher than 6 are considered as non-toxic (Laane *et al.*, 1985). However, solvents with log $P_{o/w}$ values in the range of 4 – 6 can have very different effects according to the type of molecule and the involved strain.

In some cases toxicity may be caused by impurities contained in the solvent and not by the main molecule. For example, Gao *et al.* (2009b) found that using tridecylamine (TDA) in oleyl alcohol for the extractive bioconversion of lactic acid by *Saccharomyces cerevisiase* was very toxic for the strain. However, they discovered that the toxicity was not caused by TDA as suspected initially, but to the presence of decylaldehyde at a concentration of 700 ppm. They reduced the aldehyde concentration to 33 ppm and the toxicity of the amine became insignificant. This gives awareness of the importance for amine and other solvents

purity, not only to enhance its extraction performance (Chemarin *et al.*, 2017b), but also as a method to decrease its toxicity.

Given the inherent moderate-to-high toxicity of the solvents frequently used in reactive extraction, such as tertiary amines and alcohols, direct contact with the producing cells should be avoided in order to diminish the phase-level toxicity which is the most harmful. In addition, some solvents form emulsions with the bioconversion medium which prevents the implementation of continuous processes and requires additional steps to break the emulsion. The contact between two liquid phases without dispersion can be achieved by the use of membrane contactors (Huang *et al.*, 2004).

1.3.4. Reactive extraction in membrane contactors

1.3.4.1. Characteristics of membrane contactors

Membrane contactors are devices used to put two fluids in contact, with the support of a porous membrane, promoting mass transfer between them. When both fluids are liquids, the interface formed is stabilised inside the membrane pores, which avoids mutual dispersion of the phases. In principle, the liquid with more affinity for the membrane material will wet and fill the pores of the membrane while the other fluid will remain at the pore entrance (Figure 1.12). To prevent the wetting liquid from passing through the membrane, a higher pressure must be applied by the non-wetting liquid in order to block the exit from the pores. If the difference of pressure applied on both sides is not high enough then the wetting fluid will pass through the membrane pores and disperse in the other fluid. However, if the difference of pressure is too high, the non-wetting fluid will be forced to pass through the membrane pores and will be dispersed in the wetting fluid (Prasad and Sirkar, 1987). Membranes can be either hydrophobic or hydrophilic, and this will determine which liquid will fill the pores. If the membrane is hydrophobic, an organic liquid will be the wetting phase and if the membrane is hydrophilic, an aqueous phase will fill the pores.

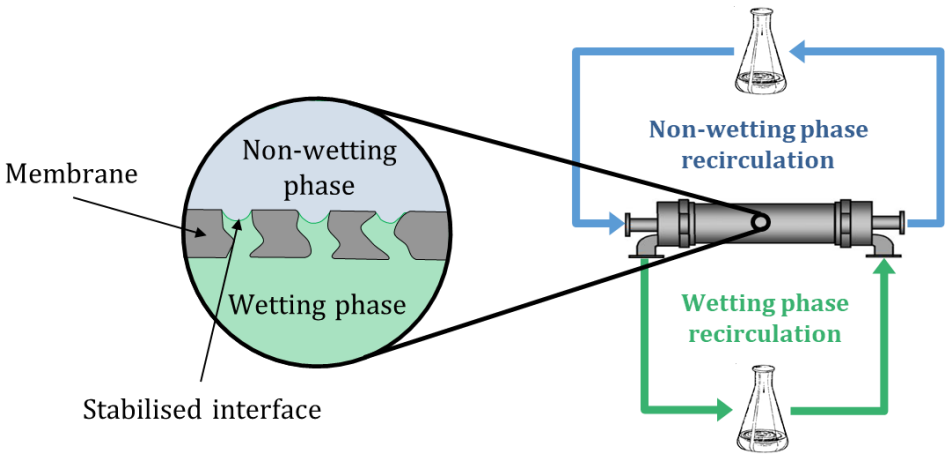


Figure 1.12 Schematic representation of a membrane contactor. The interface is stabilised inside the membrane pores.

The pressure difference at which the non-wetting fluid starts to pass through the membrane

pores is called critical pressure (ΔP_C) and can be estimated by the Laplace equation:

$$\Delta P_C = -\frac{2\gamma \cdot \cos\theta}{R} \tag{1.1}$$

where γ is the interfacial tension between both liquids, θ is the contact angle at the triple point between the two fluids and the membrane material, and R is the radius of the considered pore. In practice, it is better to consider the largest pore size and not the average value, in order not to exceed the lowest critical pressure.

Among other applications, these devices have been used for putting the organic and aqueous phases in contact for reactive extraction of several carboxylic acids (Schlosser *et al.*, 2005), revealing several advantages compared with dispersive extraction (Gabelman and Hwang, 1999; Nelson *et al.*, 2017):

i) Emulsion formation does not occur, since there is no dispersion of the liquids.

ii) For extractive bioconversions, direct contact between the microorganisms and the organic phase can be avoided, decreasing the phase-level toxicity.

iii) Fluids of identical density can be operated in any orientation.

iv) Substantially higher efficiency is achieved with membrane contactors than with dispersive contact, due to higher interfacial area.

v) The available surface area for mass transfer remains undisturbed at different flow rates of the liquids. Moreover the interfacial area is known and constant, which allows performance to be predicted more easily.

vi) Solvent holdup is low, an attractive feature when using expensive solvents.

vii) Simultaneous regeneration of the organic phase is possible with the use of two membrane modules: one module for extraction and other module for back-extraction using a stripping phase. Both modules are fed with the same organic phase which is put in contact with the acid to be extracted and the stripping phase simultaneously.

viii) They offer a great compactness (high interfacial area in relatively small volumes) generally between 1000 – 7000 m^2 m^{-3}.

ix) Scale up is more straightforward since the increase in capacity is achieved simply by adding membrane modules, while paying attention to filling volume.

These devices also present some disadvantages and potential difficulties that need to be taken into account (Gabelman and Hwang, 1999):

i) The membrane introduces another resistance to mass transfer not found in dispersive extraction. However, steps can be taken to minimise it.

ii) The system can be sensitive to pressure drop, which affects the interface stabilisation.

iii) Membranes with small and monomodal pore size are not obvious to find at commercial level

iv) Membranes have a finite life, so that the cost of periodic membrane replacement needs to be considered.

v) Some membranes use an adhesive to secure the membrane to the tube sheet that may be vulnerable to attack by organic solvents.

vi) Some membrane material is subjected to swelling behaviour in contact with solvents

Although these disadvantages are frequently overweighed by the advantages mentioned before, the successful implementation of membrane contactors remains dependent on the specifications of each application. The most studied contactor geometry is the hollow fibres membrane contactor (HFMC) because it offers the greatest compactness. HFMC have a high density of the fibre network in the module, which is advantageous for potential industrial applications (Gabelman and Hwang, 1999). The configuration is similar to a shell-and-tube heat exchanger. The fibres run axially through the shell-side. One fluid flows through the fibres, another through the shell and contact is made at the pores of the fibres membrane.

1.3.4.2. 3-HP reactive extraction assisted by membrane contactors

3-HP reactive extraction has also been evaluated in our laboratory using a hollow fibre membrane contactor (HFMC) (Burgé *et al.*, 2016; Chemarin *et al.*, 2019a). Different conditions like initial 3-HP concentration, initial pH of the aqueous phase and the presence of components found in real bioconversion media such as proteins and ions from salts were evaluated. The effects of these factors on the extraction yield at equilibrium were the same as those described in Section 1.3.2.1.2. However, changes in the extraction kinetics (evaluated using the time needed to reach 63% of the equilibrium extraction yield, $\tau_{63\%}$) were observed at different conditions. For comparison, the dimensionless 3-HP concentration (C/C_0) was calculated within time for each experimental condition:

Initial 3-HP concentration. It was observed that in the range of 0.5 – 10 g L^{-1}, the relative extraction kinetic was slower at increasing concentrations. A possible explanation is that at lower concentrations the amine is more in excess, resulting in a higher complexation yield, accelerating the extraction. Moreover, it was observed that the viscosity of the organic phase increases significantly at higher 3-HP concentrations (up to a 50% increase from 0 to 30 g L^{-1} of 3-HP for 20% TOA in n-decanol) (Chemarin, 2017). This indicates that mass transfer inside the membrane pores (which are filled with the organic phase) will be slowed down at higher 3-HP concentrations.

Initial pH of the aqueous phase. An interesting behaviour was observed: when TOA was used as the extractant, at higher initial pH the extraction yield was lower but the relative extraction kinetic was faster. The effects are comparable with the initial concentration effects. As TOA is only able to extract the non-dissociated form of the acid, the amount of acid available for extraction decreases at higher pH: 97% at pH 3.2 and only 24% at pH 5 (Chemarin *et al.*, 2019a). This results in lower extraction yields since the dissociated form of the acid remains in the aqueous phase, but also in faster kinetics because of a lower

organic phase viscosity, as suggested before.

Ions from salts. As explained in Section 1.3.2.1.2, ions such as Cl^- and $H_2PO_4^-$ hinder the extraction of 3-HP. However, their effect on the extraction kinetics is more complicated to elucidate due to the ion exchange phenomenon. 3-HP is initially extracted in the organic phase, mainly in the form of a complex with TOA. Then, the complex undergoes an ion exchange with Cl^- or $H_2PO_4^-$, liberating 3-HP again in the aqueous phase in its carboxylate form, which increases the pH of the aqueous phase (Chemarin *et al.*, 2019a). In fact, the use of salts for acid back-extraction from the organic phase is based in this phenomenon.

Organic impurities. During experiments with ion salts addition, a further increase in 3-HP concentration and pH was observed after 100 min of extraction. This increase could not be related to an anion exchange mechanism or to non-dissociated 3-HP, since their concentrations remained essentially constant over time after 100 min. So it could only be related to the carboxylate form of 3-HP. In this case, a possible explanation is that this phenomenon is mainly due to impurities present in TOA consisting of n-octylamine (OA) and di-n-octylamine (DOA) (Chemarin *et al.*, 2017b). Results showed that 3-HP was first extracted in the organic phase, forming a TOA complex which then was rearranged to form the n-octylammonium salt with the residual OA, and the salt was then released in the aqueous phase (Chemarin *et al.*, 2019a). This explains the delayed increase on 3-HP concentration and pH.

These results highlight some key aspects to take into account for the implementation of ISPR of 3-HP. For example:

i) Fast extraction could be achieved even at lower 3-HP concentrations in the aqueous phase.

ii) The pH of the bioconversion medium should be preferably lower than the pK_A of the acid.

iii) Ions from salts should be maintained at low concentrations in the bioconversion medium to avoid the competition by ion exchange.

iv) The presence of impurities from the amine must also be maintained as low as possible to avoid the release of mono- and di-octylammonium salts in the bioconversion medium.

v) The organic phase regeneration is crucial to maintain the highest possible driving force for continuous extraction.

vi) The impact of substrates in the bioconversion medium that can interfere with the extraction of the acid should be also taken into account.

1.3.4.3.Extractive fermentation and bioconversion using reactive extraction assisted by membrane contactors

Reactive extraction assisted by membrane contactors has already been implemented for extractive fermentation and bioconversion of organic acids (Table 1.6). Extractive

fermentation of lactic, propionic, butyric and hexanoic acid resulted in productivity improvement compared to the control. The authors attributed such results to an alleviation of inhibition by product since lower acid concentration were maintained in the medium. In addition, the pH of the fermentation medium was regulated by the continuous removal of the corresponding acid, without addition of a base solution. Jin and Yang (1998) showed the impact of the interfacial area available for acid extraction in the membrane modules. They increased 7.3-fold the specific contact area per fermentation volume (m^2 m^{-3}) by decreasing the fermentation volume from 2.2 to 0.3 L. A higher productivity was achieved (Table 1.6), which was attributed to a lower acid concentration maintained in the medium. It could be observed that the pH was maintained at a higher value of 5.2 instead of 4.8, which is closer to the optimum value for *Propionibacterium acidipropionici* (pH = 6.0). Lactic acid (isomer of 3-HP) extractive fermentation was achieved using an organic phase composed of an *extractant* (Alamine 336), an *active diluent* (oleyl alcohol) and an *inert diluent* (kerosene) with permitted continuous lactic acid removal during 130 h. Moreover, they used two different pH during the overall process: the pH was maintained at 5 during initial cell growth by addition of NH_4OH, and they left the pH decrease to 4.3 to start the extraction. This improved the overall productivity of the process performed at pH 4.3 from the beginning (Chen and Lee, 1997). Extractive fermentation resulted in a 1.1-fold increase productivity compared to the process without extraction (Table 1.6).

An attempt to implement 3-HP extractive bioconversion with *L. reuteri* was also performed in our laboratory (Burgé *et al.*, 2017). Unfortunately, *L. reuteri* was strongly inhibited by the reactive extraction system. This resulted in lower productivity (Table 1.6) and bioconversion stopped after 1.5 h only. The toxicity of the organic phase composed of 20% TOA in decanol towards *L. reuteri* was evaluated in the HFMC. It appeared to be very high: a significant decrease in the percentage of viable cells was observed after 30 min and almost all cells lost their membrane integrity after 3 h. Experiments with decanol alone were very similar, therefore it was identified as the main cause of cells inhibition. In fact, the membrane contactor cannot prevent the molecular-level toxicity of the solvent. Therefore, a more biocompatible composition, with an adequate extraction performance is needed. However, *L. reuteri* is not very tolerant to low pH, decreasing significantly its production performance at pH lower than 5 (Burgé *et al.*, 2017). This makes bioconversion incompatible with the needs of reactive extraction since it was observed to be very limited a pH higher than 5. Another approach is to use a more resistant microbial strain. Genetically modified yeast strains are perhaps the most promising microbial agents for 3-HP production, because of their tolerance of lower pH lower than the pKa of the acid. However, the 3-HP production performances achieved so far with yeasts are low compared with the maximum obtained using bacterial strains (Section 1.2.2.2). The use of acetic acid bacteria (AAB) capable of oxidise 1,3-PDO into 3-HP has shown remarkable performances. Although this investigation line is relatively new and more research is needed, notably the resistance of AAB to ISPR, their use represents a promising approach in the context of integrated processes design for industrial 3-HP production.

Table 1.6 Extractive fermentations using reactive extraction assisted by membrane contactors

Organic acid	Microorganism	Culture mode	pH[a]	pK_A[b]	Organic phase	Back-extraction phase	Productivity (g L^{-1} h^{-1})	Comparison with control	Reference
Lactic acid	*Lactobacillus delbrueckii*	Fed-batch (1.3 L)	4.3	3.86	20% Alamine 336 40% Oleyl alcohol 40% Kerosene	5.0 N NaOH	0.14	1.1-fold increase	(Chen and Lee, 1997)
Propionic acid	*Propionibacterium acidipropionici*	Repeated batch (0.3 L)	5.2	4.85	4% Tridecylamine 96% Oleyl alcohol	6.0 N NaOH	0.98	5-fold increase	(Jin and Yang, 1998)
		Batch (2.2 L)	4.8				0.25	2-fold increase	
Propionic acid	*Propionibacterium thoenii*	Batch (volumen not indicated)	5.5	4.85	40% Tridodecylamine 60% Oleyl alcohol	-	0.46	1.1-fold increase	(Gu *et al.*, 1999)
Butyric acid	*Clostridium tyrobutyricum*	Repeated batch (2 L)	5.5	4.81	10% Alamine 336 90% oleyl alcohol	6.0 N NaOH	7.37	1.4-fold increase	(Wu and Yang, 2003a)
Butyric and Hexanoic acid	*Megasphaera elsdenii*	Fed-batch (1 L)	6.3	4.81 4.88	10% TOA 90% Oleyl alcohol	1.0 N NaOH	0.26	No control	(Nelson *et al.*, 2017)
3-HP	*Lactobacillus reuteri*	Batch (2 L)	6	4.51	20% TOA 80% Decanol	-	0.13	0.53-fold decrease	(Burgé *et al.*, 2017)

[a] Fermentation pH
[b] pK_A of the targeted acid

1.4. Conclusion of the literature review

This literature review elucidates some key aspects involved in the production and separation of bio-based organic acids. In general, high productivities and titres (around 2.5 g L^{-1} h^{-1} and more than 100 g L^{-1}) are needed for an economically viable process. Such performances have been achieved for most of the organic acids produced at industrial scale by microbial strains that are tolerant to low pH values. Most of these strains are genetically modified. In the case of 3-hydroxypropionic acid (3-HP), remarkable advances have been made and the production performances are getting closer to the desired for its production at industrial scale. However, there are still some challenges to overcome, such as the inhibition by 3-HP accumulation in the bioconversion medium and the difficulty of extracting 3-HP from the bioconversion broth at high purity and concentration.

Liquid-liquid reactive extraction is highlighted as one promising strategy for acid recovery that has already been applied for the successful recovery of organic acid produced at industrial scale, such as citric acid and lactic acid. The implementation of this recovery method performed in a hollow fibre membrane contactor (HFMC) has been studied for the continuous acid recovery from the bioconversion medium as an approach to alleviate the inhibition by product and simplify the subsequent purification process. Important advances have been made on the understanding of the chemical reaction and mass transfer mechanisms involved in this recovery method. This has led to the successful implementation for extractive bioconversion of some organic acids, such as hexanoic, butyric, propionic and lactic acid, resulting in improved productivity. However, several aspects of the specific case of bio-based 3-HP production and recovery still remain to be better understood in order to design an efficient integrated process. For example, to find an organic phase composition more biocompatible with the strain and permits continuous 3-HP removal from the bioconversion medium.

The literature review also highlighted the conditions needed to achieve continuous 3-HP removal during its production, such as a pH lower than the pK_A of the acid (and so the interest in using a low pH tolerant microorganism), an organic phase that is biocompatible with the producing strain and also presents a good extraction performance, and that the presence of compounds that can hinder the acid extraction (ion salts, other acids than the targeted one and impurities from the organic phase) should be maintained at low concentrations. Extractive fermentation performed for other organic acids give insights of some strategies to be implemented in the case of 3-HP. For example, an organic phase composed of an *extractant*, an *active diluent* and an *inert diluent*, seems to be a promising strategy to achieve biocompatibility and good extraction performance. Also, to initiate the bioconversion process at a higher pH that is more compatible for cell growth and then let the pH decrease to start the extraction. All the identified aspects to be further optimised and strategies are addressed in this PhD work.

Chapter 2
Global experimental approach

This chapter describes the overall methodology followed, indicating the position of this work in the global project strategy. The chemicals, materials and analytical techniques used throughout this PhD work are also detailed. The specific experimental approach of each stage will be detailed in its corresponding chapter.

2.1. Overall methodology

The general methodological approach of the current PhD work was proposed in order to fulfil the specific objectives mentioned in the introduction of this PhD work. A schematic representation of the overall methodology is given in Figure 2.1, where each stage is related to its corresponding specific objective.

The first specific objective is related to a better understanding of 3-HP reactive extraction with tertiary amines. For this, an analysis of the reactive extraction mechanism at the liquid-liquid interfacial scale was performed through modelling approach. Measurements of the dynamic IFT were performed using the pendant drop method. It was assumed that this method's setup was an analogous representation of the stabilised interface inside the pores of a hollow fibres membrane contactor (HFMC). A predictive model previously developed in our laboratory (Chemarin, 2017; Chemarin *et al.*, 2017b, 2017a) was modified to account for the dynamic interfacial tension (IFT) change during 3-HP extraction and used to investigate the relation between chemical reaction, mass transfer and interfacial changes. The detailed methodology followed, and discussion of the obtained results are given in Chapter 3.

The second specific objective is to optimise the organic phase composition used for 3-HP reactive extraction. As the organic phase used in previous studies of 3-HP extraction showed a high toxicity towards the producing cells (Burgé *et al.*, 2017), a screening of different solvents was carried out in order to find a composition with a trade-off in terms of extraction performance and biocompatibility with the strain *Lactobacillus reuteri* DSM 17938. Cells physiological state was also assessed during circulation in the HFMC. The impact of the HFMC towards cell was evaluated in contact with different organic phases and in terms of shear stress. The understanding methodology and results are presented in Chapter 4.

The organic phase selected in Chapter 4 was used to evaluate the experimental feasibility of extractive bioconversion with two different 3-HP producing strains: *Lactobacillus reuteri* DSM 17938 and *Acetobacter* sp. CIP 58.66, which correspond to the third specific objective of this PhD work. The integrated experimental set-up between the reactive pertraction unit and the bioreactor determined for each 3-HP producing strain are detailed in Chapter 5.

Finally, the limiting mechanisms for the implementation of extractive bioconversion with

the 3-HP producing strain *Acetobacter* sp. CIP 58.66 where identified and different strategies for further optimisation were explored using a predictive mathematical model as a simulation tool. For this, different parameters were determined for model calibration using the organic phase selected in Chapter 4. After model validation, it was used to simulate different experimental conditions.

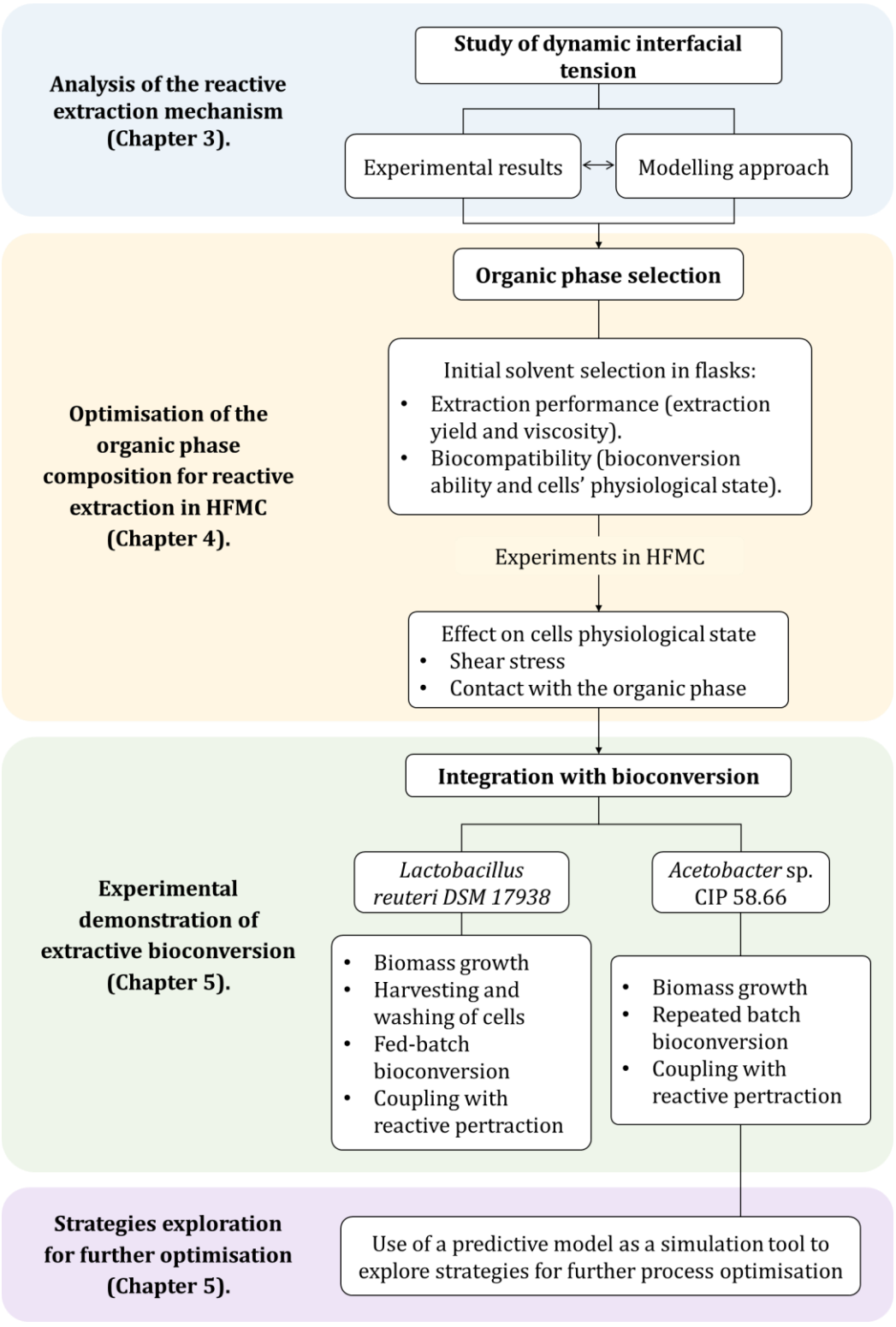


Figure 2.1 Schematic representation of the overall methodology

2.2. Bio-based 3-HP production and recovery

From observations obtained in the first attempts to couple glycerol bioconversion with a simultaneous recovery system (Burgé *et al.*, 2017), an integrated approach was envisioned in order to tackle the identified limitations. The overall strategy consists in the integration of three different processes (Figure 2.2):

1. Glycerol bioconversion into 3-HP and 1,3-PDO using resting cells of *Lactobacillus reuteri* DSM 17938. This stage includes a microbial growth step, recovery and washing of the cells, followed by a fed-batch bioconversion of glycerol.
2. 1,3-PDO bioconversion into 3-HP using resting cells of *Acetobacter* sp. CIP 58.66. In this stage, biomass growth is performed in the same bioreactor as bioconversion. The latter is triggered by 1,3-PDO addition to the medium.
3. 3-HP reactive pertraction is coupled to bioconversion. Reactive extraction and back-extraction happen simultaneously using two HFMC modules.

Each stage of the process have to take into account the needs of the other two and find the adequate trade-off for a global optimisation. In this context, the proposed experimental approach focuses on the implementation of reactive pertraction integrated to bioconversion.

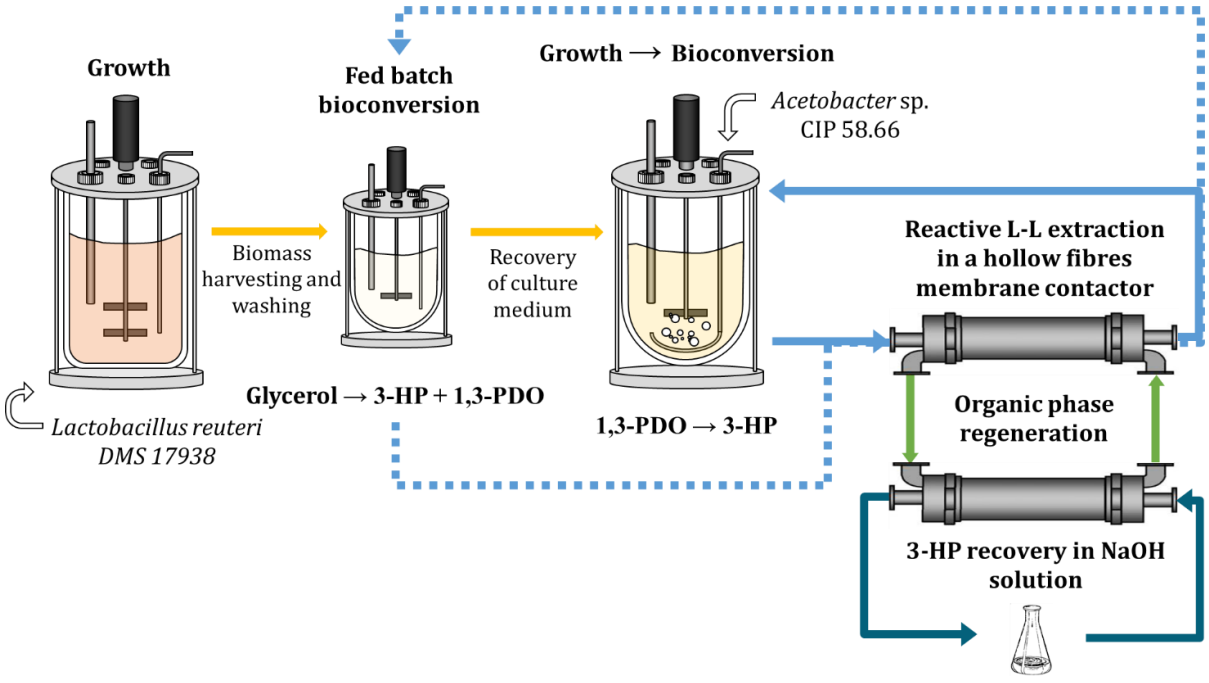


Figure 2.2 Global strategy for bio-based 3-HP production and simultaneous recovery. Dotted and continuous blue lines indicate that reactive pertraction was evaluated on bioconversion with both strains, *L. reuteri* DMS 17938 and *Acetobacter* sp. CIP 58.66.

2.3. Chemicals

All chemicals used during this work are given in Table 2.1, together with their purity, CAS number and supplier.

Table 2.1 Chemicals used during this work

Chemical	Purity %	CAS number	Supplier
Tri-n-octylamine (TOA)	99.7	1116-76-3	Sigma-Aldrich (Saint Louis, MO, USA)
Decanol	98.9	112-30-1	
Mannitol	≥ 98.0	69-65-8	
Glycerol	≥ 99.0	56-81-5	
1,3-Propanediol (1,3-PDO)	98	504-63-2	
Propidium iodide	≥ 94.0	25535-16-4	
3-Hydroxypropionic acid (3-HP)	28.9	503-66-2	TCI Europe (Zwijndrecht, Belgium)
Didodecylmethylamine (DDMA)	99.2	2915-90-4	
Octanol	99.5	111-87-5	
Dodecanol	99.6	112-53-8	
Oleyl alcohol	75.3	143-28-2	
2-Butyl-1-Octanol	99	08/02/3913	
2-Hexyl-1-Decanol	99	2425-77-6	
Decane	99	124-18-5	
Dodecane	99	112-40-3	
Isopropanol	≥ 99.5	67-63-0	
Chemchrom V8	-	-	Biomérieux (Marcy l'Étoile, France)
ChemSol B24 Buffer	-	-	
Ethanol anhydrous	≥ 99.9	64-17-5	Carlo Erba (Val de Reuil, France)
Broth MRS	-	-	Biokar Diagnostics (Beauvais, France)
3-Hydroxypropionaldehyde (3-HPA)	-	-	URD ABI (Pomacle, France)*
Sodium hydroxide	99.3	1310-73-2	VWR Chemicals (Leuven, Belgium)
Sulfuric acid	96.6	7664-93-9	
Trichloroacetic acid	≥ 96.0	76-03-9	
Glucose anhydrous	≥ 99.9	50-99-7	
K_2HPO_4	≥ 99.0	04/11/7758	Merck (Darmstadt, Germany)
Peptone	-	91079-38-8	BD-France (Le-Pont-de-Claix, France)
Yeast stract	-	02/01/8013	Organotechnie (La Courneuve, France)
Citric acid monohydrate	≥ 99.5	5949-29-1	Acros Organics (Geel, Belgium)
Na_2HPO_4	≥ 99.5	10028-24-7	Fisher Scientific (Elancourt, France)
Acetone	≥ 99.5	67-64-1	Fisher Scientific (Loughborough, UK)

*3-HPA is not commercially available, it was synthesised according to Burgé *et al*. (2015a)

2.4. Reactive pertraction set-up

Non-dispersive liquid-liquid reactive extraction was attained by using contactor modules (2.5×8, Liqui-Cel®, NC, USA) provided with X50 hollow fibres membranes (Membrana, NC, USA). Some key details of the module configuration are given in Figure 2.3. X50 membranes are distributed as a network of fibres, woven together. These fibres are hollow (internal diameter of 220 µm), so that one fluid can circulate inside and the other on the outside. The hydrophobic fibres are made of polypropylene and have a porosity of 40%, which allows contact between the two liquid phases during liquid-liquid extraction, avoiding their dispersion. With the application of an adequate pressure difference, interface can be stabilised inside the pores. In the case of 3-HP reactive extraction with trioctylamine (TOA), both molecules get in contact at this interface and form an acid-base complex that is soluble in the organic phase only. An important feature to take into account is that the

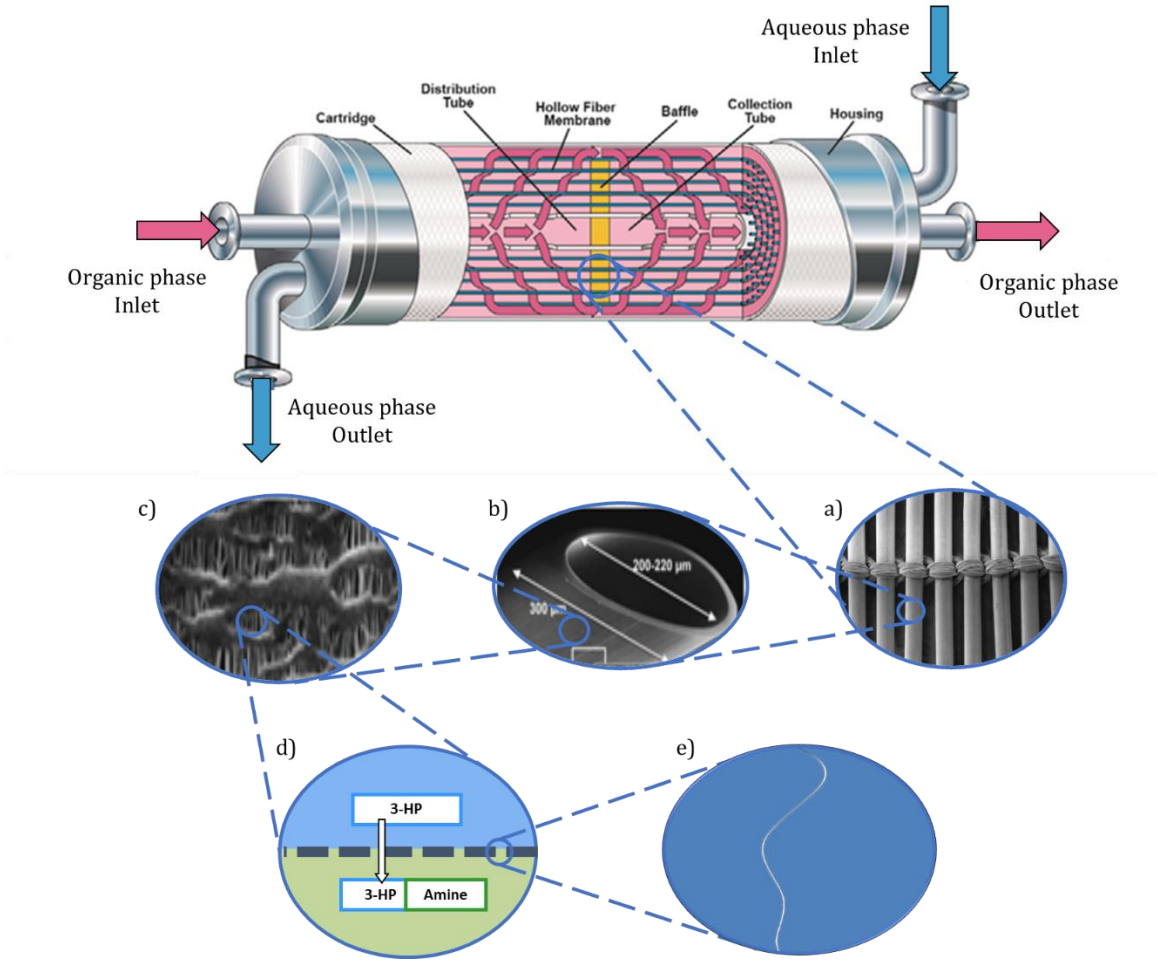


Figure 2.3 Hollow fibres membrane contactor, adapted from Liqui-Cel™ (Liqui-CelTM, 2017). a) The module is provided with a membrane consisting of a network of parallel hydrophobic fibres woven together, made of polypropylene. b) These fibres are hollow, with an internal diameter of 220 µm and a thickness of 40 µm. c) The membrane has a porosity of 40%, which allows the contact of the two liquid phases, avoiding dispersion. d) Reactive extraction happens at the stabilised interface inside the pores. e) Pores have a significant length compared with their width, representing an important resistance to mass transfer.

membrane has a thickness of 40 µm, and if we consider a tortuosity of 2.5 and an average pore size of 40 nm (data obtained from supplier), we obtain a length/width ratio higher than 3000. As the hydrophobic pores are wetted by the organic phase, the complex formed has to traverse this length, which seems to represent an important resistance to mass transfer. All modules characteristics are summarised in Table 2.2.

Table 2.2 Characteristics of the membrane contactor modules

Module 2.5 x 8 Liqui-Cel®		**Fibres X50**	
Material	Polypropylene	Material	Polypropylene
Internal diameter (mm)	58.4	Internal diameter (µm)	220
Internal length (mm)	203	External diameter (µm)	300
Number of fibres	~9800	Effective length (mm)	146
Shell side volume (mL)	400	Wall thickness (µm)	40
Fibres side volume (mL)	150	Porosity (%)	40
Contact area (m^2)	~0.4	Average pore size (nm)	40

The pertraction experimental set-up is schematically represented in Figure 2.4. Experiments were carried out using either one (extraction) of two (extraction and back-extraction) identical units. Each unit included two magnetic drive pumps (MDG-H2T, Iwaki) that allow circulation of the liquid phases inside the HFMC modules. The membrane modules were installed in vertical position, with the fibres inlet on top, and the shell side inlet on the bottom, in order to have a counter-current flow. Aqueous phases (*i.e.* 3-HP model solutions and bioconversion broth) were circulated on the lumen-side, while organic phases circulated on the shell-side. The speed of the pumps was fixed manually thanks to the use of digital rotary controllers. Aneroid manometers were installed at the inlets and outlets of the fibres and of the shell. Pressure and flow rate in each circuit were controlled by pumps speed control, and also with control valves installed in the outlets of each side.

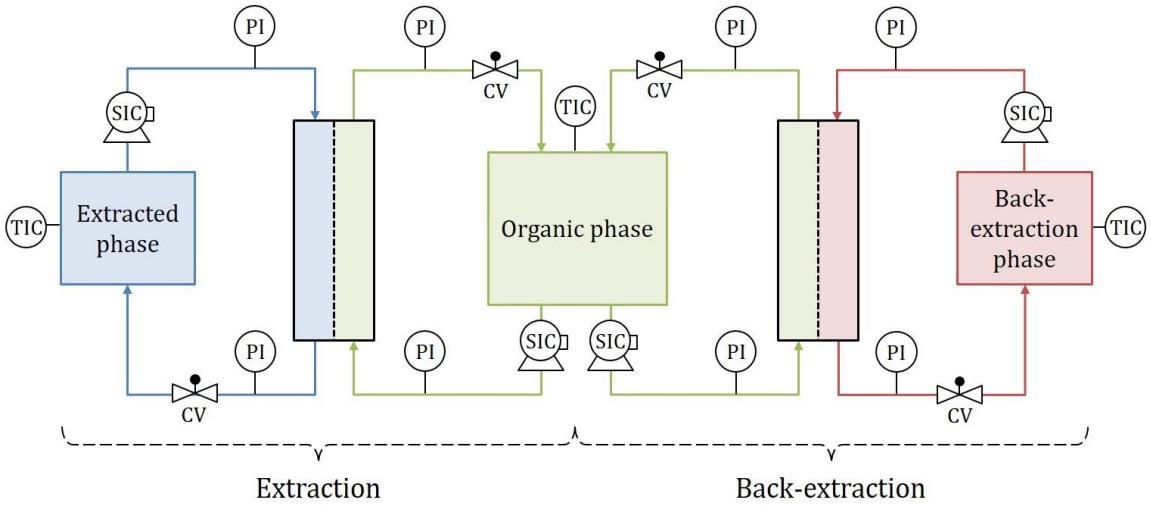


Figure 2.4 Schematic representation of the reactive pertraction set-up. Abbreviations: CV, control valve; PI: pressure indicator; SIC, speed indicator and controller; TIC, temperature indicator and controller.

2.5. Analytical techniques

The following analytical techniques were used in the processing of samples obtained from all the different studies carried out during the present PhD work.

2.5.1. HPLC analysis

All HPLC analysis were performed using a Biorad Aminex HPX-87H column (300 mm × 7.8 mm; Biorad, Richmond, VA, USA) and a refractive index detector (Waters, Guyancourt, France). Samples containing 3-HP, 1,3-PDO and glycerol were analysed with a column temperature of 65°C, using a H_2SO_4 0.5 mmol L^{-1} solution as mobile phase with a flow rate of 0.4 mL min^{-1}. Samples containing 3-HPA were analysed with a column temperature of 35°C and using a H_2SO_4 5 mmol L^{-1} solution as mobile phase with a flow rate of 0.6 mL min^{-1}.

2.5.1.1. Samples from model solutions

Samples taken from experiments using model solutions were filtered through a nylon membrane filter (pore size: 0.2 µm). Then, 750 µL were mixed with an equal volume of a filtered citric acid solution at 10 g L^{-1} (internal standard) just before analysis.

3-HP in the organic phase samples were analysed by initial back-extraction mixing 2 mL of the organic phase with an equal volume of a NaOH 0.5 M solution. They were mixed during 3 min and put at 25°C to reach equilibrium overnight, then separated by centrifugation at 8 228×g and 25°C. The aqueous phase was carefully recovered and filtered before addition of the internal standard.

2.5.1.2. Samples from extractive bioconversion with L. reuteri DSM 17938

Samples taken from bioconversion broth were filtered, then centrifuged at 10 000×g during 10 min. An equal volume of a citric acid solution at 5 g L^{-1} was added to the sample and the mixture was put at 4°C overnight for proteins precipitation. A final centrifugation step was performed before analysis. Samples from the organic phase and back-extraction solution were prepared as samples from model solutions.

2.5.1.3. Samples from extractive bioconversion with Acetobacter sp. CIP 58.66

Samples taken from the bioconversion broth were first diluted into an equal volume (750 µl) of trichloroacetic acid (TCA) at 6% (w/v), in order to precipitate proteins. They were put immediately at 0 °C for at least 45 min. Samples were then centrifuged at 10 000×g and 4°C, during 10 min. Supernatant was recovered and filtered before analysis. No internal standard was added to these samples. Samples from the organic phase and back-extraction solution were prepared as samples from model solutions but without addition of internal standard.

2.5.2. Assessment of cells' physiological state by flow cytometry

The physiological state of bacteria was assessed by dual fluorescent staining and subsequent analysis with a Cyflow® Space cytometer (Sysmex-Partec, Villepinte, France). The protocol was adapted from Rault *et al.* (Rault *et al.*, 2007). Carboxyfluorescein diacetate (cFDA) was diluted in acetone at 10 % (v/v) and used to assess the esterase activity of cells, reflecting their basal enzymatic activity. The propidium iodide dye was diluted at 1 mg mL^{-1} in deionised water and used for membrane integrity assessment. Data were acquired and analysed with the FlowMax software (Sysmex-Partec, Villepinte, France). The detected particles were gated based on forward scatter (FSC) and side scatter (SSC) values, in order to select only those corresponding to bacteria and to reduce background noise. This method made it possible to distinguish between dead cells (Q1, stained with PI), altered cells (Q2, stained with cFDA and PI) and viable cells (Q4, stained with cFDA), as shown in Figure 2.5. The quadrant Q3 corresponds to unstained particles, however in the case of *Acetobacter* sp., dead cells were considered as the combination between Q1 and Q3 (Figure 2.5b).

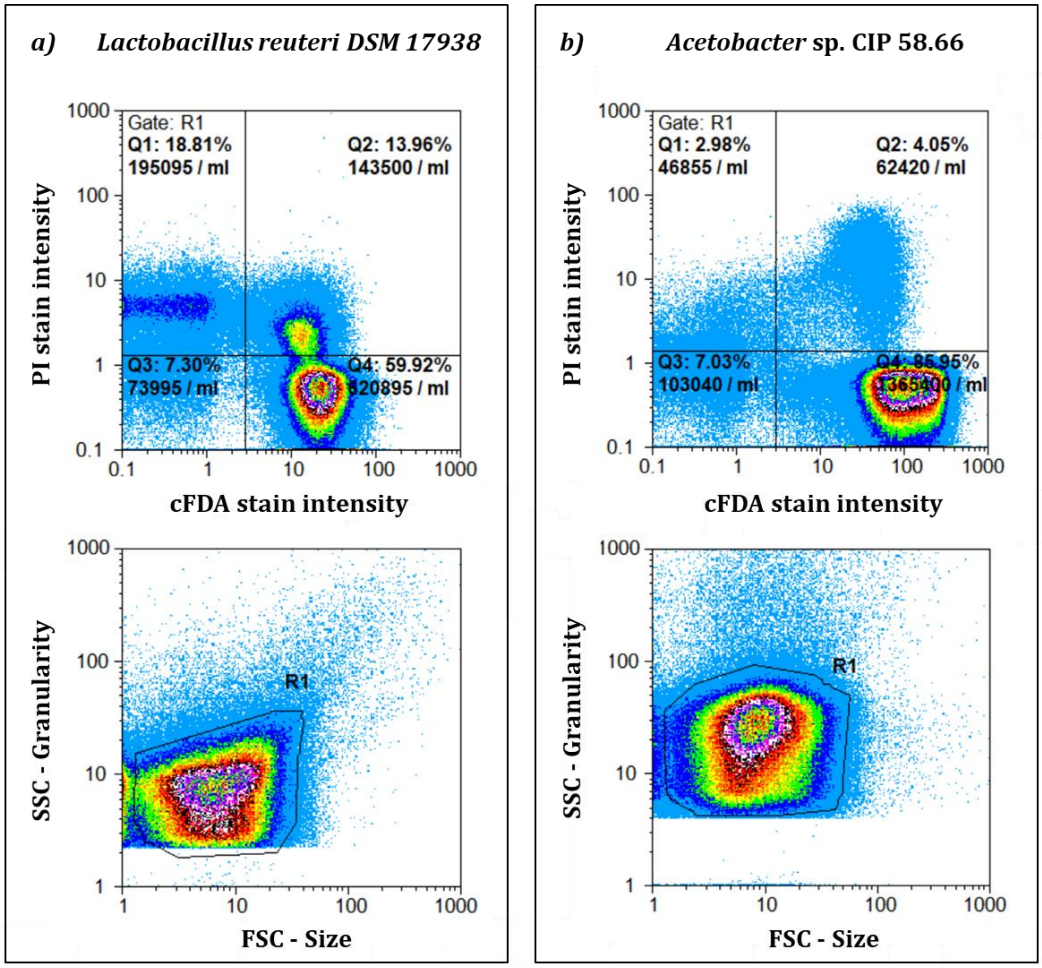


Figure 2.5 Example of cytograms for a) *Lactobacillus reuteri* and b) *Acetobacter* sp. using a dual stain with propidium iodide (PI) and carboxy fluorescein diacetate (cFDA). Q1: Dead cells (stained with PI); Q2: altered cells (PI and cFDA); Q3: unstained particles; and Q4: viable cells (cFDA). For Acetobacter sp. dead cells were considered as a combination of Q1 and Q3.

2.5.2.1. Staining of L. reuteri cells DSM 17938

Cell suspension samples were diluted in Mac Ilvaine (MI) buffer (0.1 M citric acid and 0.2 M disodium dihydrogenophosphate, pH = 7.3) to reach approximately 10^6 particles mL^{-1}. Dual staining was done by adding 10 µL of the cFDA solution and 5 µL of the PI solution to 1 mL of the diluted bacterial cell solution. After thorough mixing, samples were incubated for 15 min in a bath at 40 °C before flow cytometry analysis.

2.5.2.2. Staining of Acetobacter sp. CIP 58.66 cells

Cell suspension samples were diluted in ChemSol B24 buffer to a concentration of about 10^6 particles mL^{-1}. Dual staining was done by adding 100 µL of the cFDA solution and 5 µL of the PI solution to 1 mL of the diluted bacterial cell solution. After thorough mixing, samples were incubated for 15 min in a bath at 40 °C, and then centrifuged for 90 s at 14 100×*g*. Next, the supernatant was discarded and the cell pellet was re-suspended in 1 mL of ChemSol B24 buffer before flow cytometry analysis. Physiological state assessment was performed on samples from two of the three replicates of extractive bioconversion.

2.5.3. Biomass quantification by optical density (OD)

2.5.3.1. Lactobacillus reuteri DSM 17938

L. reuteri cells concentration was determined by optical density measurements at a wavelength of 600 nm (OD600nm), using an Evolution 201 spectrophotometer (ThermoScientific, Madison, USA). A correlation (Equation 2.1) between OD_{600nm} and cell dry weight (CDW, g L^{-1}) was used for CDW calculation:

$$CDW = 0.38 \times OD_{600nm} \quad (2.1)$$

2.5.3.2. Acetobacter sp. CIP 58.66

Previous 1,3-PDO bioconversion into 3-HP results, demonstrated that *Acetobacter* sp. had further growth after addition of 1,3-PDO to resting cells (de Fouchécour, 2019). Therefore, cells concentration was monitored all along experiments (growth and bioconversion) by off-line OD_{600nm} measurements. For each sample, OD_{600nm} was first measured for the native sample, as well as for its filtrate (PES filter, pore size: 0.22 µm). The filtrate's OD_{600nm} was subtracted from the native sample's OD_{600nm}, so that variation of the medium colour would be taken into account. A previously established correlation (Equation 2.2) between OD_{600nm} and cell dry weight (CDW, g L^{-1}) (de Fouchécour, 2019) was used for CDW calculation:

$$CDW = 0.59 \times OD_{600nm} \quad (2.2)$$

Chapter 3

Study of 3-HP reactive extraction mechanisms *via* dynamic interfacial tension measurement

This chapter describes the investigation of 3-HP and TOA/decanol reaction and mass transfer mechanisms in the vicinity of the aqueous-organic interface, in order to elucidate rate-limiting phenomena and pave the route to process optimisation. Dynamic interfacial tension (IFT) measurements gave direct access to interfacial phenomena during 3-HP reactive extraction, which is not feasible in membrane contactor experiments. The obtained data was used to explore different mass transfer configurations using a mathematical model capable of describing 3-HP chemical and mass transfer dynamics. It was found that the purely diffusive transfer model was not compatible with the observed IFT dynamics, suggesting that phenomena such as concentration-induced density gradients and associated buoyancy forces, or concentration-induced interfacial tension gradients and associated Marangoni convection significantly enhance local mass transfer.

Nomenclature

Symbol	Units	Significance
A^-	kmol m^{-3}	Dissociated acid in the aqueous phase
AH	kmol m^{-3}	Non-dissociated acid in the aqueous phase
AH_{org}	kmol m^{-3}	Non-dissociated acid in the organic phase
C	kmol m^{-3}	Molar concentration of a generic chemical species
CPX	kmol m^{-3}	Acid-amine complex in the organic phase
D	m^2 s^{-1}	Diffusion coefficient
IFT	N m^{-1}	Interfacial tension
k	kmol^{-n} m^{3n} s^{-1}	Kinetic rate constant
K	kmol^{-n} m^{3n}	Equilibrium constant
m	-	3-HP partition coefficient in decanol
M	kg kmol^{-1}	Molar mass
n	-	Number of reacting molecules in Freundlich isotherm
n	1	Unit normal vector
p	Pa	Pressure field
r_1, r_4	kmol m^{-3} s^{-1}	Volume reaction rates
r_2, r_3	kmol m^{-2} s^{-1}	Surface reaction rates
R	M	Radius, radial position
R_g	J kmol^{-1} K^{-1}	Ideal gas constant
S_{ad}	kmol m^{-2}	Free sites for acid-amine complex adsorption at the interface
t	S	Time
T	K	Absolute temperature
TOA	kmol m^{-3}	Tri-n-octylamine in the organic phase
u	m s^{-1}	Velocity field
v	dm^3 kmol^{-1}	Molar volume at the normal boiling temperature
z	M	Vertical position

Symbol	Units	Significance
Greek letters		
ϕ	-	Association coefficient
γ	N m^{-1}	Interfacial tension between the aqueous and the organic phase
Γ	kmol m^{-2}	Excess surface concentration of the acid-amine complex
μ	Pa s	Fluid viscosity
Subscripts		
aq		Aqueous phase
d		Droplet
i		Interface
N		Needle
org		Organic phase
out		Outer boundary of the modelling region
S		Subsurface concentration

3.1. Introduction

As mentioned in Chapter 1, reactive extraction is a promising method for *in situ* organic acids recovery from bioconversion/fermentation media. However, extraction media can be toxic towards acid producing microorganisms. Beyond the selection of extractants and diluents with high biocompatibility towards the microorganisms, direct contact should be avoided during in stream extraction. Among other devices, hollow fibres membrane contactors (HFMC) achieve this goal by stabilising the liquid-liquid interface in the membrane pores, with the added advantages of avoiding emulsion formation and continuous solvent regeneration using a stripping solution (Schlosser *et al.*, 2005).

Mass transfer in traditional liquid-liquid extraction using HFMC is usually considered to be governed by diffusion in membrane pores (Prasad and Sirkar, 1987) but reactive extraction implies additional phenomena such as (i) reagent transport towards the liquid-liquid interface, (ii) interfacial and bulk reactions and (iii) product transport back in the bulk phase (Djas and Henczka, 2018). Modelling of mass transfer often involves additional "lumped" resistances reflecting the kinetics of interfacial reactions of the complex formation or decomposition, equilibrium and the kinetics of competitive complex adsorption or desorption and the free extractant molecules on/from the interface (Schlosser *et al.*, 2005). Experimental measurements restricted to bulk concentrations only can presumably accommodate a variety of detailed interfacial mechanisms (Juang and Chen, 2000; Marti *et al.*, 2011; Wasewar *et al.*, 2002) even those assuming constant interfacial concentrations (Pursell *et al.*, 2003).

Changes in the liquid-liquid interface generated by solute concentration, chemical kinetics and diffusion during reactive extraction can give essential information to understand mass transfer. Observations of the dynamic IFT have permitted to identify the governing factors on surface-active species transfer between two immiscible liquids and facilitated modelling of the transfer kinetics for systems with non-reactive surfactants (Gassin *et al.*, 2012, 2013; Liggieri *et al.*, 1997; Rubin and Radke, 1980) and surfactants created *in situ* by chemical

reaction (Lee and Wang, 1995; Martin-Gassin *et al.*, 2011, 2012; Vidyalakshmi *et al.*, 2003). In the case of reactive interfaces, spontaneous natural convection driven by chemical reaction has been observed (Eckert *et al.*, 2004; Eckert and Grahn, 1999; Sczech *et al.*, 2008; Sherwood and Wei, 1957) and found to have an important impact on mass transfer. This flow motion around the interface can be generated by local density and surface tension changes (Almarcha *et al.*, 2011).

Based on this, dynamic IFT observation can give valuable information for better understanding of carboxylic acids reactive extraction with solvents. Reaction mechanisms and acid-amine complex formation of a 3-HP and TOA/decanol system has been studied in detail previously (Chemarin *et al.*, 2017b, 2017a). A mathematical model that takes into account chemical equilibria and mass transfer dynamics during 3-HP reactive extraction with a TOA/decanol solution has been developed and was shown to predict experimental results with good accuracy (Chemarin, 2017). Such a model is adapted to extraction assisted by HFMC conditions, but in that configuration there is no direct access to the interface changes during reactive extraction. The pendant drop method is a technique widely used for IFT measurement, one of its advantages is its non-invasive character, which makes it an ideal tool for interfacial phenomena observation (Berry *et al.*, 2015; Lin *et al.*, 1990; Saad *et al.*, 2011). During reactive extraction, an acid-base complex is formed when TOA in the organic phase gets in contact with 3-HP (aqueous phase). Because of the very low solubility of TOA in water ($1.4 \cdot 10^{-7}$ mol L^{-1} (U.S. Environmental Protection Agency, 1992)), and the small partition coefficient of 3-HP in decanol (0.02, expressed as molar concentration ratio (Chemarin *et al.*, 2017a)), the chemical reaction takes place essentially at the interface. In addition, the complex formed has an amphiphilic structure and it has a direct impact on IFT. Therefore, the idea of studying the IFT evolution during reactive extraction emerged as a way of having access to interfacial phenomena. Figure 3.1 illustrates the proposed analogy of interfacial reaction inside the membrane pores of the HFMC, with the configuration of the pendant drop method.

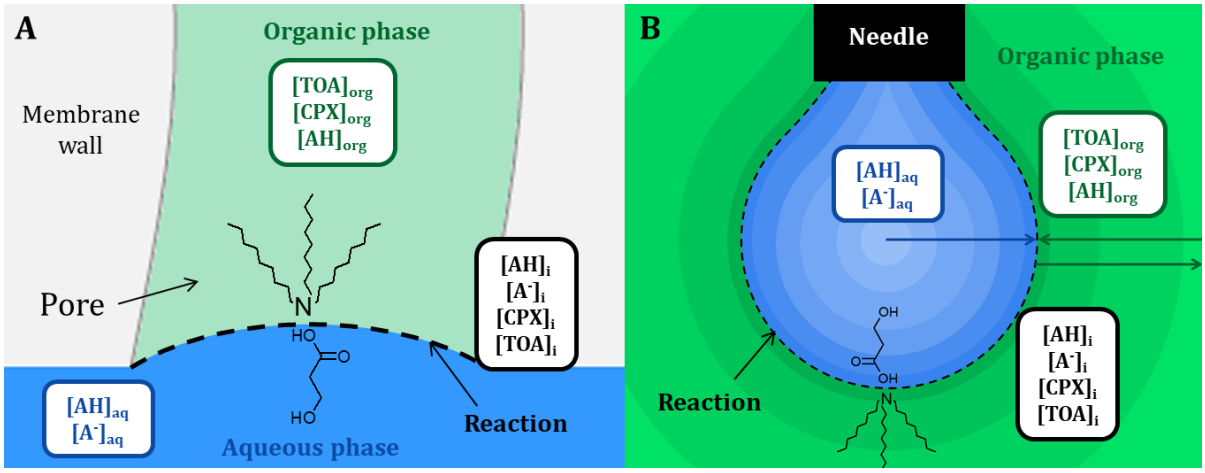


Figure 3.1 Main chemical species involved in 3-HP reactive extraction with TOA. Comparison between configurations A) inside the membrane pores and B) in the pendant drop set-up. AH: Non-dissociated acid, A-: dissociated acid, TOA: trioctylamine, CPX: acid-base complex. Subscripts, aq: bulk aqueous phase, org: bulk organic phase, i: interface.

The goal of the present study is to investigate local reaction and mass transfer mechanisms near the liquid-liquid interface by dynamic IFT measurement. The acid-amine complex formed in the reactive extraction of carboxylic acids with amines is suspected to be surface-active because of its chemical structure: one side that contains the acid molecule (hydrophilic) and other side with the carbon chains of the amine (hydrophobic). The Gibbs theory relates surface excess of such species to their subsurface concentration and interfacial tension. Dynamic measurement of interfacial tension, combined with mass transfer and reaction kinetic modelling is thus expected to provide insight into the rate-limiting mechanisms near the interface (Giustiniani *et al.*, 2017). The pendant drop tensiometer is a convenient classical tool for such dynamic studies in liquid-liquid systems (Beverung *et al.*, 1999; Gassin *et al.*, 2012, 2013; Lin *et al.*, 1990; Vidyalakshmi *et al.*, 2003).

The main bulk and interfacial chemical reactions were combined with three different adsorption models and three mass transfer models. Quantitative predictions of those models were compared to measured time evolutions of the IFT for different initial acid concentrations, in order to find the most plausible combination of adsorption and mass transfer model. This permitted further insights into governing mechanisms, based on the selected model combination.

3.2. Materials and methods

3.2.1. Description of the reaction system

The reaction system is composed of i) the molecule of interest, 3-hydroxypropionic acid (3-HP), in aqueous solution and ii) an organic phase consisting of a tertiary amine, tri-n-octylamine (TOA), used as *extractant* and n-decanol, used as an *active diluent*. When both phases are put in contact, an acid-amine complex is formed (Tamada *et al.*, 1990). Since the aqueous and the organic phase are practically immiscible, the reaction takes place at the interface between these liquid phases. The resulting complex consists of dissociated acid, protonated amine and water, which is stabilised by the active diluent through H-bonding and is soluble in the organic phase. Only non-dissociated acid in the aqueous phase can be extracted in this way (Chemarin *et al.*, 2017a; Tamada and King, 1990a).

In the pendant drop tensiometer used in this study the aqueous phase initially containing 3-HP was used to form a droplet that was immersed in the extracting organic phase composed of TOA and decanol (Figure 3.2). In this work, surface concentrations of adsorbed acid-amine complex Γ as well as adsorption and desorption reactions are considered explicitly. Previously (Chemarin *et al.*, 2017b, 2017a), these reactions were considered fast compared to other mass transfer phenomena and local equilibrium was assumed between subsurface compartments on both sides of the liquid-liquid interface. It should be also noted that instead of the term "interfacial" (volumetric) concentration CPX_i (mol m^{-3}) employed in those previous studies, we use the term "subsurface" (volumetric) concentration CPX_s (mol m^{-3}) in this work; in contrast, we set here the term "interfacial concentration" aside for surface concentration of adsorbed species Γ (mol m^{-2}) (Figure 3.2).

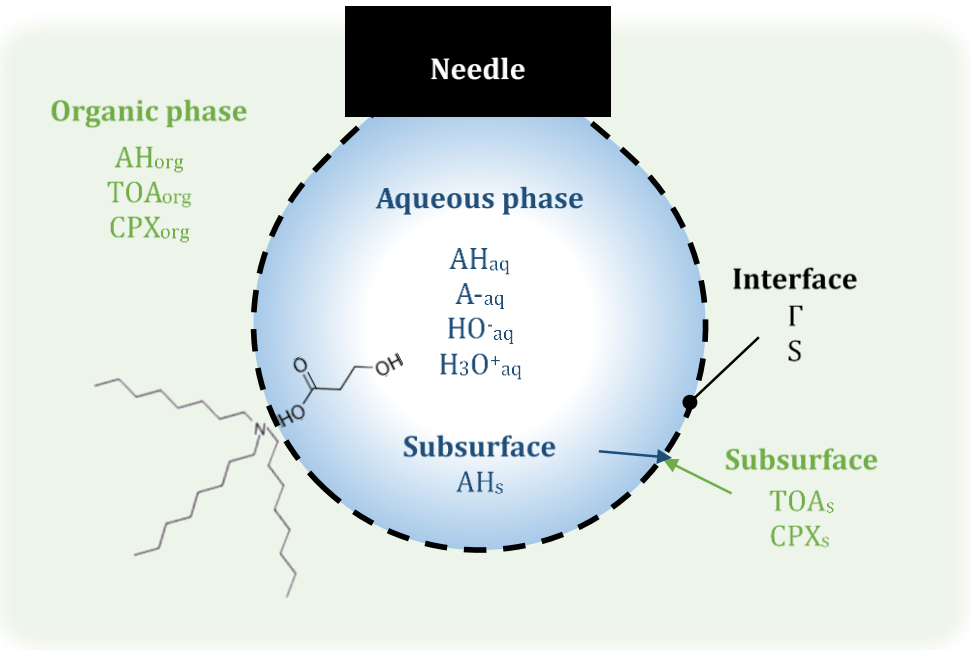


Figure 3.2 Schematic representation of the pendant drop and main reacting species. AH: non-dissociated acid, A-: dissociated acid, TOA: tri-n-octylamine, CPX: acid-amine complex, Γ: adsorbed complex, S: adsorption sites, index s: subsurface, org: organic phase, aq: aqueous phase.

3.2.2. Mathematical model of the dynamic interfacial tension related to 3-HP reactive extraction

The mathematical model developed by Chemarin (2017) takes into account chemical equilibria of species complexation and dissociation, as well as the species physical partitioning and mass transfer dynamics. The model is based on boundary layers and adapted to be used in a HFMC configuration. It was modified to take into account the dynamic IFT change during 3-HP reactive extraction and to adapt the mass transfer mechanisms to the geometry of the pendant drop method used in this study.

3.2.2.1. Chemical reactions

3.2.2.1.1. Aqueous phase

The considered weak acid can be extracted by the amine in the organic phase in its non-dissociated form only (Chemarin *et al.*, 2017a; Tamada *et al.*, 1990). Hence, acid dissociation equilibrium in the aqueous phase has to be considered to ensure correct reagent concentrations, *AH*, for the extraction reaction:

$$AH_{aq} + H_2O_{aq} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A^-{}_{aq} + H_3O^+{}_{aq} \qquad \text{Reaction 1}$$

The net reaction rate (forward rate minus backward rate) can be expressed considering the acid equilibrium constant (K_1):

$$K_1 = \frac{A \cdot H_3O}{AH}\Big|_{eq} \qquad r_1 = k_{-1}(K_1 \cdot AH - A \cdot H_3O) \tag{3.1}$$

The equilibrium constant of this reaction corresponds to the dissociation constant of the considered acid, $K_1 = 10^{-pK_A}$. pK_A of 3-HP is 4.51 (Haynes *et al.*, 2017).

3.2.2.1.2. Aqueous-organic interface

At the interface, non-dissociated acid in the subsurface of the aqueous phase (AH_s) reacts with the amine in the subsurface of the organic side (TOA_s) to give the acid-amine complex, which is first adsorbed at the interface (Γ) and subsequently desorbed in the subsurface of the organic phase (CPX_s). Three commonly used adsorption-desorption models were investigated in this work, as described below.

Gibbs equation (Gassin *et al.*, 2013; Rubin and Radke, 1980) was used to relate the excess interfacial concentration (Γ) of the acid-amine complex, considered as the only surface-active species, to the measured interface tension IFT (γ):

$$\Gamma = -\frac{1}{R_g T}\left(\frac{d\,\gamma}{d \ln CPX_s}\right)_T \tag{3.2}$$

where R_g is the ideal gas constant and T is the temperature of the system. Gibbs equation assumes a quasi-static equilibrium at the interface. It was combined with each of the following adsorption-desorption models to calculate the corresponding IFT.

a) Langmuir model (L)

Langmuir model sets an upper limit (Γ_m) on the number of the surfactant adsorption sites (S_{ad}) at the interface. One kinetic expression of the Langmuir model is (Rubin and Radke, 1980):

$$AH_s + TOA_s + S_{ad} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \Gamma \qquad \text{Reaction 2L}$$

$$\Gamma \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} CPX_s + S_{ad} \qquad \text{Reaction 3L}$$

Equilibrium constants and net (forward minus backward) reaction rates are thus defined as follows:

$$K_2 = \frac{\Gamma}{AH_s \cdot TOA_s \cdot S_{ad}}\Big|_{eq} \qquad r_2 = k_{-2}(K_2 \cdot AH_s \cdot TOA_s \cdot S_{ad} - \Gamma) \tag{3.3}$$

$$K_3 = \frac{CPX_s \cdot S_{ad}}{\Gamma}\Big|_{eq} \qquad r_3 = k_{-3}(K_3 \cdot \Gamma - CPX_s \cdot S_{ad}) \tag{3.4}$$

IFT calculated using the Langmuir model combined with the Gibbs equation (Equation 3.2) is:

$$\gamma = \gamma_0 + R_g T \Gamma_m \ln\left(1 - \frac{\Gamma}{\Gamma_m}\right) \tag{3.5}$$

where γ_0 is the IFT between the considered aqueous and organic phases without the considered surface-active species ($\Gamma = 0$).

b) Freundlich model (F)

Freundlich model assumes that the number of available adsorption sites at the interface is very large compared to the considered concentrations of surface-active molecules and remains approximately constant ($\Gamma \ll S \cong \Gamma_m$). It is an empirical model that can be formally derived considering that a certain number (n) of reacting molecules are combined in a single adsorbed entity. The special case when $n = 1$ corresponds to the linear Henry adsorption-desorption model. Interfacial reactions in the case of the Freundlich model read:

$$nAH_s + nTOA_s \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \Gamma \qquad \text{Reaction 2F}$$

$$\Gamma \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} nCPX_s \qquad \text{Reaction 3F}$$

Equilibrium constants and net reaction rates are in this case:

$$K_2 = \left.\frac{\Gamma}{AH_s^n \cdot TOA_s^n}\right|_{\text{eq}} \qquad r_2 = k_{-2}(K_2 \cdot AH_s^n \cdot TOA_s^n - \Gamma) \tag{3.6}$$

$$K_3 = \left.\frac{CPX_s^n}{\Gamma}\right|_{eq} \qquad r_3 = k_{-3}(K_3 \cdot \Gamma - CPX_s^n) \tag{3.7}$$

Combining Freundlich model with Gibbs equation (Equation 3.2) gives:

$$\gamma = \gamma_0 - R_g T \frac{\Gamma}{n} \tag{3.8}$$

c) Langmuir-Freundlich model (LF)

A more general adsorption-desorption model can be obtained assuming both a limitation on the number of available adsorption sites as in the Langmuir model and an adsorption-desorption reaction involving n molecules as in the Freundlich model. The reaction system is in this case:

$$nAH_s + nTOA_s + S_{ad} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \Gamma \qquad \text{Reaction 2LF}$$

$$\Gamma \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} nCPX_s + S_{ad} \qquad \text{Reaction 3LF}$$

Equilibrium constants and net reaction rates are:

$$K_2 = \left.\frac{\Gamma}{AH_s^n \cdot TOA_s^n \cdot S_{ad}}\right|_{eq} \qquad r_2 = k_{-2}(K_2 \cdot AH_s^n \cdot TOA_s^n \cdot S_{ad} - \Gamma) \tag{3.9}$$

$$K_3 = \left.\frac{CPX_s^n \cdot S_{ad}}{\Gamma}\right|_{eq} \qquad r_3 = k_{-3}(K_3 \cdot \Gamma - CPX_s^n \cdot S_{ad}) \tag{3.10}$$

IFT calculated using this model is:

$$\gamma = \gamma_0 + R_g T \frac{\Gamma_m}{n} \ln\left(1 - \frac{\Gamma}{\Gamma_m}\right) \tag{3.11}$$

In the case $n = 1$ Langmuir-Freundlich model reduces to the Langmuir model, and in the case when the adsorbed concentrations are very small compared to the maximum one ($\Gamma \ll \Gamma_m$) it reduces to the Freundlich model because $\ln(1 + x) \cong x$ for $|x| \ll 1$.

3.2.2.1.3. Organic phase

A small amount of acid can be dissolved in the organic phase (AH_{org}) by physical partition and reacts with the amine therein. This is taken into account via the following reaction:

$$AH_{org} + TOA \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} CPX \qquad \text{Reaction 4}$$

The equilibrium constant and the reaction rate were expressed as:

$$K_4 = \left.\frac{CPX}{AH_{org} \cdot TOA}\right|_{eq} \qquad r_4 = k_{-4}\left(K_4 \cdot AH_{org} \cdot TOA - CPX\right) \tag{3.12}$$

Physical partition of the acid in the organic phase being small, concentration (AH_{org}) is also expected to be small and bulk acid-amine reaction (Reaction 4) could be omitted in a simplified version of the model. It was included here for completeness, however, because for high acid concentrations in the aqueous phase amine concentrations in the subsurface could be significantly decreased and local acid-amine-complex equilibrium in the subsurface (Reaction 2 + Reaction 3) could be achieved for concentrations different from the bulk. Reaction 4 allows for the restoration of the bulk equilibrium.

3.2.2.2. Geometries and mass transfer models

In order to assess the relative importance of various involved phenomena, several configurations of increasing complexity have been considered (Figure 3.3).

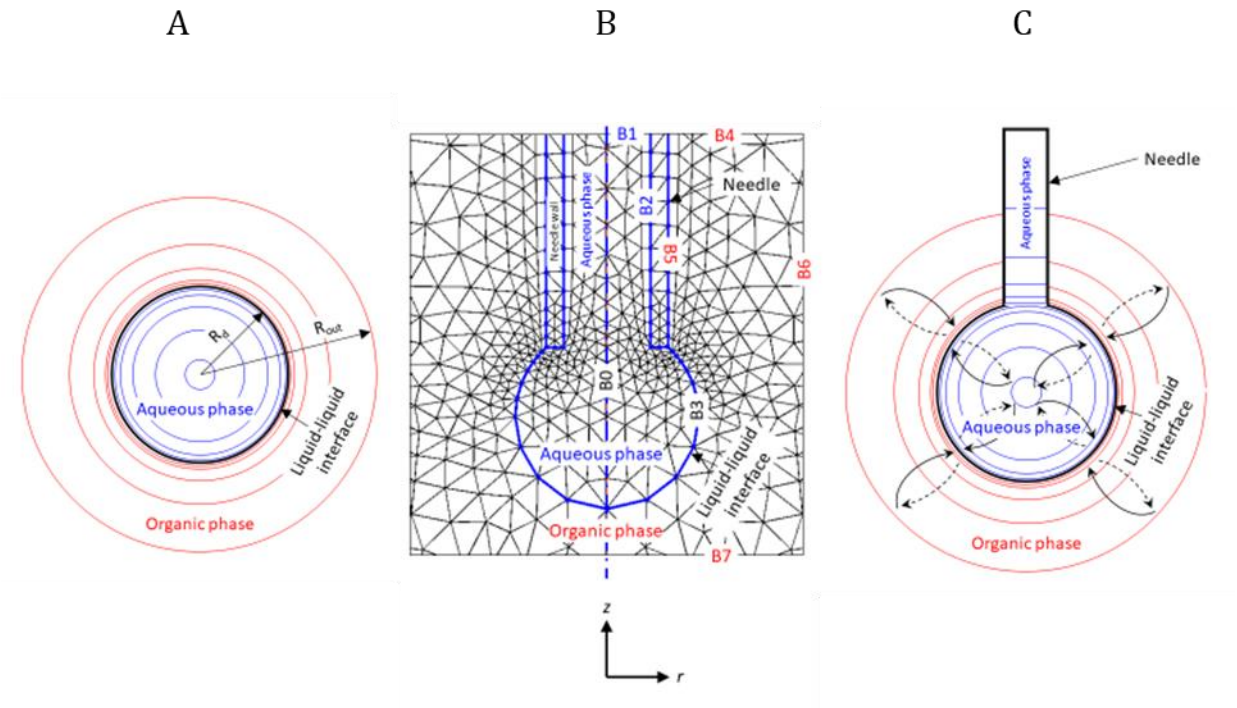


Figure 3.3 Schematic view of the geometries used in the considered mass transfer models, not in scale. (A) Droplet only, spherical symmetry, 1D structured spherical grid. (B) Droplet and needle, axial symmetry, 2D unstructured triangular grid. (C) Droplet (spherical symmetry) and needle (plane symmetry), 1D+1D structured spherical and plane grids.

3.2.2.2.1. Droplet only, diffusive mass transfer (D)

The simplest mass transfer model was based on the assumption of spherical symmetry of the droplet and transport of all involved chemical species by diffusion (Figure 3.3A). It thus neglected the effect of the needle and assumed no other motion or convection in any of the fluids since no mechanical stirring or pumping was applied during the experiments.

In the bulk of the aqueous and organic phases, the governing equation for a given involved species i states that local accumulation of this species (first term) is due to Fickian diffusion (second term) and to production or consumption via chemical reactions (source term) listed in Table 3.1:

$$\frac{\partial C_i}{\partial t} + \nabla \cdot (-D_i \nabla C_i) = r_i \tag{3.13}$$

Diffusion coefficients for each considered species and corresponding reaction terms have been calculated using the classical Wilke-Chang relation (Bird *et al.*, 2007):

$$D_i = 7.4 \cdot 10^{-12} \frac{(\phi M)^{0.5} T}{\mu v_i^{0.6}} \tag{3.14}$$

In this equation D_i (m^2 s^{-1}) is the diffusion coefficient of species i, T (K) the absolute temperature, M (kg $kmol^{-1}$) the molar mass of the solvent, μ (mPa s) the solvent viscosity,

v_i (dm^3 kmol^{-1}) the molar volume of the solvent at its normal boiling temperature and the numeric coefficient accommodates various units. The dimensionless coefficient ϕ is the association factor of the solvent, with a value of 1 for non-associated solvents and 2.6 for water. For the organic solvent used, the association factor is not known but is expected to be close to 1 and in all cases less than 2.6.

Table 3.1 Reaction terms used in the mass transfer models

	Species	Reaction term
Bulk of aqueous phase: volume reaction term	AH	$-r_1$
	A^-	r_1
	H_3O^+	r_1
Aqueous-organic interface: surface reaction term	AH_s	$-r_2$
	TOA_s	$-r_2$
	CPX_s	r_3
	Γ	$r_2 - r_3$
Bulk of organic phase: volume reaction term	AH_{org}	$-r_4$
	TOA	$-r_4$
	CPX	r_4

The viscosity of the organic solvent was found to vary significantly (+50%) for the considered range of acid concentrations. This variation was taken into account via the following empirical linear relationship (Chemarin, 2017).

$$\mu_{org} = \mu_0 \left(1 + 1.593\left(CPX + AH_{org}\right)\right) \tag{3.15}$$

where μ_0 is the viscosity of the organic phase without acid and $CPX + AH_{org}$ is the local concentration of acid (kmol m^{-3}), including both forms present in the organic phase.

The boundary condition at the centre of the aqueous phase is zero flux for all species because of the spherical symmetry assumption:

$$-D_i \nabla C_i|_{R=0} = 0 \tag{3.16}$$

The boundary condition at the aqueous-organic interface involves the adsorption-desorption reactions:

$$-D_i \nabla C_{si}|_{R=R_d} = r_i \tag{3.17}$$

Surface reactions considered for each species at the interface are also listed in Table 3.1.

The outer boundary condition for the organic phase is also taken as zero flux for all species since the volume of the organic phase considered for modelling was assumed large enough to make leakage outside this volume negligible:

$$-D_i \nabla C_i|_{R=R_{out}} = 0 \tag{3.18}$$

The model was solved numerically with the finite volume method. A structured 1-dimensional spherical grid was used. Near the aqueous-organic interface the grid

(difference between 2 successive radii) was 10 times finer than near the centre and the outer boundary, to improve accuracy in the region with the highest concentration gradients. Between these limits, the size of the grid was increased uniformly on a logarithmic scale. The number of finite elements was increased until relative variations in model predictions were less than 10^{-4}. As a result, 10 finite volumes in the aqueous phase and 20 in the organic phase were selected. Matlab® 2018b (The MathWorks Inc., Natick, MA) equipped with the Statistics Toolbox was used for numeric calculations. A typical model simulation took between 1 and 2 seconds and a parameter estimation run several minutes on a laptop computer with an Intel i7 processor (4 physical cores) at 2.11 GHz with 32GB of RAM.

3.2.2.2.2. Droplet and needle, diffusive-convective mass transfer with global convection (GC)

Two extensions of the previous model were considered (Figure 3.3B). (i) Since the diameter of the needle is not negligible compared to that of the droplet, the possibility of diffusion of chemical species in the aqueous phase in and from the needle was included. One can namely expect that some acid diffuses from the needle into the droplet when the acid in the droplet is extracted in the organic phase. (ii) The possibility of some residual convection in both liquid phases, either due to thermal gradient, mechanical vibration or other causes.

In the organic phase, residual uncontrolled fluid convection was modelled by an essentially vertical, top-down, slow fluid motion with velocity field $\mathbf{u}$. At the liquid-liquid interface an equality constraint for the velocities in the organic and aqueous phase was imposed, thus inducing convection in the aqueous phase also. Since the fluid velocity is expected to be very low in both phases, a simplified version of the Navier-Stokes equations was solved, assuming an incompressible Newtonian fluid, laminar flow and the inertia term negligible compared to the viscous term (Stokes flow):

$$\nabla p = \nabla \cdot \left(\mu(\nabla \mathbf{u} + (\nabla \mathbf{u})^T)\right), \qquad \nabla \cdot \mathbf{u} = 0 \tag{3.19}$$

Along the vertical and on the axis of symmetry (B0) and on the outer boundary (B6) a non-penetration condition was set because the flow in the organic phase was assumed essentially vertical. The same condition was applied at the top of the modeled part of the aqueous phase in the needle (B1) and also the aqueous-organic interface (B3) because the fluids are not miscible:

$$\mathbf{n} \cdot \mathbf{u}|_{\mathrm{B0 \cup B1 \cup B3 \cup B6}} = 0 \tag{3.20}$$

In addition, the continuity of the velocity field was imposed along the liquid-liquid interface (B3):

$$\mathbf{u}_{aq}\big|_{\mathrm{B3}} = \mathbf{u}_{org}\big|_{\mathrm{B3}} \tag{3.21}$$

Along the metallic wall of the needle (B2 and B5), a no-slip condition was applied:

$$\mathbf{u}|_{\mathrm{B2 \cup B5}} = 0 \tag{3.22}$$

On the top boundary of the organic phase (B4) the velocity was set to a fixed value u_0

oriented downward along the z axis:

$$\mathbf{u}|_{B4} = -u_0 \cdot \mathbf{n} \tag{3.23}$$

On the bottom boundary (B7) an "outlet" condition was set as zero pressure. To determine the pressure field in the aqueous phase uniquely, the same condition was set point-wise at the intersection of boundaries B1 and B2:

$$p|_{\mathrm{B7}\cup(B1\cap B2)} = 0 \tag{3.24}$$

Transport of chemical species by advection was considered in addition to Fickian diffusion:

$$\frac{\partial C_i}{\partial t} + \nabla \cdot (-D_i \nabla C_i + \mathbf{u} C_i) = r_i \tag{3.25}$$

Reaction terms are listed in Table, with the aqueous-organic interface corresponding to boundary B3:

$$\mathbf{n} \cdot (-D_i \nabla C_i + \mathbf{u} C_i)|_{B3} = r_i \tag{3.26}$$

Zero normal flux boundary condition for all species was applied on the axis of symmetry (B0) and on the metallic walls of the needle (B2 and B5). The same condition was applied on the top of aqueous phase in the needle (B1) and on the outer boundary of the organic phase (B6) because the modelled part of the system was taken large enough to make leakage through these boundaries negligible:

$$\mathbf{n} \cdot (-D_i \nabla C_i + \mathbf{u} C_i)|_{B0\cup\mathrm{B1}\cup\mathrm{B2}\cup\mathrm{B5}\cup\mathrm{B6}} = 0 \tag{3.27}$$

On the inlet boundary (B4) the concentrations of all relevant species were considered constant and equal to their initial values in the organic phase:

$$C_i|_{B4} = C_{i0} \tag{3.28}$$

On the outlet boundary (B7) it was assumed that all species were transported out of the model domain mainly by the fluid motion and diffusive transport was neglected:

$$\mathbf{n} \cdot (-D_i \nabla C_i)|_{B7} = 0 \tag{3.29}$$

The model was solved numerically with the finite element method. A non-structured 2-dimensional triangular grid was used. The number of finite elements was increased until relative variations in model predictions were less than 10^{-4}. 1700 finite elements for a total of 6000 degrees of freedom were generated. Preliminary full dynamic simulations showed that the velocity field reached steady state in less than 2 seconds, which is very short compared to the typical duration of an experiment (between 6 000 and 15 000 s). To save computation time a steady-state solution was first computed for the velocity field and used for the dynamic calculations of the chemical species transport and reactions. Comsol Multiphysics® 5.3a (Comsol Group, Stockholm, Sweden) was used for numeric calculations, equipped with the Computational Fluid Dynamics, Chemical Reaction Engineering and Optimization modules. A typical simulation took between 1 and 2 hours and a parameter estimation run more than 10 days on a desktop computer with an Intel i7 processor (4 physical cores) at 3.60 GHz with 32GB of RAM.

3.2.2.2.3. Droplet and needle, diffusive-convective mass transfer with local convection (LC)

A variant of the previous model was tested replacing the "global", essentially top-down flow in the organic phase by local stirring at a millimetre scale. Such local convection could be generated by differences in organic phase density due to gradients in concentrations of chemical species and/or to the Marangoni effect, i.e. IFT gradient along the aqueous-organic interface (Sternling and Scriven, 1959). Full physical modelling of such a local convection remains a challenging task (Mao and Chen, 2004; Wang *et al.*, 2011). The authors did not attempt to model the unsteady velocity field generated by such phenomena but assumed a global mixing flow rate with velocity essentially normal to the interface. Moreover, simulations of the axisymmetric model with global convection (GC), described above, showed that the concentration field of relevant species had an effectively planar symmetry in the needle and spherical symmetry inside and around the droplet. The main exception was a small region at the contact between the needle and the droplet, as one might expect. Also, convection inside the needle was neglected.

The calculations were thus substantially simplified by using a 1D plane structured mesh in the needle and a spherical 1D structured mesh inside and around the droplet (Figure 3.C). Governing equations were thus the same as for the model GC, except that in both phases fluid velocity was assumed normal to droplet interface, oriented towards the interface, with magnitude given by:

$$u = \frac{Q}{4\pi R^2} \tag{3.30}$$

instead of being the solution of the Stokes flow equations ((3.19)-3.24). Q represents a mixing flow rate and the magnitude of the velocity derives from the continuity equation for an incompressible fluid ($\nabla \cdot \mathbf{u} = 0$), with R being the radial position from droplet centre. To ensure correct mass balances an outflow of magnitude Q was introduced from the interface to outer region of the modelled domain in the organic phase and from the interface to the center of the droplet in the aqueous phase. A variant of the model without convection inside the droplet was also tested to check the assumption of the mass transfer resistance mainly situated in the organic phase. Numeric calculations were performed similarly to the diffusive mass transfer model (D).

3.2.2.3. Model parameters

Some model parameters were fixed by the experimental setting or measured independently. Their values were fixed throughout simulations and are listed in Table 3.2. Other parameters had to be estimated from experimental IFT measurements, as indicated in the results section. In all cases, experiments with initial concentration of 3-HP in the aqueous phase of 0.5, 5 and 50 kg m^{-3} were used for model calibration, while the remaining experiments with initial concentrations of 1, 10 and 25 kg m^{-3} were used for model validation only.

Table 3.2. Fixed model parameters

Parameter	Value	Significance
R_d	$1.24 \cdot 10^{-3}$ m	Droplet radius
R_{out}	$12.4 \cdot 10^{-3}$ m	Outer radius of the modelled region
R_n	$0.595 \cdot 10^{-3}$ m	Internal needle radius
R_{nout}	$0.825 \cdot 10^{-3}$ m	External needle radius
l_n	$12.4 \cdot 10^{-3}$ m	Length of the modelled part of the needle
TOA_0	0.457 kmol m^{-3}	Initial TOA concentration in the organic phase
$(K_2K_3)^{1/n}$	8.17 kmol^{-1} m^3	Equilibrium constant between subsurface concentrations $\frac{CPX_s}{AH_s \cdot TOA_s}\Big\vert_{eq} = K_{11}$ (Chemarin *et al.*, 2017a)
K_1	$10^{-4.51}$ kmol m^{-3}	Acid dissociation constant in aqueous phase
K_4	408.5 kmol^{-1} m^3	Equilibrium constant in organic phase $\frac{CPX}{AH_{org} \cdot TOA}\Big\vert_{eq} = \frac{K_{11}}{m}$ (Chemarin *et al.*, 2017a)
k_{-1}	10^6 kmol^{-1} m^3 s^{-1}	Rate constant for Reaction 1. Arbitrary large value to make Reaction 1 not rate limiting
k_{-4}	10^6 s^{-1}	Rate constant for Reaction 4. Arbitrary large value to make Reaction 4 not rate limiting
μ_{aq}	$0.89 \cdot 10^{-3}$ Pa s	Aqueous phase viscosity
μ_0	$12.1 \cdot 10^{-3}$ Pa s	Organic phase viscosity without acid
γ_0	$10.23 \cdot 10^{-3}$ N m^{-1}	Interfacial tension without acid
D_{AH}	$1.044 \cdot 10^{-9}$ m^2 s^{-1}	Diffusivity of AH in aqueous phase
D_{A^-}	$1.096 \cdot 10^{-9}$ m^2 s^{-1}	Diffusivity of A^- in aqueous phase
$D_{AH_{org0}}$	$1.677 \cdot 10^{-10}$ m^2 s^{-1}	Diffusivity of AH in organic phase, without acid
D_{TOA0}	$5.814 \cdot 10^{-11}$ m^2 s^{-1}	Diffusivity of TOA in organic phase, without acid
D_{CPX0}	$5.325 \cdot 10^{-11}$ m^2 s^{-1}	Diffusivity of CPX in organic phase, without acid

3.2.3. Dynamic IFT measurements

3.2.3.1. Chemicals

3-HP was used as received without further treatment. It was diluted in deionised water at different concentrations (aqueous phase). All solutions were filtered through nylon filters (pore size: 0.22 µm). The tertiary amine used was tri-n-octylamine (TOA), which was further purified following a protocol used in a previous study (Chemarin *et al.*, 2017b): the amine was washed with an equal volume of H_2SO_4 0.1 M, then centrifuged at 8228×g and 25 °C. The upper phase formed after centrifugation was then recovered and washed with

an equal volume of NaOH 0.3 M, then centrifuged again. This was repeated twice and two final washes with deionized water were made, verifying that water pH was the same before and after the last washing. The purified TOA was diluted in decanol, at a proportion of 20% v/v TOA, 80% decanol. The mixture was washed with an equal volume of deionised water, in order to decrease the amount of water-soluble impurities contained. Water was separated by centrifugation at 8228×g and 25°C for 15 min and the organic phase was then recovered and filtered through a PTFE filter (pore size: 0.2 µm).

3.2.3.2. Pendant drop method

Experiments were carried out using an automated drop tensiometer Tracker (Teclis, France). Different 3-HP solutions from 0 to 50 kg m^{-3} were used to from a drop inside a transparent cell containing 25 mL of the organic phase (Figure 3.2), at a controlled temperature of 25°C. When the drop achieved a determined volume of 8 µL, interfacial tension (γ) was registered as a function of time until no change on γ values was observed (steady-state). Experiments were performed in triplicate.

The obtained experimental data was used together with the mathematical model to obtain further insights in interfacial tension phenomena and its relation with chemical equilibrium and mass transfer.

3.2.4. Statistical analysis

R^2 values (Equation 3.31) and the root mean square deviation (RMSD) (Equation 3.32) were calculated in order to measure the prediction accuracy of each model and be able to determine which model describes better the reaction system.

$$R^2 = 1 - \frac{\sum_{i=1}^{N}\left(\gamma_{exp,i} - \gamma_{mod,i}\right)^2}{\sum_{i=1}^{N}\left(\gamma_{exp,i} - \bar{\gamma}_{exp}\right)^2} \tag{3.31}$$

$$RMSD = \sqrt{\frac{\sum_{i=1}^{N}\left(\gamma_{mod,i} - \gamma_{exp,i}\right)^2}{N}} \tag{3.32}$$

where $\gamma_{mod,i}$ and $\gamma_{exp,i}$ are the predicted and measured IFT values, respectively; $\bar{\gamma}_{exp}$ is the average IFT value, and N is the number of experimental values.

3.3. Results

Dynamic IFT measurement results from the performed experiments are summarized in Figure 3.4 (symbols). Data from the 3 replicates of each experiment are superposed, indicating a good repeatability.

The interfacial tension between pure water and the TOA-decanol organic phase is around 10.23 mN m^{-1} and stable with time. The presence of the acid (3-HP) in the organic phase produces a sharp decrease in the interfacial tension followed by a slow increase. The decrease is faster than the first available measurements (10^{-2} s after droplet formation) and

is interpreted as the result of the accumulation of the surface-active complex (CPX) at the interface. Subsequent increase in IFT is interpreted as complex desorption and dilution in the bulk of the organic phase and takes up to 10^4 s to reach a pseudo-steady state. Thus complexation and adsorption reaction (Reaction 2) appears very fast compared to desorption reaction (Reaction 3) and the transport of the complex away from the interface in the organic phase. Existence of dynamic IFT minima is typical of situations when the mass transfer resistance in the phase towards which the surface-active compound moves is large, and the minimum is accentuated by the large volume of the extracting phase (Gassin *et al.*, 2013; Rubin and Radke, 1980). Both conditions are met here, since the viscosity of the organic phase is at least 10 times higher than of the aqueous phase and the volume more than 3000 times larger.

Maximum IFT decrease is higher when initial acid concentration is higher, but this relation is far from proportionality: a 100 times increase in 3-HP concentration (50 *vs.* 0.5 kg m^{-3}) induces a 4.56 times higher IFT variation (3.88 *vs.* 0.85 mN m^{-1}). At long times (several hours) IFT appears to stabilize at lower values for higher initial acid concentrations and in all case does not return to the clean interface value. Various phenomena could be invoked to explain this observation (Gassin *et al.*, 2013), for example:

- an irreversible adsorption of surface-active molecules
- reversible but very slow desorption and transport, at time scales much longer than the performed experiments
- a slow but continuous supply of acid from the needle, the acid in the droplet being never completely exhausted

Modelling results given below bring additional insight in the observed phenomena and further comments are given in the discussion section. The estimated parameters of each model are summarised in Table 3.3 and the prediction accuracy of each model is indicated in Table 3.4.

Simulation results for the purely diffusive mass transfer model (D) are shown in Figure 3.4. Adequate fit with experimental measurements of interfacial tension could not be found, for any of the adsorption-desorption models tested (Langmuir, Freundlich or Langmuir-Freundlich). If the association coefficient ϕ in Equation 3.14, which is not precisely known for the considered solvent, was allowed to vary, reasonable fit could be obtained for the highest initial acid concentration tested (Figure 3.4, solid lines) but at the price of a value of ϕ of 59.9 (Table 3.3) which is much higher than the largest value reported in the literature of 2.6. This indicates that measurements could be better described with much higher (about 7 times) value of the diffusion coefficients in the organic phase than given by Equation 3.14. Such values are unphysical but suggest the presence of convection. In the following, the association coefficient was fixed to a reasonable value of 1.2 (Wilke and Chang, 1955) and models including global and local convection were explored.

Table 3.3 Estimated model parameters

Parameter ± standard error	Model					
	D-LF	D-LF estimated ϕ	GC-LF[c]	LC-L	LC-F	LC-LF
Γ_m (10^{-9} kmol m^{-2})	1.5 ± 1.4	2.6 ± 2.4	3.34	0.246 ± 0.007	n. a.	1.75 ± 0.87
n	0.365 ± 0.033	0.32 ± 0.02	0.391	n. a.	0.321 ± 0.066	0.351 ± 0.018
k_{-2} (s^{-1})	(7.1 ± 6.3)·10^{8} [a]	3610 ± 650	5854	1570 ± 510	7550 ± 970	7300 ± 1200
k_{-3} (kmol^{-n} m^{3n} s^{-1})	390 ± 480	400 ± 450	2957	(7.2 ± 2.2)·10^{5} (kmol^{-1} m^3 s^{-1})	(2.41 ± 0.61)·10^{-6} (kmol^{1-n} m^{3n-2} s^{-1})	1900 ± 1400
K_3 (kmol^{n} m^{-3n})	3.1 ± 3.7	4.4 ± 4.8	2.54	(2.29 ± 0.19)·10^{-3} (kmol m^{-3})	(1.77 ± 0.09)·10^{9} (kmol^{n-1} m^{2-3n})	2.4 ± 1.5
ϕ	1.2[b]	59.9 ± 9.3	1.2 [b]	1.2 [b]	1.2 [b]	1.2 [b]
u_0 (10^{-6} m s^{-1})	n. a.	n. a.	5.99	n. a.	n. a.	n. a.
Q (10^{-12} m^3 s^{-1})	n. a.	n. a.	n. a.	4.78 ± 0.17·	5.13 ± 0.18	5.06 ± 0.18

n. a. not applicable
(a) Very high value that cannot be estimated exactly, indicates that Reaction 2 is not rate limiting
(b) Fixed value
(c) Standard error estimates are not available for this model
D-LF = Diffusion model with Langmuir-Freundlich isotherm (ϕ = 1.2)
D-LF estimated ϕ = Diffusion model with Langmuir-Freundlich isotherm (ϕ = 59.9)
GC-LF = Global convection model with Langmuir-Freundlich
LC-L = Local convection model with Langmuir isotherm
LC-F = Local convection model with Freundlich isotherm
LC-LF = Local convection model with Langmuir-Freundlich isotherm

Table 3.4 Models predictions accuracy

$[3\text{-HP}]_0$ (kg m^{-3})	Model					
	D-LF ϕ = 1.2	**D-LF ϕ = 59.6**	**GC-LF**	**LC-L**	**LC-F**	**LC-LF**
R^2						
0.5	0.814	0.890	0.896	0.749	0.962	0.959
1	0.844	0.862	0.851	0.583	0.947	0.940
5	0.888	0.950	0.928	0.888	0.981	0.980
10	0.913	0.957	0.910	0.914	0.981	0.977
25	0.923	0.996	0.973	0.978	0.993	0.993
50	0.889	0.997	0.941	0.968	0.994	0.994
RMSD						
0.5	0.140	0.085	0.081	0.162	0.066	0.059
1	0.208	0.128	0.130	0.230	0.079	0.087
5	0.237	0.146	0.158	0.196	0.091	0.090
10	0.214	0.149	0.213	0.212	0.099	0.107
25	0.280	0.126	0.177	0.154	0.139	0.131
50	0.440	0.081	0.298	0.219	0.094	0.095

D-LF = Diffusion model with Langmuir-Freundlich isotherm
GC-LF = Global convection model with Langmuir-Freundlich
LC-L = Local convection model with Langmuir isotherm
LC-F = Local convection model with Freundlich isotherm
LC-LF = Local convection model with Langmuir-Freundlich isotherm

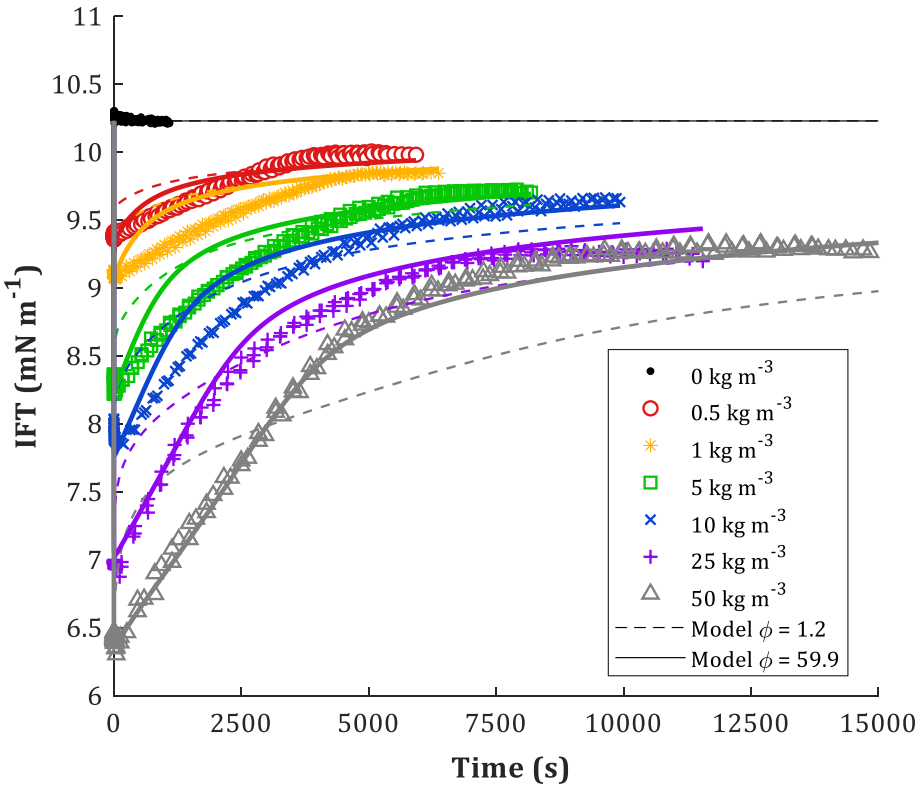


Figure 3.4 Dynamic interfacial tension for indicated initial 3-HP acid concentrations in the aqueous phase. Symbols: experimental measurements, lines: simulations for purely diffusive mass transfer combined with the Langmuir-Freundlich adsorption-desorption model (D-LF). Dashed lines: association coefficient $\phi = 1.2$, solid lines: $\phi = 59.9$.

3.3.1. Global convection mass transfer model (GC)

Simulation results with the global convection model (GC) are given in Figure 3.5 and estimated model parameters values in Table 3.3. The convection velocity in the organic phase is essentially vertical and oriented downward, with magnitude close to u_0 except near the needle wall and the droplet, as expected (Figure 3.5A). At the liquid-liquid interface velocities in both phases are equal and tangent to the interface, according to the modelling assumptions. Fluid movement in the organic phase induces a recirculation in the droplet with velocity oriented downward near the interface and upward along the axis of symmetry. In the needle, the velocity appears extremely low, suggesting an essentially diffusive mass transfer mechanism.

Shortly after the beginning of the experiment an acid concentration gradient is established near the interface between the aqueous and organic phases (Figure 3.5B). Near the needle, fluid velocity is close to zero and acid transport is essentially diffusive. Although the estimated fluid velocity is of order of only 20 mm h^{-1} (Table 3.3) the acid recovered in the organic phase is gradually swept away from the droplet as illustrated by the iso-concentration contours. At 10 s, however, the acid concentration in the bulk of the droplet is still essentially equal to the initial one. At 100 s the acid concentration in the droplet decreases significantly (Figure 3.5C) while at 1000 s there is very little acid left in the

droplet and it is mostly supplied from the needle (Figure 3.5D).

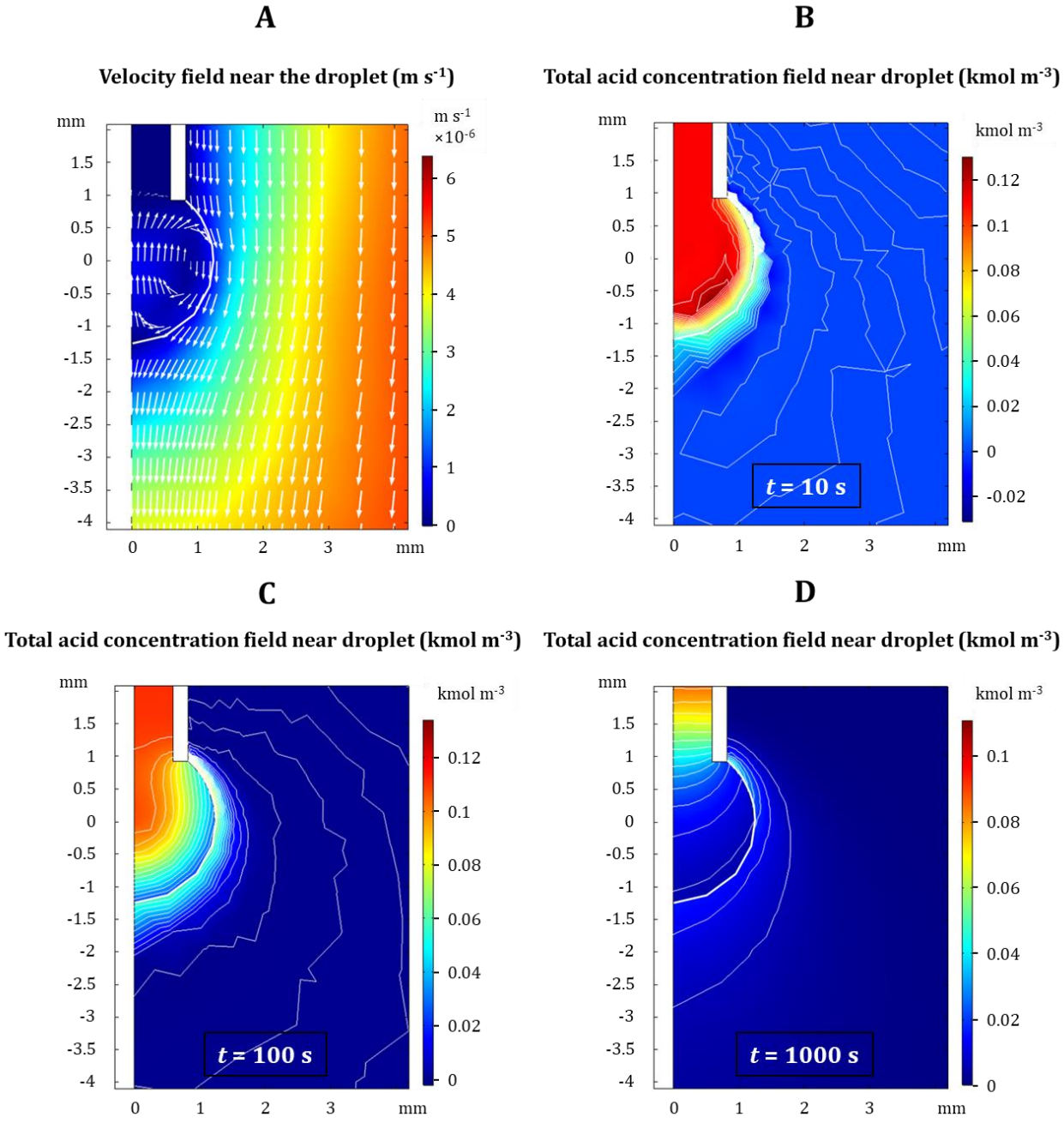


Figure 3.5 Simulations of the global convection mass transfer combined with the Langmuir-Freundlich adsorption-desorption model (GC-LF), for an initial acid concentration in the aqueous phase of 10 kg m^{-3} (0.11 kmol m^{-3}). A portion of the simulated domain situated around the droplet is shown. A) Fluid velocity, arrows indicate velocity magnitude on a logarithmic scale. B-D) 3-HP acid concentration in all forms: AH + A- in aqueous phase, AHorg + CPX in organic phase, at 10, 100 and 1000 s respectively.

The global convection mass transfer model accounts for the dynamically measured IFT (Figure 3.6) better than the purely diffusive model (Table 3.4) with realistic diffusion coefficients (Figure 3.4, dashed lines). Although the general trends are correct, simulated IFT increases faster than the measured one at short times (before 1000 to 3000 s, depending on the experiment) and slower at longer times, whatever the initial acid concentration and

adsorption-desorption model tested. This suggests that the acid removal pattern from the interface toward the bulk of the organic phase, via fluid motion along the interface (Figure 3.5A), is still not satisfactory. Hence, the local convection model, with fluid motion essentially normal to the interface, was explored next.

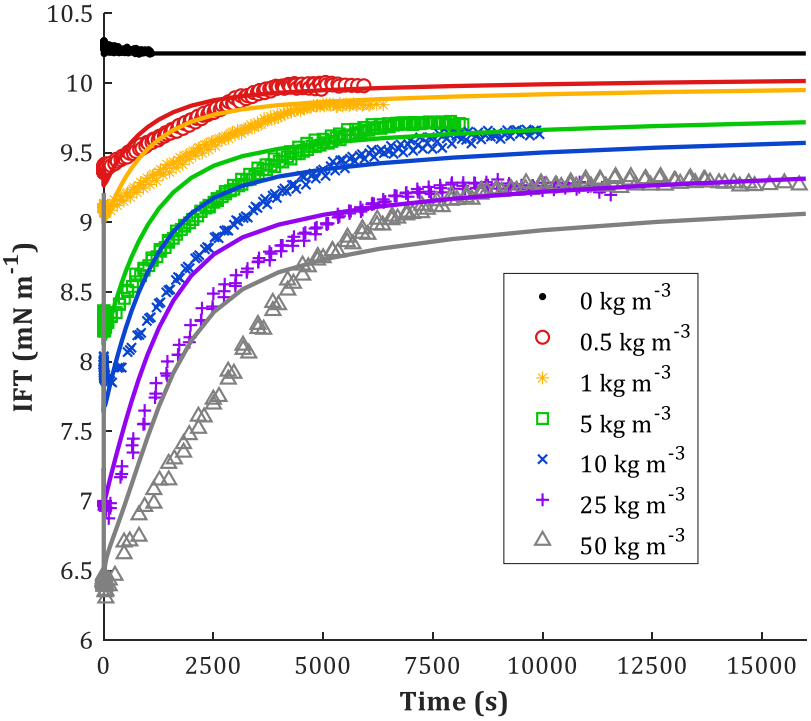


Figure 3.6 Dynamic interfacial tension for indicated initial 3-HP acid concentrations in the aqueous phase. Symbols: experimental measurements, lines: simulations for global convection mass transfer combined with the Langmuir-Freundlich adsorption-desorption model (GC-LF).

3.3.2. Local convection mass transfer model (LC)

We will show now that among the tested models, the best simulation of the observed dynamic interfacial tension was obtained with the local mass transfer model (Figure 3.7). Recall that data from experiments with initial concentration of 3-HP in the aqueous phase of 0.5, 5 and 50 kg m^{-3} were used for model calibration, while the remaining experiments with initial concentrations of 1, 10 and 25 kg m^{-3} were used for model validation only.

As previously observed, the relation between the initial acid concentration and the maximum decrease of IFT (compared to the case with no acid present) is highly nonlinear. In Figure 3.7A this nonlinearity appears to be better accounted for by the Freundlich model (LC-F, $0.947 \leq R^2 \leq 0.994$, Table 3.4) than by the Langmuir model (LC-L, $0.583 \leq R^2 \leq 0.978$, Table 3.4), especially for small acid concentrations: predictions for an initial 3-HP concentration of 0.5 kg m^{-3} had a $R^2 = 0.749$ with the LC-L model and a $R^2 = 0.962$ with the LC-F model; while predictions for 1 kg m^{-3} had a $R^2 = 0.583$ with the LC-L model and a $R^2 = 0.947$ with the LC-F model (Table 3.4). With the Langmuir model the adsorbed acid-amine complex concentrations (Γ) are very close to the maximum estimated adsorption capacity of the interface (Γ_m) for initial acid concentrations of 5 kg m^{-3} and higher (Figure

3.7C, dashed lines). With the Langmuir model, the nonlinearity between IFT and Γ mainly comes from the logarithm function (Equation 3.5). With the Freundlich model, no such adsorption limit is defined and the nonlinearity mainly comes from the order of the adsorption-desorption reaction (n). The estimated value of $n \cong 1/3$ (Table 3.3) suggests that one acid-amine complex occupies three adsorption sites on the average (Reactions 2F and 3F).

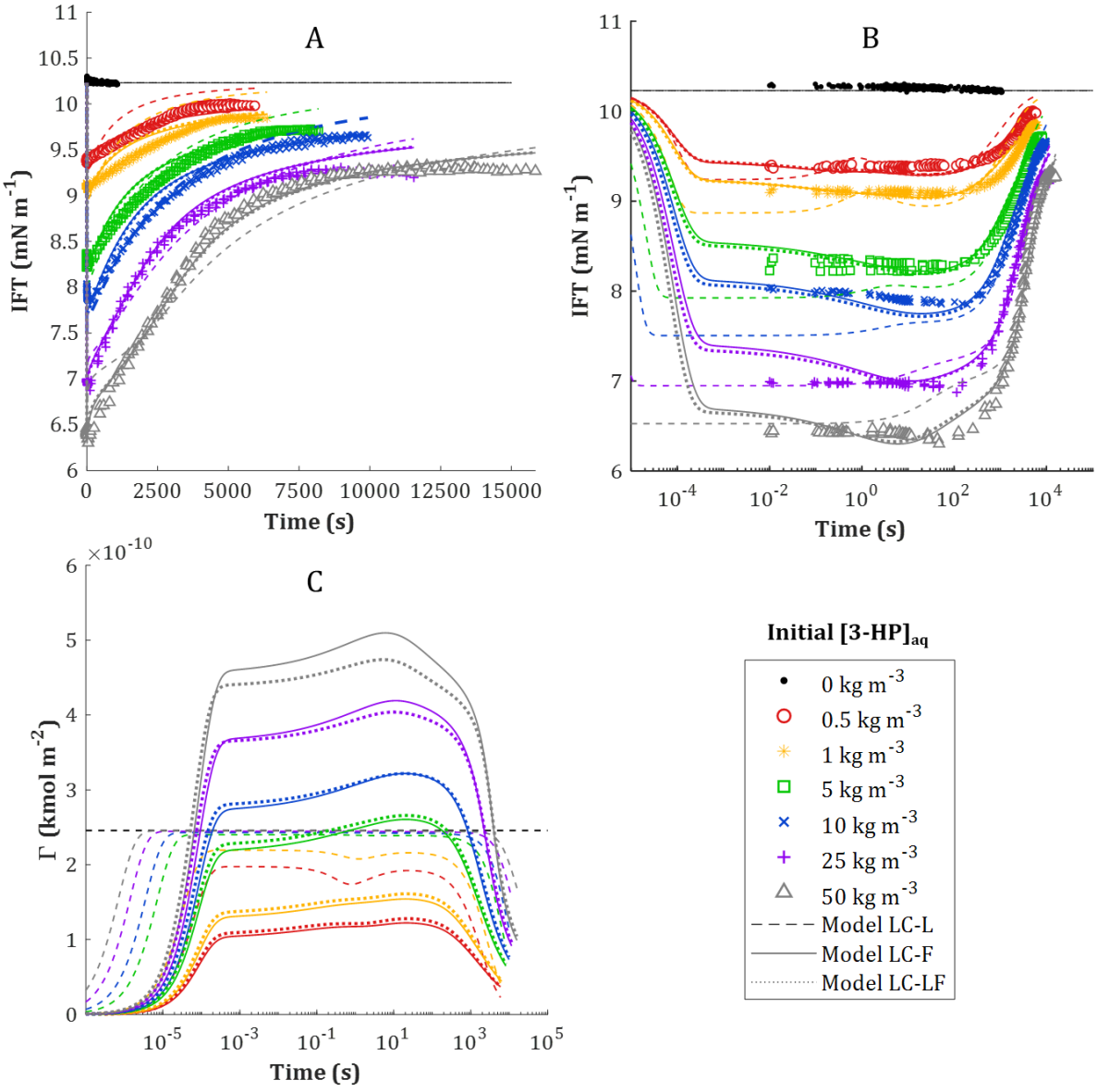


Figure 3.7 Simulations of the local convection mass transfer combined with Langmuir (LC-L), Freundlich (LC-F) and Langmuir-Freundlich (LC-LF) adsorption-desorption models. A) Interfacial tension, linear time scale. B) Interfacial tension, logarithmic time scale. C) Interfacial acid-amine complex concentration. Horizontal line: maximum interfacial concentration in the case of the Langmuir adsorption-desorption model (LC-L). The estimated maximum interfacial concentration in the case of the Langmuir-Freundlich adsorption-desorption model (LC-LF) is out of the figure scale.

The combination of the Langmuir and Freundlich models (LC-LF) gives no further advantage in terms of IFT prediction accuracy (Figures 3.7A and B). The finally best

predicted curves are nearly superimposed. Indeed, in the case of the Langmuir-Freundlich model, the estimated adsorption capacity of the interface (Γ_m, Table 3.3) is 3.5 times higher than the highest predicted interfacial complex concentrations (Figure 3.7C), meaning that Equation 3.11 can be reasonably well approximated by the linear Equation 3.8 for $\Gamma \ll \Gamma_m$.

The uncertainty in the value of Γ_m is relatively large for all LF models (Table 3.3) while the Freundlich exponent n is estimated to stay remarkably close to 1/3 in all F and LF models. The Freundlich adsorption-desorption model should be thus preferred for both accuracy and parsimony reasons. The following discussion thus concerns the LC-F model.

3.4. Discussion

Let us first note that, although Figures 3.7B and C focus on short time scales, experimental data are only available after 10^{-2} s. Therefore, acid-amine complex adsorption kinetics, predicted by the LC-F model to take place between 10^{-6} and 10^{-4} s cannot be validated directly. As a consequence, model parameter k_{-2} which governs the adsorption kinetics, can only be estimated with a relatively large uncertainty (Table 3.3). Between 10^{-4} and 10^{1} or 10^{2} s (depending on the initial acid concentration) adsorption and desorption reactions are roughly at a dynamic equilibrium. The slight increase of the interfacial complex concentration, and related slight IFT decrease, are due to gradual complex accumulation in the organic phase near the interface, which slows down the desorption reaction. This can be qualitatively observed in Figures 3.5B and C. At longer times, the stock of acid in the droplet is gradually exhausted (Figure 3.5D) and desorption reaction dominates, decreasing the interfacial complex concentration.

At long times, the developed LC-F model tends to predict faster IFT increase than actually observed (Figures 3.7A and B). In the model, this is associated to the rate of complex removal from the interface, which is related to the assumption of constant convection flowrate Q near the interface. If this convection is mainly due to a concentration gradient near the interface that vanishes over time, the local convection phenomenon is expected to die out at long times, leaving diffusion as the only mass transfer mechanism and explaining the observed slowdown in complex removal.

Figure 3.8 shows the concentrations of the considered species at various locations in the aqueous and organic phases. It is thus confirmed that the length of the needle (l_n) and the radius of the organic phase (R_{out}) considered in the model are sufficiently large to leave the concentrations in the farthest compartments constant at the experiment time scale. In the aqueous phase, the concentration gradients in the droplet are very small but are high in the needle, suggesting that some acid is supplied from there to the droplet by diffusion. Recall that convection in the needle is expected to be negligible (Figure 3.5A) but was considered in the droplet (Mao and Chen, 2004).

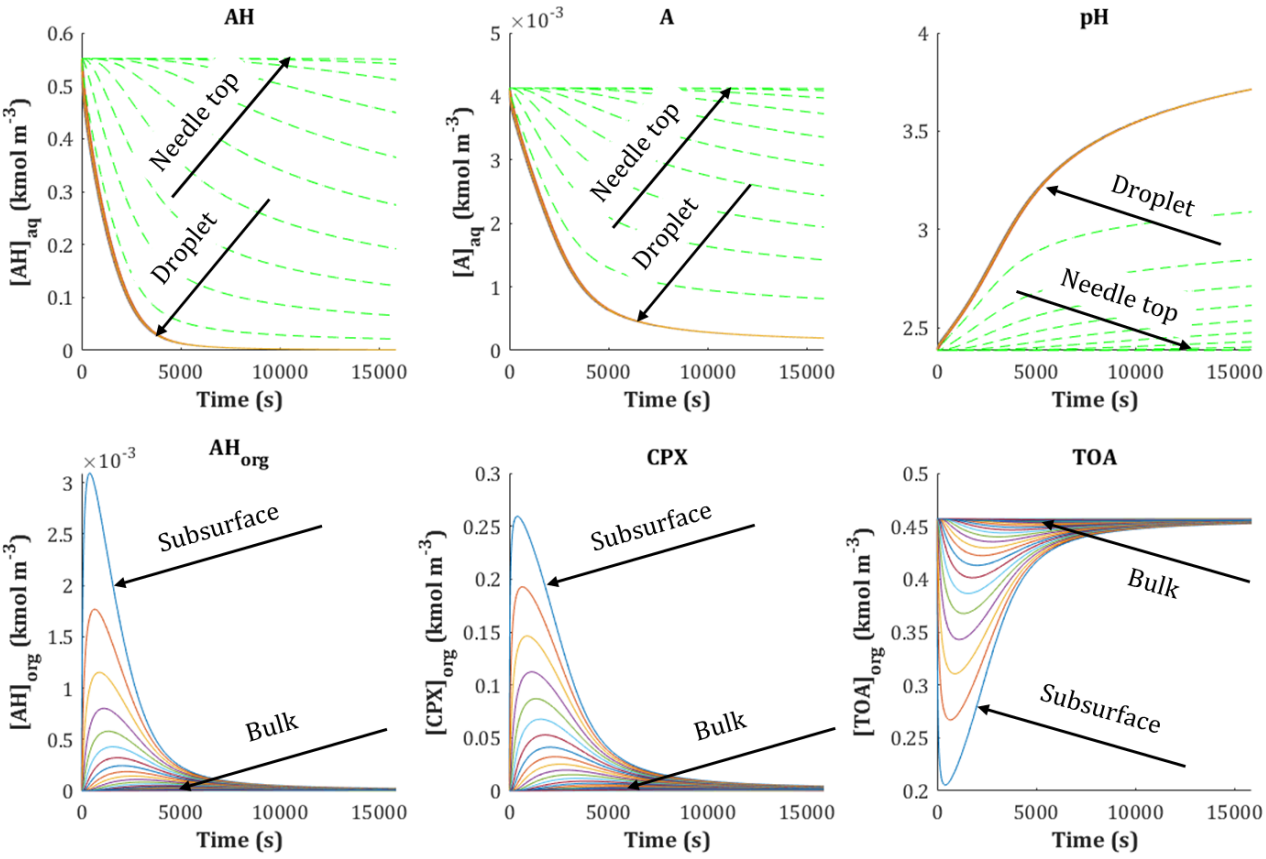


Figure 3.8 Dynamic concentrations of the considered chemical species as simulated by model LC-F for an initial acid concentration in the aqueous phase of 50 kg m^{-3} (0.56 kmol m^{-3}). Top: aqueous phase, solid lines: droplet, dashed lines: needle. Bottom: organic phase. Each line represents concentration at different distances from the interface.

3-HP acid in the aqueous phase is mostly present in its non-dissociated form (AH), the pH being always below 4 and the $pK_A = 4.5$. At long times, however, pH increases and the fraction of dissociated acid (A^-) that cannot be extracted by amine, increases, contributing to the slowdown of the extraction process.

In the organic phase, 3-HP, a hydrophilic compound, is mainly present in the acid-amine complex (CPX). The free form (AH_{org}) follows the same pattern with a roughly 100 times lower concentration (Reaction 4). For a short period after the beginning of the extraction process, subsurface TOA concentrations descends below half of its initial value, introducing some limitation in the complex formation process (Reaction 2). Recall that Figure 3.8 corresponds to the highest acid concentration tested, however; TOA limitation is thus expected to be minor for lower initial acid concentrations.

3.5. Conclusions

The present experimental and modelling study of the liquid-liquid extraction of an organic acid with an amine-alcohol mixture, conducted in a pendant drop interfacial tensiometer, gives insights in the involved interfacial reaction mechanisms and suggests some useful

guidelines for implementation in the contactor.

Comparison of diffusive and convective mass transfer models shows that the rate of acid-amine complex removal from the interface cannot be explained by molecular diffusion only and convection should be involved, even if no mechanical stirring was applied and the temperature of the system was controlled. Comparison of global and local convection models suggests that convection appears to be essentially normal to the liquid-liquid interface. The exact mechanisms of such local convection were not investigated in this work, but phenomena such as concentration-induced density gradients and associated buoyancy forces, or concentration-induced interfacial tension gradients and associated Marangoni convection could be suspected (Bekki *et al.*, 1990, 1992; Martin and Hudson, 2009). Study of these mechanisms is subject of future work.

Comparison of adsorption-desorption models did not clearly show a maximum interfacial acid-amine complex concentration as assumed by the Langmuir model, at least in the studied range of initial acid concentrations. The interfacial complex concentration varied as the power 1/3 of the subsurface acid concentration and this phenomenon was well accounted for by the Freundlich model. With this model, adsorption and desorption rates and equilibrium constants could be estimated with reasonable accuracy.

As a practical consequence for the extraction in membrane contactor, this study shows that high acid concentrations in the aqueous phase can reduce an already low interfacial tension which can cause difficulties in stabilizing the liquid-liquid interface. As a practical rule, reduction of the interfacial tension is roughly proportional to the power 1/3 of the acid concentration. At the highest acid concentration studied (50 kg m^{-3}) interfacial tension in the considered system was reduced by 40%. At higher acid concentrations amine availability near the interface may also become a limiting factor. The impact of the concentration-induced convection on mass transfer in a membrane contactor remains to be studied, but it could enhance transfer compared to molecular diffusion, e.g. in membrane pores.

Chapter 4

Organic phase selection for membrane-based reactive extraction: biocompatibility and extraction performance

This chapter describes a comprehensive methodology for the selection of an organic phase composition for 3-HP reactive extraction, based on both extraction performance and biocompatibility. Mixtures containing the three necessary types of solvents – extractants, active diluents and inert diluents – were evaluated first for 3-HP extraction performance (*i.e.*, 3-HP distribution coefficient and viscosity of the organic phase) and biocompatibility towards the 3-HP producing strain *Lactobacillus reuteri* DSM 17938, at flask-scale. Biocompatibility was evaluated by molecular level toxicity assessment. This was performed by determination of the cells physiological state through double fluorescent staining and subsequent analysis by flow cytometry, as well as bioconversion ability of glycerol into 3-HP. Finally, the impact of circulation through the membrane contactor towards cells was explored in different conditions, towards the possible implementation of an integrated process of in stream extractive bioconversion.

Most parts of this chapter were published as a research article:

Sánchez-Castañeda, A. K., Moussa, M., Ngansop, L., Trelea, I. C., and Athès, V. (2019). Organic phase screening for in-stream reactive extraction of bio-based 3-hydroxypropionic acid: biocompatibility and extraction performances. *Journal of Chemical Technology & Biotechnology*, in press. https://doi.org/10.1002/jctb.6284

4.1. Introduction

As mentioned in Chapter 1, *in-situ* or in stream product recovery (ISPR) is a promising strategy for the intensification of processes affected by end-product inhibition (Moussa *et al.*, 2016; Van Hecke *et al.*, 2014). Indeed, one of the main drawbacks of the bioprocess using *Lactobacillus reuteri* is inhibition by 3-HP accumulation (Burgé *et al.*, 2017), resulting in low bioconversion performance with the best results so far being an overall process productivity of 0.25 g L^{-1} h^{-1} and a final titre of 14 g L^{-1} (Dishisha *et al.*, 2015), which is insufficient for industrial-scale production (Werpy and Petersen, 2004). Reactive liquid-liquid extraction is thought to be well-adapted for removal of organic acids from aqueous media (Datta *et al.*, 2015; Djas and Henczka, 2018). The organic phase contains an *extractant* molecule able to react with the organic acid, allowing a selective recovery. Tertiary amines are among the most suitable *extractants*, thanks to their high extraction capacity, high selectivity and low water solubility (Kertes and King, 1986; Krzyzaniak *et al.*, 2011; Li *et al.*, 2016b; Tamada *et al.*, 1990). Tertiary amines react with the non-dissociated form of the acid and create an acid-base complex that is insoluble in the aqueous media. In order to stabilise the complex in the organic phase and enhance the extraction yield, an *active diluent* with a functional group able to interact with the acid-base complex is needed. Long-chain alcohols are among the best *active diluents* due to their polarity and specific H-bond donor character that favours their complex formation and solvation. This

has already been validated for 3-HP extraction (Chemarin *et al.*, 2017b) and other organic acids (Tamada *et al.*, 1990; Tamada and King, 1990a; Uslu *et al.*, 2009). The feasibility of 3-HP reactive extraction with different mixtures of extractants and active diluents has been demonstrated and the extraction mechanism has been thoroughly studied (Burgé *et al.*, 2016; Chemarin *et al.*, 2017b, 2019a), and details are given in Chapter 1. Previous studies of 3-HP reactive extraction showed that an organic phase made up of 20% (v/v) TOA and 80% decanol provided high extraction yield and selectivity (Burgé *et al.*, 2016; Moussa *et al.*, 2016). However, its application for the ISPR of 3-HP during its production by *L. reuteri* had a strong inhibitory effect on the cells, resulting in reduced 3-HP production (56% of the total production compared to the process without ISPR), even though the liquid-liquid extraction was assisted by a Hollow Fibres Membrane Contactor (HFMC) that avoids the direct contact of the organic phase with the cells (Burgé *et al.*, 2017). The toxicity of solvents used for reactive extraction is often reported in the literature concerning bacteria (Choudhury *et al.*, 1998; Matsumoto *et al.*, 2004; Yamamoto *et al.*, 2011). It has been suggested that the addition of a biocompatible but poor extractive solvent (*inert solvent*) to the active alcohol-type diluent can improve the biocompatibility of the extracting phase while maintaining an adequate extraction performance (Kumar *et al.*, 2012; Pérez-Ávila *et al.*, 2018; Wasewar *et al.*, 2011; Yabannavar and Wang, 1991a; Yamamoto *et al.*, 2011).

Solvents may affect cells at two different levels: by direct contact with the immiscible part of the solvent (phase-level toxicity) and interaction with the water-soluble solvent molecules (molecular-level toxicity) (Bar and Gainer, 1987; Marinova and Yankov, 2009; Yabannavar and Wang, 1991a). The toxicity of several organic solvents commonly used for the reactive extraction of carboxylic acids has been assessed on different strains of microorganisms (Chen and Lee, 1997; Choudhury *et al.*, 1998; Marinova and Yankov, 2009; Pérez-Ávila *et al.*, 2018; Seevaratnam *et al.*, 1991), but the variable and often contradictory results suggest that the selection of a biocompatible extraction phase strongly depends on the microorganism strain used. Solvent selection according to the particular needs of an ISPR strategy is therefore a key issue.

In comparison to other solvent screening studies, where the effect of different types of organic solvents has been studied, either on the extraction yield (Chemarin *et al.*, 2017b; Malinowski, 2001) or its toxicity towards the producing microorganism only (Marinova and Yankov, 2009; Pérez-Ávila *et al.*, 2018), this chapter describes a comprehensive approach that considers the extraction performance (extraction yield and viscosity) and toxicity of the extraction phase towards microorganisms. The strategy consisted first in evaluating the extraction yield at a given temperature and fixed initial 3-HP concentration. The viscosity of the organic phases was then measured and taken into account in the screening strategy because it affects mass transfer and, consequently, extraction process performance. Since the mass transfer in a HFMC is mainly governed by diffusion in membrane pores (Prasad and Sirkar, 1987), a low viscosity would lead to faster 3-HP extraction (Coelhoso *et al.*, 1997). This first approach provided a rapid screening of many solvents, the most promising of which were retained for toxicity studies. Solvent toxicity was evaluated by molecular-level toxicity assessment, like for in stream extraction, since the use of a HFMC avoids direct contact between the solvent and the microorganisms (Gao

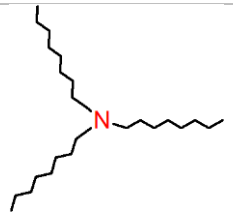
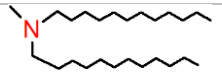
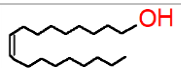
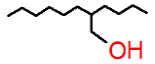
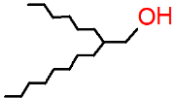
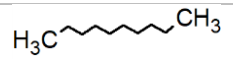
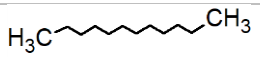
et al., 2009a; Huang *et al.*, 2004). Two approaches were proposed: 1) flow cytometry was used with the dual staining of cells as a quick method to evaluate the molecular-level toxicity of the solvents, through enzymatic activity and membrane integrity assessment; and 2) the ability of *L. reuteri* to convert glycerol into 3-HP was tested in an aqueous phase previously saturated with the soluble fraction of the studied solvents. This strategy is proposed as a tool to find a compromise between extraction performance and solvent toxicity in order to select an organic phase that allows continuous recovery of 3-HP produced by bioconversion in an in stream process. Beside evaluation of some solvents impact on cell physiological state, the effect of circulation through the membrane contactor was also evaluated, using cells suspended in water to feed membrane contactor fibres, to evaluate *L. reuteri* sensitivity to shearing alone. Finally, the selected organic phase composition was used in experiments of extraction and back-extraction in the HFMC modules, to test 3-HP extraction performance in these conditions. These experiments were also used to determine some parameters required to adjust a previously developed mathematical model, and also implement in this model the newly selected organic phase characteristics.

4.2. Materials and methods

4.2.1. Organic solvents for reactive extraction

Mixtures of three different types of organic solvents were evaluated: *extractants*, *active diluents* and *inert diluents*. TOA was purified as described in Chapter 3 (Section 3.2.3.1), and used as *extractant*. It was mixed with different long-chain alcohols as *active diluents*. Linear alcohols with carbon (C) chain lengths from 8 to 12 C were selected: octanol, decanol and dodecanol. Alcohols with shorter C chains present a high solubility in water and are expected to be toxic for the producing cells (Matsumoto *et al.*, 2004; Vermuë *et al.*, 1993), while longer linear C chain alcohols (*i.e.* tetradecanol), remained at a solid state at 25°C even in a mixture with the *extractant* and the *inert diluents*. In order to test longer C chains at a liquid state, two ramified alcohols (2-butyl-1-octanol and 2-hexyl-1-decanol) and one unsaturated alcohol (oleyl alcohol) were selected. Finally, two alkanes were tested as *inert diluents*: decane and dodecane. Didodecylmethylamine (DDMA) was also evaluated as *extractant*, purified in the same way as TOA and diluted at different concentrations with decanol in order to compare its extraction performance with TOA. Table 4.1 shows the physicochemical properties of the extractants and solvents used in this study.

Table 4.1 Physicochemical properties of tested extractants and solvents[a]

Compound	Purity %	Number of carbons	log $P_{o/w}$*	Solubility in water (mg L^{-1}) at 25°C	Molecular weight (g mol^{-1})	Density (g mL^{-1})	Chemical structure
Extractants							
Trioctylamine	99.7	24	11.22	0.05	353.7	0.809	
Didodecylmethylamine	99.2	25	8.76	-	367.7	0.820	
Active diluents							
Octanol	99.5	8	2.68	540	130.2	0.826	
Decanol	98.9	10	4.57	37	158.3	0.829	
Dodecanol	99.6	12	5.13	4	186.3	0.831	
Oleyl alcohol	75.3	18	7.40	0.07	268.5	0.848	
2-Butyl-1-Octanol	99.0	12	4.80	16.18	186.3	0.833	
2-Hexyl-1-Decanol	99.0	16	7.10	0.1727	242.4	0.836	
Inert diluents							
Decane	99.0	10	5.01	0.052	142.3	0.730	
Dodecane	99.0	12	6.10	0.0037	170.3	0.748	

* Logarithm of the partition coefficient in an n-octanol/water system

[a] From the National Center for Biotechnology Information (2018)

4.2.2. Extraction performance evaluation

4.2.2.1. Extraction yield

Different compositions of the organic phase were prepared by mixing the purified TOA with the different alcohols and alkanes, then washed with an equal volume of deionised water and separated by centrifugation at 8 228×g and 25°C for 20 min. Then, 10 mL of each organic phase were put in contact with an equal volume of a 3-HP solution at 5 g L^{-1} (0.055 mol L^{-1}) and mixed vigorously during 3 min. They were left to achieve equilibrium at 25°C for 48 h and subsequently separated by centrifugation. The volume ratio between the aqueous and organic phases for yield determination was 1:1 throughout this study.

In addition, an aqueous phase composed of 5 g L^{-1} 3-HP, 5 g L^{-1} 1,3-PDO and *L. reuteri* cells was prepared. This composition mimics the real medium in glycerol bioconversion into 3-HP and 1,3-PDO by *L. reuteri* (Burgé *et al.*, 2017). This aqueous phase was put in contact with the selected organic phase, for 3-HP extraction yield determination.

The aqueous phase was recovered and analysed by High Performance Liquid Chromatography (HPLC) as described in Chapter 2 (Section 2.5.1.1). Extraction yield was calculated with Equation 4.1:

$$Y = \frac{[AH]_{HPLC}^{ini} - [AH]_{HPLC}^{eq}}{[AH]_{HPLC}^{ini}} \times 100 \quad (4.1)$$

where $[AH]_{HPLC}^{ini}$ is the total initial acid concentration in the aqueous phase and $[AH]_{HPLC}^{eq}$ the acid concentration at equilibrium, as measured by HPLC. This value is related to the distribution coefficient K_D of the acid according to Equations 4.2 and 4.3.

$$K_D = \frac{[AH]_{org}^{eq}}{[AH]_{aq}^{eq}} \quad (4.2)$$

$$Y = \frac{K_D \times \frac{V_{org}}{V_{aq}}}{1 + K_D \times \frac{V_{org}}{V_{aq}}} \times 100 \quad (4.3)$$

where the volume ratio of the organic and the aqueous phase was $\frac{V_{org}}{V_{aq}} = 1$ in all experiments.

4.2.2.2. Viscosity of the organic mixtures

The viscosity of each organic phase mixture was measured with a cone and plate rheometer (Rheostress 600, Thermo Scientific), using a linear increase of shear rate from 0 to 100 s^{-1} at 25°C. Measurements were made in triplicate.

4.2.3. Solvent toxicity evaluation

4.2.3.1. Microorganism

The strain *Lactobacillus reuteri* DSM 17938 was obtained from BioGaia AB (Stockolm, Sweden). Before utilisation, cells were preserved in cryotubes containing 1 mL of Man, Rogosa and Sharpe (MRS) broth with 20% (v/v) of glycerol at -80°C. For every test, a new cryotube was activated by diluting 0.5 mL of the cells suspension in 9 mL of MRS broth contained in a test tube, then incubated during 30 min. All incubations were carried out at 37°C and 100 rpm. Then, 1 mL was taken from the test tube and diluted in 9 mL of fresh MRS broth and incubated during 8 h.

This preculture was used for biomass production in Schott bottles with 250 mL of MRS broth, supplemented with 20 g L^{-1} of glucose (initial pH = 6.2). The medium was inoculated at an initial optical density (OD_{600nm}) of $1.67 \cdot 10^{-5}$ (corresponding to a cell concentration of around $4 \cdot 10^{3}$ cells mL^{-1}), then incubated during 16 h. Once cells arrived to early stationary phase, they were harvested by centrifugation at 5000×g and 4°C, for 10 min, and washed once with Reverse Osmosis (RO) water to decrease growth metabolites concentration. Cells were re-suspended in RO water to a final OD_{600nm} of 70 ($1.8 \cdot 10^{10}$ cells mL^{-1}) in RO water.

4.2.3.2. Assessment of cells' physiological state

4.2.3.2.1. Experiments in Schott bottles

Equal volumes of sterilised RO water and of each organic phase were mixed and left to achieve equilibrium at 25°C for 48 h. The phases were then separated by centrifugation at 8 228×*g* for 20 min at 25°C and the aqueous phase saturated with the soluble fraction of the solvents was recovered. Around 1.2 mL of the washed cell suspension obtained as described in Section 4.2.3.1 was diluted in 20 mL of the solvent-saturated aqueous phase contained in 50 mL Schott bottles, at an OD_{600nm} of 4 (around $1.5 \cdot 10^{9}$ cells mL^{-1}). A control was prepared in parallel with every batch of experiments. It consisted in a cell suspension in RO water, without contact with organic molecules. All Schott bottles were put at 37°C and 100 rpm. Samples were taken periodically during 5 h and a last sample was taken at 24 h, to evaluate the physiological state of the cells by flow cytometry as detailed in Chapter 2 (Section 2.5.2.1). Experiments were made in duplicate.

4.2.3.2.2. Experiments in HFMC module

The effect of cells circulation through the HFMC on *L. reuteri* was also evaluated. Shear stress caused only by circulation through the modules was evaluated by recirculating 500 mL of a cell suspension at an OD_{600nm} of 4 (around $1.5 \cdot 10^{9}$ cells mL^{-1}) at different flow rates and inlet pressures to simulate high (630 mL min^{-1} and 1 bar), and low shear stress conditions (330 mL min^{-1} and 0.1 bar); RO water was circulated in the shell side for these experiments (Table 4.4). Flow rates were controlled by the rotation speed of the pump only,

in order to decrease the difference in the shear stress caused by the control valves (Figure 4.1).

Furthermore, two selected organic phase's compositions were tested over 500 mL of a cell suspension. The organic phase composed of 20% v/v TOA, 40% decanol and 40% dodecane was recirculated at a flow rate of 500 mL min^{-1} and an inlet pressure of 0.3 bar; while the organic phase with 20% v/v TOA and 80% oleyl alcohol was recirculated at 450 mL min^{-1} and 0.4 bar. The cell suspensions were recirculated at 620 mL min^{-1} and 0.9 bar for both organic phases.

A control consisting of 500 mL of a cell suspension in pure RO water and gently agitated by a magnetic stirrer was prepared simultaneously with each experiment. Samples were taken periodically during 5 h and a last sample was taken at 24 h. Cells physiological state was assessed by flow cytometry. Experiments were made in duplicate.

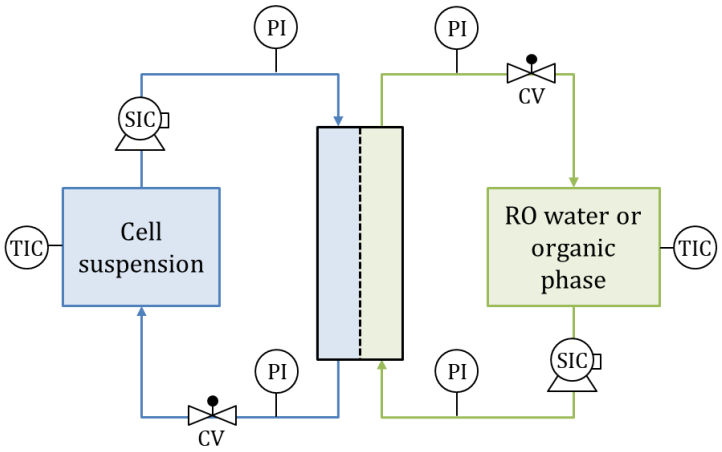


Figure 4.1 Schematic representation of the HFMC configuration to evaluate its impact towards cells physiological state. Abbreviations: CV, control valve; PI: pressure indicator; SIC, speed indicator and controller; TIC, temperature indicator and controller.

4.2.3.2.3. Sample analysis and calculations

Cells physiological state was evaluated with a dual fluorescent staining for (i) esterase activity assessment, using Carboxyfluorescein diacetate (cFDA), and (ii) membrane integrity, using the dye propidium iodide (PI), followed by analysis with flow cytometry (method detailed in Chapter 2, Section 2.5.2).

In order to compare the impact of the different solvents on the physiological state of the cells, a parameter referred to as Excess Mortality Rate (EMR) compared to control conditions was calculated (h^{-1}). To this end, the concentration of viable cells (stained with cFDA) in the solvent-saturated aqueous phase (X) was divided by the concentration of viable cells in the control (X^*). These values were plotted *vs.* time, and Equation 4.4 was adjusted to the data:

$$\frac{X}{X^*} = \frac{X_0}{X^*_0} e^{-EMR \cdot t} \quad (4.4)$$

where $\frac{X_0}{X^*_0}$ is the ratio of viable cells at time 0.

4.2.3.3. Assessment of 3-HP production ability

Bioconversions of 5 g L^{-1} of glycerol solubilised in 100 mL of RO water previously saturated with the organic phases were performed in 100 mL Schott bottles at 37°C and 100 rpm. Aqueous solutions were prepared as described in the molecular-level toxicity evaluation. For each set of experiments, a control was performed simultaneously, consisting in a bioconversion with glycerol at 5 g L^{-1} in RO water. Samples were taken periodically during 5 h. Experiments were made in triplicate, samples were analysed by HPLC to measure glycerol, 3-HP, 1,3-PDO and 3-HPA concentrations, as described in Section 2.5.1.2.

4.2.3.4. Bioconversion parameter determination

Data regarding glycerol consumption and products formation (3-HP, 1,3-PDO and 3-HPA) were fitted to an exponential function according to Equation 4.5:

$$P = a(1 - e^{-kt}) \quad (4.5)$$

This equation describes an increasingly asymptotic behavior that approaches an upper limit. It appears appropriate to describe a production affected by inhibition, as is the case of glycerol bioconversion to 3-HP. The parameter a represents the maximum produced concentration (mol L^{-1}), and k (h^{-1}) is related to the maximum production rate: the first derivative of Equation 3 gives the production/consumption rate r, which is maximal at t=0 (Equation 4.6):

$$r_0 = P'(0) = ak \quad (4.6)$$

In order to compare the different sets of experiments, the parameters a, k and r_0 for each tested organic phase were divided by their respective values obtained for the control (a*, k* and r_0*), corresponding to bioconversion without contact with solvents.

4.3. Results and discussion

4.3.1. Effect of inert diluent addition on extraction performances and toxicity towards L. reuteri cells

The first step in solvent screening was to determine the effect of concentration and the type of *inert diluent* addition on 3-HP extraction performance and toxicity. The organic phase was composed of 20% (v/v) of TOA as the extractant, diluted in 80% decanol as the *active diluent* or in a mixture containing 20%, 40% or 60% of decanol, where the remaining part was either decane or dodecane as the *inert diluent*.

4.3.1.1. *Extraction performance*

It appears from Figure 4.2a that increasing decane and dodecane concentration dramatically decreases the extraction yield. There is no significant difference ($p>0.05$) in extraction yield using either of the two inert diluents at the same concentration. Figure 4.2b shows that decane or dodecane addition reduces the viscosity of the mixture, with relatively minor differences between decane and dodecane. It should be recalled that low viscosities are desirable for accelerating mass transfer (Coelhoso *et al.*, 1997). A compromise between low viscosities and high extraction yield is quite difficult to find when looking at Figure 4.2b. Nevertheless, following the ideal tendency of the solvent properties (high extraction yield and low viscosity) shown on Figure 4.3b, the use of either decane or dodecane at 20 and 40% seems to be the most promising composition. Indeed, the extraction yield of the mixture with 60% of inert diluent is very low, and the mixture of 20% TOA and 80% decanol has proven to be toxic for *L. reuteri* (Burgé *et al.*, 2017). For this reason, toxicity to the producing strain was further studied.

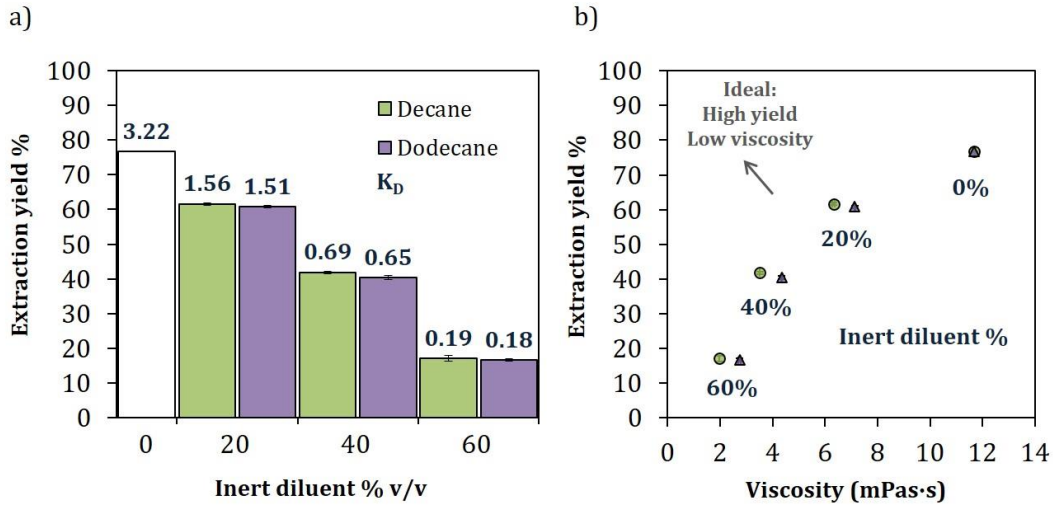


Figure 4.2 Effect of inert diluent addition to a TOA-decanol mixture on 3-HP extraction yield and organic phase viscosity. (a) Extraction yield (volume ratio 1:1) with corresponding distribution coefficient K_D, and (b) relationship between extraction yield and viscosity for decane (●) and dodecane (▲) at different concentrations (% v/v). Initial [3-HP] = 5 g L^{-1}. Error bars are masked by symbols.

4.3.1.2. *Solvent toxicity*

For this study, dodecane was selected as the inert diluent regarding its Log P value (6.10), which is higher than that of decane (log $P_{o/w}$ = 5.01, Table 4.1). Indeed, the results in the literature on solvent toxicity towards microorganisms often link the toxicity level to the log $P_{o/w}$ of the solvent (Bruce and Daugulis, 1991), and dodecane is therefore expected to be less toxic. In order to choose the adequate dodecane concentration, the molecular toxicity of the mixtures was evaluated by flow cytometry. Figure 4.3 shows the physiological state of *L. reuteri* in contact with TOA-decanol mixtures containing 0, 20 and 40% of dodecane. Figure 4.3a shows how the number of *L. reuteri* cells with different physiological states

after molecular contact with the mixture of 20% (v/v) TOA, 40% decanol and 40% dodecane varies over time compared to the control. It appears that the number of viable cells X^* essentially remains constant in the control, whereas the number of viable cells X decreases when in contact with the solvent molecules. Figure 4.3b shows the X/X^* ratio vs. time for three dodecane concentrations between 0 and 40% in mixture with TOA and decanol, and compares them using the EMR value. As expected, the addition of dodecane to the TOA-decanol mixture makes it possible to significantly decrease its toxicity towards *L. reuteri*. However, even with 40% dodecane, a significant viability loss after 5 h was observed, even though the EMR value is lower compared to the other conditions. Decanol seems to be responsible for this loss in viability since the TOA concentration is the same in all of these experiments.

Based on these results, the mixture of 20% TOA, 40% decanol and 40% dodecane was chosen as the reference composition for the next step in order to compare different active diluents to replace decanol.

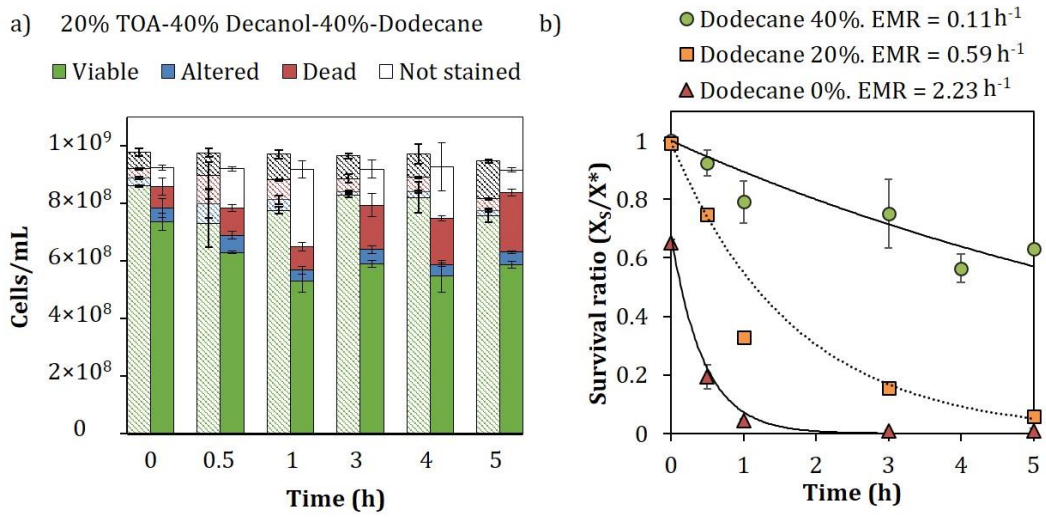


Figure 4.3 Evolution of the physiological state of *L. reuteri* in contact with three organic phases. (a) Number of stained cells analysed by flow cytometry: Control with L. reuteri suspended in RO water (striped bars, X^*) and molecular-level toxicity of a 20% TOA-40% decanol-40% dodecane mixture (plain bars, X_S). (b) Effect of dodecane addition to the mixture of TOA and decanol on cell viability.

4.3.2. Evaluation of the replacement of decanol with alternative long-chain active diluents

In a previous study it was determined that long-chain alcohols were the best active diluents for 3-HP reactive extraction with TOA thanks to their H-bond donor characteristic that provides good stabilisation of the acid-amine complex in the organic phase (Chemarin *et al.*, 2017b). Thus, alcohols of different carbon chain lengths were evaluated while maintaining the same TOA/alcohol molar proportion as in the reference composition to make a stoichiometric comparison of extraction yields, the rest being completed with dodecane (Table 4.2). Oleyl alcohol was also evaluated at 80% with TOA without the addition of dodecane.

Table 4.2 Organic phase composition for active diluent evaluation

#	Active diluent				TOA		Dodecane	
	Name	Carbon number	% (v/v)	mol L^{-1}	% (v/v)	mol L^{-1}	% (v/v)	mol L^{-1}
1	Octanol	8	33	2.10	20	0.46	47	2.06
2	Decanol	10	40	2.10	20	0.46	40	1.75
3	Dodecanol	12	47	2.10	20	0.46	33	1.45
4	2-Butyl-1-octanol	12	47	2.10	20	0.46	33	1.45
5	2-Hexyl-1-decanol	16	61	2.10	20	0.46	19	0.85
6	Decanol	10	80	4.20	20	0.46	-	-
7	Oleyl alcohol	18	80	2.51	20	0.46	-	-

Table 4.3 shows the molecular toxicity of each mixture sorted according to its EMR value and its survival ratio after 5 and 24 h of contact with aqueous solutions equilibrated with the selected solvent mixtures. After 24 h, cells survival ratio decreased significantly for almost all organic phases. Only 2-hexyl-decanol maintained a high value, being the most biocompatible solution. For comparison purposes, we can consider a biocompatible solvent with a survival ratio at 5 h higher than 0.75, a toxic solvent with a value lower than 0.25 and a medium toxicity in between. Similar to the classification proposed by Marták *et al.* (1997).

Table 4.3 Molecular-level toxicity of the selected solvent mixture with 20% TOA and dodecane

#	**Active diluent**	**% (v/v)**	**mol L^{-1}**	**Excess Mortality Rate (EMR) h^{-1}**	**Survival ratio X/X^* at 5 h**	**Survival ratio X/X^* at 24 h**	**Toxicity classification**
5	2-Hexyl-1 decanol	61	2.1	0.004 ± 0.002	0.833 ± 0.027	0.820 ± 0.047	Low
7	Oleyl alcohol	80	2.5	0.039 ± 0.011	0.867 ± 0.126	0.107 ± 0.044	Low
2	Decanol	40	2.1	0.112 ± 0.004	0.566 ± 0.011	0.016 ± 0.001	Medium
3	Dodecanol	47	2.1	0.146 ± 0.019	0.501 ± 0.007	0.014 ± 0.008	Medium
6	Decanol	80	4.2	2.231 ± 0.205	0.013 ± 0.006	0.006 ± 0.001	High
1	Octanol	33	2.1	2.319 ± 0.242	0.002 ± 0.001	0.002 ± 0.001	High

The selected markers for flow cytometry provide valuable information about the physiological state of the cells based on esterase activity and membrane integrity. This information is relevant in terms of the ultimate goal of identifying biocompatible organic phases that do not hinder the bioconversion step during the integrated process. In addition, it is necessary to determine if there is a correlation with their bioconversion capacity. Therefore, solvent toxicity was also evaluated by comparing the bioconversion of 5 g L^{-1} of glycerol in contact with the soluble fraction of the organic phases. To do this, three

bioconversion parameters were calculated for glycerol consumption: 3-HP, 1-3-PDO production and their metabolic intermediate (3-HPA) accumulation. All parameters were expressed as ratios relative to a control without contact with the solvents (Figure 4.4).

These parameters provide an interesting description of metabolite production and glycerol consumption in the presence of the different solvent molecules. Results for the ratio a/a^* show that the mixture with octanol is the most toxic of all the organic phases. This can be explained by its relatively high solubility value (540 mg L^{-1}; Table 4.1). The ratio k/k^* shows an interesting behaviour for mixtures with decanol. It appears that the 3-HP production rate is higher than the control at the beginning of the bioconversion, as confirmed by the ratio of initial production rates ($r_0/r_0{}^*$), but it is inhibited by the solvent molecules later on. This phenomenon is not observed with the octanol mixture, however, probably because of its very high toxicity that does not allow much metabolic activity. Bioconversion parameters indicate that the mixtures with 2-hexyl-1-decanol, oleyl alcohol and dodecanol are not toxic since they have bioconversion indicators similar to the control.

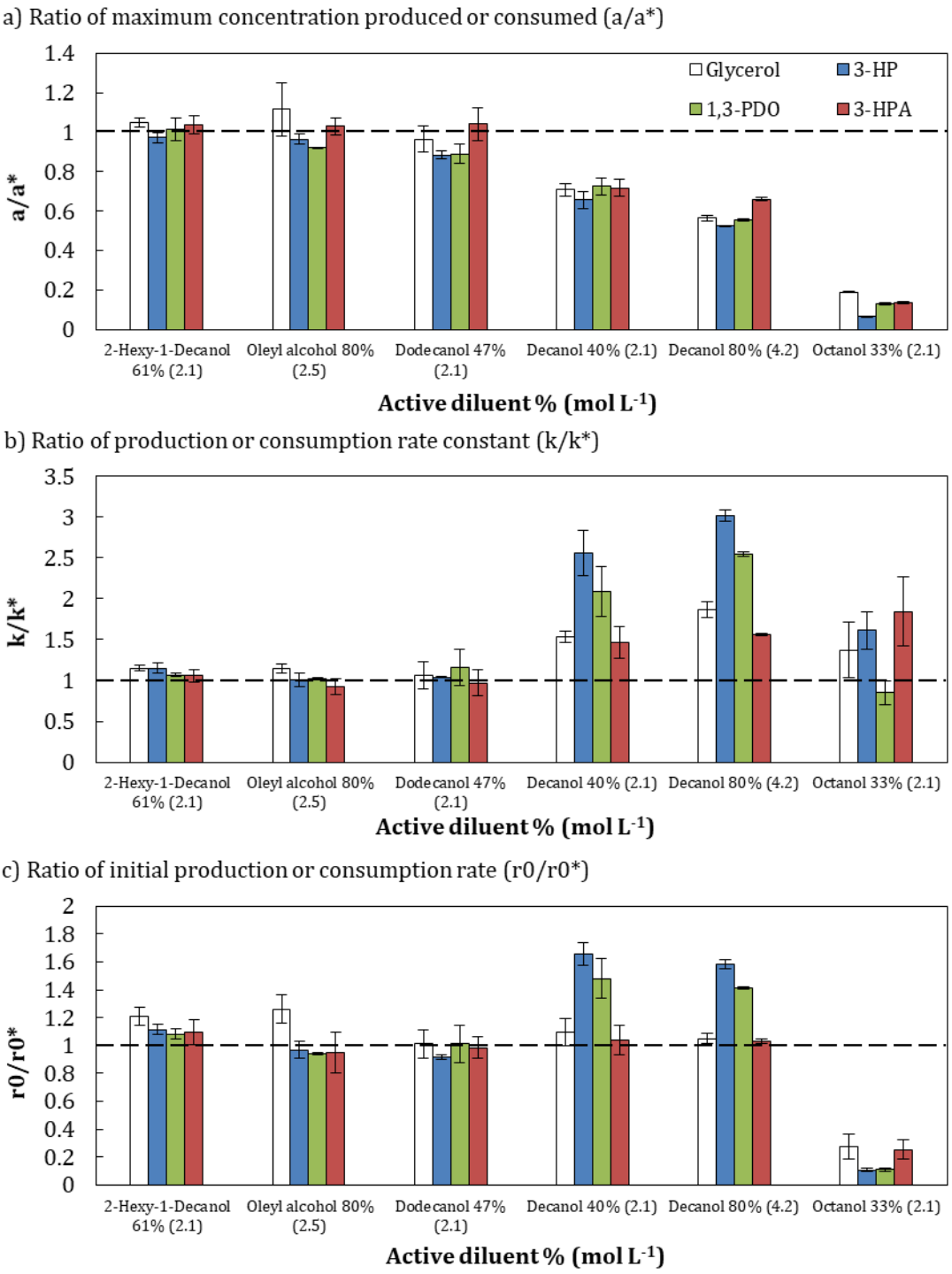


Figure 4.4 Bioconversion parameters of *L. reuteri* in contact with the soluble fraction of the different solvents, compared to a control (glycerol bioconversion in pure water)

Figure 4.5 shows the time evolution of the ratio of 3-HP production in contact with the soluble fraction of the solvent ($[3-HP]_S$) compared to the control ($[3-HP]^*$), represented by the ratio $[3-HP]_S/[3-HP]^*$. Once again, the difference between octanol and the other alcohols is clear. In the case of the two mixtures with decanol, there is a higher initial production of 3-HP than in the control, which is consistent with the bioconversion

indicators represented in Figure 4.4. However, this increase in production rate is followed by an inhibition effect during the first 2 – 3 hours before the production rate stabilises at 0.70 for 40% decanol and 0.55 for 80% decanol. The mixtures with oleyl alcohol and 2-hexyl-1-decanol seem to be quite biocompatible, while the mixture with dodecanol shows a slight reduction of 3-HP production after 4 h, compared to the control. However, a similar trend between the impact of solvents on cell viability and their bioconversion ability was observed overall.

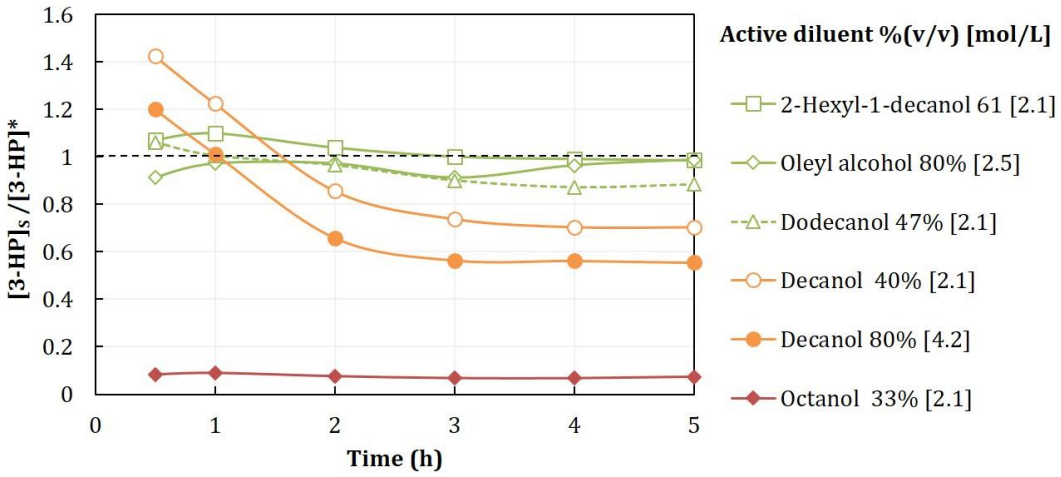


Figure 4.5 Evolution of 3-HP concentration ratio between bioconversion in contact with the solvent's soluble fraction $[3-HP]_S$ and control $[3-HP]^*$

4.3.3. Selection of the organic phase

Figure 4.6 shows the extraction yield and viscosity of the organic phases presented in Table 4.3 and includes information about their toxicity towards *L. reuteri*. For the same alcohol molar concentration, extraction yield decreases with the number of Cs in the alcohol chain, whereas viscosity increases. The log $P_{o/w}$ value also increases with the C number (Table 4.1), which may be related to a better biocompatibility, as observed in Table 4.3. This is consistent with what has been widely described in the literature, *i.e.*, that attempts to improve the biocompatibility of the extraction phase usually decrease its extraction performance (Datta *et al.*, 2015; Morales *et al.*, 2003; Tamada and King, 1990a). Thus, the evaluation of the solvent toxicity on the studied microorganism is mandatory in order to find a good compromise. Extraction performance of 2-butyl-1-octanol was tested to determine the difference between linear and ramified alcohol of the same carbon length (dodecanol) on 3-HP extraction. The extraction yield is lower with the ramified alcohol and the viscosity is similar. If the log $P_{o/w}$ value of both alcohols (dodecanol log $P_{o/w}$ = 5.13; 2-butyl-1-octanol log $P_{o/w}$ = 4.8, Table 4.1) is considered, the toxicity of the ramified alcohol can be similar to that of the linear alcohol or even higher. Therefore, 2-butyl-1-octanol was not considered for solvent toxicity tests.

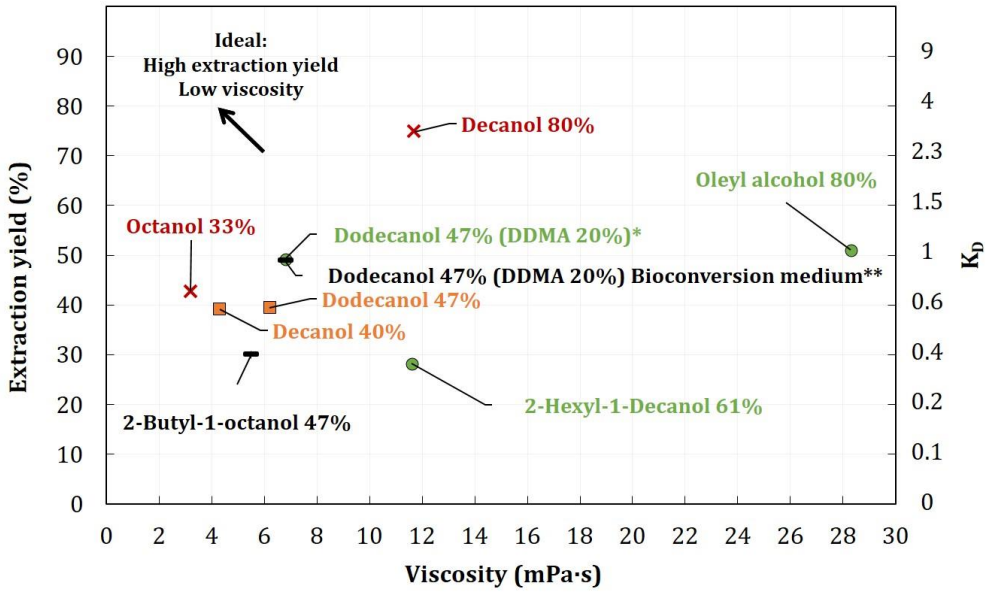


Figure 4.6 Extraction performance: extraction yields (volume ratio 1:1) and corresponding partition coefficients K_D vs. viscosity. Solvent toxicity classification of mixtures containing 20% v/v TOA (unless otherwise specified) with different active diluents and dodecane concentrations. Initial [3-HP] = 5 g L^{-1}. Classification was according to survival ratio reported in Table 4.3. Green (●): low toxicity; orange (■): medium toxicity; red (×): high toxicity; black (—): toxicity was not determined. *Didodecylmethylamine (DDMA) as the extractant. **3-HP in bioconversion-like medium.

Flow cytometry results gave consistent information with 3-HP bioconversion ability. However, solvents with medium toxicity as the mixture with dodecanol, showed a stronger effect on cells viability and integrity (Table 4.3) that on 3-HP bioconversion ability, since it was very similar to the control (Figure 4.5). The same behaviour was observed with 20% (v/v) TOA in decanol, implying that even if the physiological state of cells is affected by the solvents, they are still capable of producing 3-HP. Bioconversion in contact with this organic phase in the ISPR system, gave lower 3-HP production than bioconversion without extraction (Burgé *et al.*, 2017). This concordant and complementary information given by flow cytometry suggests that it is a relevant and quick method to monitor bioconversion with *L. reuteri* DSM 17938.

Taking into account the compromise between the extraction yield, viscosity and solvent toxicity related to cell viability and 3-HP production ability, the mixture of 20% (v/v) TOA, 47% dodecanol and 33% dodecane seems to be the best choice for the in stream 3-HP recovery from bioconversion by *L. reuteri*. However, this mixture shows relatively low extraction performance (extraction yield = 39.5 ± 0.5%). A possible strategy to solve this issue is the use of an alternative *extractant* while maintaining the biocompatibility of the organic phase. This strategy was investigated using didodecylmethylamine (DDMA) to replace TOA.

4.3.3.1. Impact of the amine extractant: comparison between DDMA and TOA

DDMA consists of an amine group linked to two chains of 12 carbons each and one methyl

group (Table 4.1). It was chosen because the N atom in this configuration is expected to exhibit better reactivity than in the case of TOA where the amine group is more affected by steric hindrance of the three chains of eight carbons. In addition, having a similar number of carbon atoms as TOA and a log $P_{o/w}$ value of 8.76 is expected to result in low water solubility and low molecular level toxicity.

Figure 4.7 shows the extraction yield of DDMA diluted in decanol compared with TOA at the same molar concentrations determined by Chemarin *et al*. (Chemarin *et al.*, 2017b) A similar bell-shaped behaviour is observed for both extractants, with higher extraction yield for DDMA than for TOA. Others studies that tested different amines as extractants have shown a decrease in the extraction yield caused by steric hindrance of the carbon chains linked to the N atom. For example, the results of Kyuchoukov and Yankov (Kyuchoukov and Yankov, 2012) show that when different amines are used for lactic acid extraction, yields decrease when the steric hindrance of amine increases. The presence of longer alkyl chains next to the N atom hinders the access of the H atom from the acid to form the ion pair in the acid-base complex. Matsumoto *et al*. (Matsumoto *et al.*, 2003) also observed a decrease in extraction yields of TOA compared with two other tertiary amines with the same carbon number but in ramified configurations. They mentioned that the stability of the complex becomes impaired due to steric hindrance.

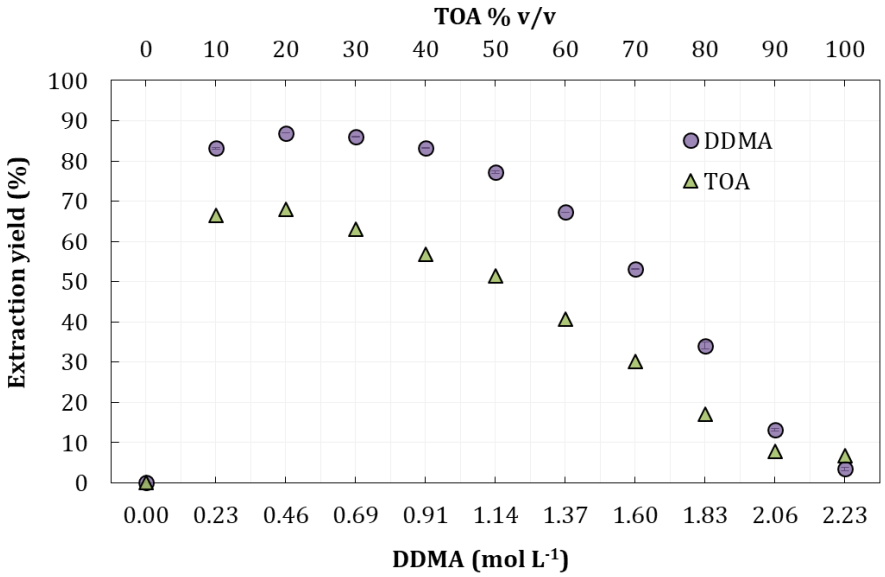


Figure 4.7 Extraction yields (volume ratio 1:1) of DDMA diluted in decanol at different concentrations compared with TOA with a 1 g L^{-1} 3-HP solution. (●) DDMA, (▲) TOA. Error bars masked by symbols.

Therefore, the replacement of TOA with DDMA was evaluated in the selected composition of the organic phase with 20% TOA, 47% dodecanol and 33% dodecane. The extraction yield, viscosity and solvent toxicity of the mixture are given in Figure 4.6. As expected, a higher extraction yield is obtained with DDMA at the same diluent concentrations, with a slightly higher viscosity. Toxicity evaluated by flow cytometry was very low (EMR = 0.004 ± 0.001 h^{-1}, survival ratio at 5 h = 0.905 ± 0.041) and the bioconversion parameters were very similar to the control since this solvent mixture was classified as biocompatible.

Although it was possible to find an organic phase composition with a higher extraction yield and low toxicity, it was observed that dispersive extraction using DDMA formed a very stable emulsion (even after centrifugation at 8 228×g for 15 min) where the stability of the emulsion increases with the amine concentration. This behaviour is probably caused by the strong surfactant properties of the acid-base complex formed by DDMA. This issue can be solved using a non-dispersive in stream extraction system like HFMC with careful interface stabilisation, which avoids the formation of emulsion.

4.3.3.2. Extraction performance of the selected organic phase with a bioconversion-like medium

Real bioconversion broth composition has an important effect on 3-HP reactive extraction (Chemarin *et al.*, 2019a). However, one of the advantages of a fed-batch glycerol bioconversion into 3-HP by resting cells of *L. reuteri*, is the simplicity of the medium: a glycerol solution is fed to a cell suspension in RO water only. The use of physiological water is avoided because the presence of ions from dissolved salts decreases the distribution coefficient of the acid (Chemarin *et al.*, 2019a; Reyhanitash *et al.*, 2016). 3-HP and 1,3-PDO are the only products, at equimolar proportions (Burgé *et al.*, 2017). The feeding rate is adjusted to maintain a low glycerol concentration and avoid 3-hydroxypropionaldehyde (3-HPA) accumulation, which is highly toxic for the strain (Dishisha *et al.*, 2015; Schaefer *et al.*, 2010). It was previously observed that glycerol and 3-HPA did not have a significant effect on 3-HP reactive extraction yield (Burgé *et al.*, 2016; Moussa *et al.*, 2016). In addition, as continuous removal of the acid is expected, no base solution for pH control will be used. Therefore, the only components considered to have a significant effect on extraction performance were 1,3- PDO and *L. reuteri* cells.

In order to mimic the bioconversion medium, *L. reuteri* cells and 5 g L^{-1} of 1,3-PDO were added to a 5 g L^{-1} 3-HP solution. This aqueous phase was put in contact with the selected organic phase consisting of 20% DDMA, 47% dodecanol and 33% dodecane. As expected, the obtained extraction yield (48.8 ± 0.1 %) was similar to the value with the model solution at 5 g L^{-1} (49.3 ± 0.2 %, Figure 4.6). In addition, 1,3-PDO was extracted only at a yield of 1.8 ± 0.5 %, proving that this organic phase has a high selectivity for 3-HP, even with the addition of dodecane to the organic mixture.

The presence of cells did not have an important effect on extraction yield, even though there was an increase in the initial pH from 2.89 to 3.16, compared to the model 3-HP solution. This is expected at values below the pKa of the acid (4.51) (Chemarin *et al.*, 2019a). However, it has been reported that cells decrease the mass transfer coefficient, acting as a physical barrier at the liquid-liquid interface (Pursell *et al.*, 2004). In this respect, low viscosity is an advantageous property than can alleviate this effect for the global mass transfer coefficient.

Chen and Lee (1997) used a biocompatible organic phase composed of 20% Alamine 336, 40% oleyl alcohol and 40% kerosene by volume, for extractive fermentation of lactic acid. They obtained a partition coefficient of 0.30, lower than the one obtained in this study with the selected organic phase (K_D= 0.95 ± 0.004), but it allowed continuous acid extraction.

Other successful studies of extractive fermentations assisted by HFMC used biocompatible organic phases composed of a tertiary amine at concentrations from 4 to 10% (v/v), diluted in oleyl alcohol (Jin and Yang, 1998; Nelson *et al.*, 2017; Wu and Yang, 2003a), similar to the composition of 20% (v/v) TOA diluted in oleyl alcohol tested in this study (K_D = 1.04 ± 0.007). Comparisons with different systems in the literature is delicate because distribution coefficient depends on initial acid concentration, pH, the nature of the acid and microbial medium composition (Chemarin *et al.*, 2019a; Moussa *et al.*, 2016). However, assuming that distribution coefficient could be similar to the one obtained in this study, the selected organic phase had a significantly lower viscosity (6.4 *vs.* 28.3 mPa·s). As the partition coefficients are similar, a higher mass transfer coefficient can be expected in extractive bioconversion.

4.3.4. Experiments in HFMC

Assessment of cells' physiological state by flow cytometry was used to evaluate the effect of HFMC towards *L. reuteri* DSM 17938 at different conditions. Operational conditions and results are summarised in Table 4.4 and discussed below.

4.3.4.1. Shear stress evaluation

Shear stress in HFMC was previously evaluated in relation with an extractive bioconversion of glycerol into 3-HP and 1,3-PDO by *L. reuteri* DSM 17938 which had a duration of less than 3 h in a batch-mode bioreactor (Burgé *et al.*, 2017). Results showed no significant contribution to inhibition phenomena towards cells. As fed-batch mode allows longer bioconversion time (Dishisha *et al.*, 2015), it is therefore necessary to evaluate shear stress for longer than 3 h. To this end, cells' physiological state was monitored during 24 h. Two different flow rate conditions were tested. First, cells were circulated at a flow rate of 630 mL min^{-1} and 1 bar of inlet pressure on the fibres-side (condition #1). Survival ratio was maintained relatively constant during the first 5 h of monitoring, which was consistent with the previous study where no significant inhibitory effect was observed during 3 h of circulation (Burgé *et al.*, 2017). Nevertheless, survival ratio decreased significantly after 24 h (0.061 ± 0.031, Table 4.4), meaning that *L. reuteri* is sensitive when exposed to longer time for these circulation conditions. A lower flow rate was then tested (330 mL min^{-1} with 0.1 bar of inlet pressure, condition #2). For these conditions the EMR was lower (Table 4.4), maintaining a survival ratio of 0.805 ± 0.003 after 24 h, preserving cells from shearing stress.

These results show that *L. reuteri* cells are sensitive to the shear stress caused by circulation through the HFMC during extended periods of time and it depends on the flow rate and the inlet pressure value. Adequate circulation conditions have to be determined in order to ensure an adequate interface stabilisation with the organic phase, while choosing conditions leading to a minimal shear stress.

Table 4.4 Cells' physiological state during experiments in HFMC

#	Fibres side			Shell side			Excess Mortality Rate (EMR) h^{-1}	Survival ratio at 5 h	Survival ratio at 24 h
	Phase	**Pressure (bar)**	**Flow rate (mL min^{-1})**	**Phase**	**Pressure (bar)**	**Flow rate (mL min^{-1})**			
				Effect of shear stress					
1	Cells in RO water	1	630	RO water	0.7	580	0.137 ± 0.016	0.895 ± 0.182	0.061 ± 0.031
2	Cells in RO water	0.1	330	RO water	0.05	350	0.005 ± 0.003	0.842 ± 0.028	0.805 ± 0.003
				Effect of the organic phase					
3	Cells in RO water	0.9	620	40% decanol 40% dodecane	0.3	500	0.513 ± 0.064	0.116 ± 0.040	N.D.
4	Cells in RO water	0.9	620	20% TOA 80% oleyl alcohol	0.4	450	0.103 ± 0.003	0.348 ± 0.075	0.154 ± 0.075
5	Cells in a 3-HP solution (1 g L^{-1})	0.9	620	20% TOA 80% oleyl alcohol	0.4	450	2.335 ± 0.395	0.047 ± 0.009	0.035 ± 0.003

N.D. = Not determined

4.3.4.2. Effect of the organic phase on cells' physiological state during circulation in the HFMC

The organic phase consisting of 20% (v/v) TOA, 40% decanol and 40% dodecane, selected as a reference composition in Section 4.3.1.2, was also tested in the HFMC (condition #3). The obtained EMR value (0.513 ± 0.064 h^{-1}, Table 4.4) was higher than the one obtained in the experiments in Schott bottles (0.112 ± 0.004 h^{-1}, Table 4.3). Also, survival ratio was significantly lower after 5 h only (0.116 ± 0.040 h^{-1}). This may reaffirm that shear stress due to circulation through the HFMC represent an additional inhibitory factor towards cells. However, results in Section 4.3.4.1 show that cells are not affected significantly during the first 5 h of circulation. Another explanation for the higher inhibitory effect on the HFMC conditions can be that the organic phase circulating on the shell-side is continuously feeding the cells suspension with water-soluble solvent molecules. Contrary to experiments in Schott bottles, where the organic phase is completely separated from the aqueous phase, leaving a fixed concentration of the water-soluble solvent molecules.

A more biocompatible mixture, consisting of 20% TOA and 80% oleyl alcohol, was also tested in the HFMC (condition #4). A higher EMR value (0.103 ± 0.003 h^{-1}, Table 4.4) was also obtained in comparison with the experiments in Schott bottle (0.039 ± 0.011 h^{-1}, Table 4.3) using the same organic phase composition. Also, the EMR value was lower than the one obtained with 20% (v/v) TOA, 40% decanol and 40% dodecane, as expected. Interestingly, the EMR value was also slightly lower than in condition #1 using only water in the shell-side (Table 4.4). The flow rate and pressure used for interface stabilisation in condition #4 were lower than in condition #1. This might explain the lower inhibitory effect towards cells, suggesting that *L. reuteri* is particularly sensitive to changes in the flow rate and pressure.

Finally, the mixture of 20% TOA and 80% oleyl alcohol was used for the reactive extraction of a 3-HP solution at 1 g L^{-1} containing *L. reuteri* cells. The presence of 3-HP together with circulation through the HFMC and contact with the organic phase resulted to be highly detrimental towards cells. An EMR value of 2.335 ± 0.395 h^{-1} was obtained and the survival ration was only 0.047 ± 0.009 after 5 h (Table 4.4). The 3-HP extraction yield at equilibrium was of 43.8 ± 1.7 %. These results suggest that *L. reuteri* is very sensitive to the presence of 3-HP in the medium and more generally to extractive bioconversion conditions, despite the different efforts for lowering the toxicity of the organic phase. All these experiments were performed using washed resting cells without substrate addition, in order to decrease the variability on the medium composition within time and be able to compare the different organic phase compositions. However, the lack of a carbon source could represent an additional stress, rending the cells more sensitive to experimental conditions. Therefore, the effect of the organic phase selected in Section 4.3.3 consisting of 20% (v/v) DDMA, 47% dodecanol and 40% dodecane, was tested in real glycerol bioconversion conditions. Results are presented and discussed in Chapter 5.

4.4. Conclusion

Different solvent mixtures (*extractants*, *active diluents* and *inert diluents*) were evaluated in terms of their extraction performance and toxicity in order to select an adequate composition of the organic phase for in stream extraction of 3-HP produced by *L. reuteri* DMS 17938. Inert diluent addition to a 20% TOA – decanol mixture decreased its toxicity and viscosity. However, it also decreased extraction yield, so it was necessary to determine a compromise between the extraction performance and the overall solvent toxicity towards cells. The combination of the extraction yield, viscosity and solvent toxicity criteria provided valuable information for the evaluation of different long-chain alcohols as active diluents. For linear alcohols, extraction performance decreased with the number of carbons (extraction yield decreased and viscosity increased). As for solvent toxicity, the opposite behaviour was observed. Ramified alcohols resulted in the lowest extraction yields, which can be explained by the steric hindrance of the OH group.

Two different methods for the evaluation of solvent toxicity were tested: assessment of the physiological state of the cells by flow cytometry and evaluation of glycerol bioconversion ability. Both methods provided consistent and complementary information about solvent toxicity and were found to be relevant and quick.

Among the most biocompatible solvent mixtures, the 20% TOA – 47% dodecanol – 33% dodecane solution gave an adequate trade-off between extraction yield and viscosity. Replacing TOA with DDMA, a tertiary amine with a higher reactivity, made it possible to improve the extraction yield while maintaining the benefit of low solvent toxicity. It was verified that the extraction performance of the selected organic phase is not expected to be highly affected by the components of the real bioconversion broth. These results were observed in bioconversion-like conditions that mimic the broth composition. They open promising prospects towards a coming study on the integrated extractive bioconversion process for the fed-batch production of 3-HP with *L. reuteri* from glycerol.

Finally, experiments in the HFMC revealed the high sensitivity of *L. reuteri* DSM 17938 to extractive bioconversion operating conditions, despite the favourable decrease of the organic phase toxicity. Cells' physiological state was significantly affected by the cumulated inhibitory factors such as shear stress due to circulation through the fibres-side, continuous feeding of water-soluble solvent molecules to the bioconversion medium and 3-HP concentration.

Chapter 5

Coupling of reactive pertraction assisted by hollow fibre membrane contactors (HFMC) to bioconversion

This chapter describes the adaptation of the reactive extraction and back-extraction assisted by a HFMC, also known as reactive pertraction, to two different bioconversion processes. First, a preliminary experiment with glycerol bioconversion into 3-HP by *Lactobacillus reuteri* DSM 17938 is presented. The strain used is a gram-positive and anaerobic bacterium. Then, 1,3-PDO bioconversion into 3-HP by *Acetobacter* sp. CIP 58.66 was also coupled with reactive pertraction. This strain is gram-negative and obligate aerobic, which represents an important change on process' conditions concerning interface stabilisation and cells resistance to the 3-HP recovery conditions. Bioconversion ability, biocompatibility and extraction performances are analysed and discussed for each process.

This work was carried out in collaboration (equal contributions) with the PhD project of Thi Lan Phuong Nguyen for experiments with *L. reuteri*. Experiments with *Acetobacter* sp. and data analysis were carried out in collaboration (equal contributions) with the PhD project of Florence de Fouchécour. Some parts in this chapter are therefore common with Florence de Fouchécour thesis manuscript (2019).

A patent application concerning the extractive bioconversion by *Acetobacter* sp. CIP 58.66 is under the second stage assessment by the "*Biorefinning and biobased product*" review board in charge of technology transfer in INRAE. Once the patent is published, this chapter will be submitted for publication in the journal *Bioresource Technology*.

5.1. Introduction

Numerous studies have focused on 3-HP microbial production, in particular on genetic engineering aspects, and high performances are now achieved at laboratory-scale (de Fouchécour *et al.*, 2018). Nevertheless, important hurdles still remain and bio-based 3-HP production has not yet reached industrial scale. In particular, significant challenges still lie in downstream processes for 3-HP recovery. Indeed, 3-HP is very soluble in aqueous media, it breaks down at high temperatures and dimerises at high concentrations (Hoppe *et al.*, 2015). As mentioned in Chapter 1, reactive extraction and back-extraction assisted by a HFMC, or reactive pertraction, is a promising method for 3-HP recovery, because of its advantages: i) an adequate integration of this method with bioconversion can reduce product inhibition and improve the overall productivity (Gao *et al.*, 2009a; Jin and Yang, 1998; Nelson *et al.*, 2017; Wu and Yang, 2003b), ii) pH can be self-regulated by continuous acid removal from the fermentation medium, avoiding base addition, iii) HFMC reduces the toxicity of the solvents used for reactive extraction thanks to the non-dispersive liquid-liquid contact (Yang *et al.*, 2007), and iv) a concentrated aqueous solution of the purified acid can be obtained after back-extraction (Chemarin *et al.*, 2019b).

Regarding 3-HP bioproduction, one of the dominant approaches so far has been its

production from glycerol (de Fouchécour *et al.*, 2018). Yet, 1,3-propanediol (1,3-PDO) is an obligate by-product of glycerol conversion to 3-HP, and multi-step approaches have thus been undertaken for subsequent 1,3-PDO oxidation into 3-HP (Dishisha *et al.*, 2015; Zhao *et al.*, 2015), in order to maximise the overall 3-HP yield from glycerol. This last conversion step was notably performed with acetic acid bacteria (AAB) (de Fouchécour, 2019; Dishisha *et al.*, 2015; Li *et al.*, 2016a; Zhao *et al.*, 2015), which are well known for their ability to incompletely oxidise alcohols into acids. AAB were shown to quantitatively oxidise 1,3-PDO into 3-HP, through a two-step pathway, with 3-hydroxypropanaldehyde (3-HPA) as intermediate. Both steps are catalysed in the periplasm by membrane-bound enzymes, the second one being the rate limiting (Zhu *et al.*, 2018). In our laboratory, this multi-step approach is being studied using the strain *L. reuteri* DSM 17938 for initial glycerol bioconversion into 3-HP and 1,3-PDO and subsequent 1,3-PDO bioconversion into 3-HP with the strain *Acetobacter* sp. CIP 58.66. In particular, a sequential fed-batch strategy was recently developed with *Acetobacter* sp. CIP 58.66 (de Fouchécour, 2019): cells were first grown on glycerol in batch mode, then fed-batch bioconversion of 1,3-PDO was initiated, using a pH control-based feeding strategy.

Moreover, using an AAB can have additional advantages for the combination of bioconversion with reactive pertraction, for in stream 3-HP recovery. One is that gram-negative bacteria are generally more resistant to organic solvents than gram-positive bacteria (Harrop *et al.*, 1989; Inoue and Horikoshi, 1991; Vermuë *et al.*, 1993). In particular, *L. reuteri* DSM 17938 showed to be very sensitive toward toxicity of the organic phase used for 3-HP reactive extraction (Burgé *et al.*, 2017). Other advantage is that AAB are likely to be resistant to acidic environments (Raspor and Goranovič, 2008; Wang *et al.*, 2015b; Yang *et al.*, 2019), which is in accordance with the requirement of a low pH for reactive extraction with amines.

The present chapter explores the feasibility of integrating reactive pertraction, using the organic phase selected in Chapter 4, with bioconversion for 3-HP production. Coupling was made with bioconversion processes of both 3-HP producing strains, in order to compare the effect of reactive pertraction on their production ability. Various bacterial stresses might arise during such a process, such as solvent toxicity and shear stresses (Jin and Yang, 1998). In addition, contrary to strains like *L. reuteri* DSM 17938, *Acetobacter* sp. CIP 58.66 is obligate aerobe. This raises potential new challenges for process integration, such as lack of oxygen during bacteria circulation through the extraction circuit and interface stabilisation issues, due to the presence of air bubbles. This chapter provides a first experimental demonstration of 3-HP microbial production associated with continuous reactive pertraction, based on reactive liquid-liquid product recovery in a HFMC. Both aspects, microbial production and product recovery, were studied here in order to identify the main associated difficulties to future process scale-up.

5.2. Materials and methods

In the global strategy for 3-HP production from glycerol, reactive pertraction is envisaged to be integrated with bioconversion by *Acetobacter* sp. CIP 58.66. However, a preliminary

extractive bioconversion experiment with *L. reuteri* DSM 17938 was also performed using the organic phase selected in Chapter 4. This was done with the aim of comparing the effect of reactive pertraction on the bioconversion ability of both strains, and also to identify and compare the potential difficulties that may arise under different experimental conditions.

5.2.1. Reactive pertraction preparation

5.2.1.1. Chemicals for reactive pertraction

The organic phase used for the reactive pertraction coupled with bioconversion by *L. reuteri* DSM 17938 was selected in Chapter 4 (20% v/v DDMA, 47% dodecanol and 33% dodecane). This composition presented a distribution coefficient K_D of 0.95, a viscosity of 6.4 mPa·s and showed to be biocompatible with *L. reuteri* DSM 17938. This constituted an adequate trade-off between extraction performance and biocompatibility.

The organic phase composition was slightly modified to be used in the extractive bioconversion by *Acetobacter* sp. CIP 58.66: 20 % v/v DDMA, 40% dodecanol and 40% dodecane. The K_D value of this composition was 0.78 and presented a viscosity of 4.7 mPa·s. Both properties were measured as described in Chapter 4 (Section 4.2.2). This second organic phase had a similar distribution coefficient as the previous one, but a lower viscosity. Therefore, a higher mass transfer rate was expected in the organic phase (Coelhoso *et al.*, 1997), as well as a better biocompatibility due to the lower dodecanol to decane ratio (Kumar *et al.*, 2012; Wasewar *et al.*, 2011; Yamamoto *et al.*, 2011).

For back-extraction, a NaOH solution was used. A 1 M solution was used on experiments with *L. reuteri* DSM 17938, and a 0.5 M concentration was used for *Acetobacter* sp. CIP 58.66 (Table 5.1).

5.2.1.2. Interface stabilisation

The reactive pertraction set-up (Figure 5.1) consisted in two HFMC (described in Chapter 2, Section 2.4) for 3-HP extraction and back-extraction. Before each experiment, the modules were washed by circulating a 60% v/v isopropanol solution for two hours through the fibres and the shell side, then drained and rinsed with sterilised RO water and finally dried overnight by flushing compressed air.

Interface stabilisation in the extraction module was first performed by circulating 1 L of sterilised RO water inside the fibres and the organic phase in the shell side. Initial operating conditions for each bioconversion are summarised in Table 5.1. A three-way valve was installed at the fibres inlet to switch the feed from the RO water to the bioreactor medium. For the back-extraction module, the NaOH solution at the corresponding concentration was circulated inside the fibres, while the extraction phase was circulated on the shell side. Both modules were fed with organic phase from the same flask, allowing extractant regeneration.

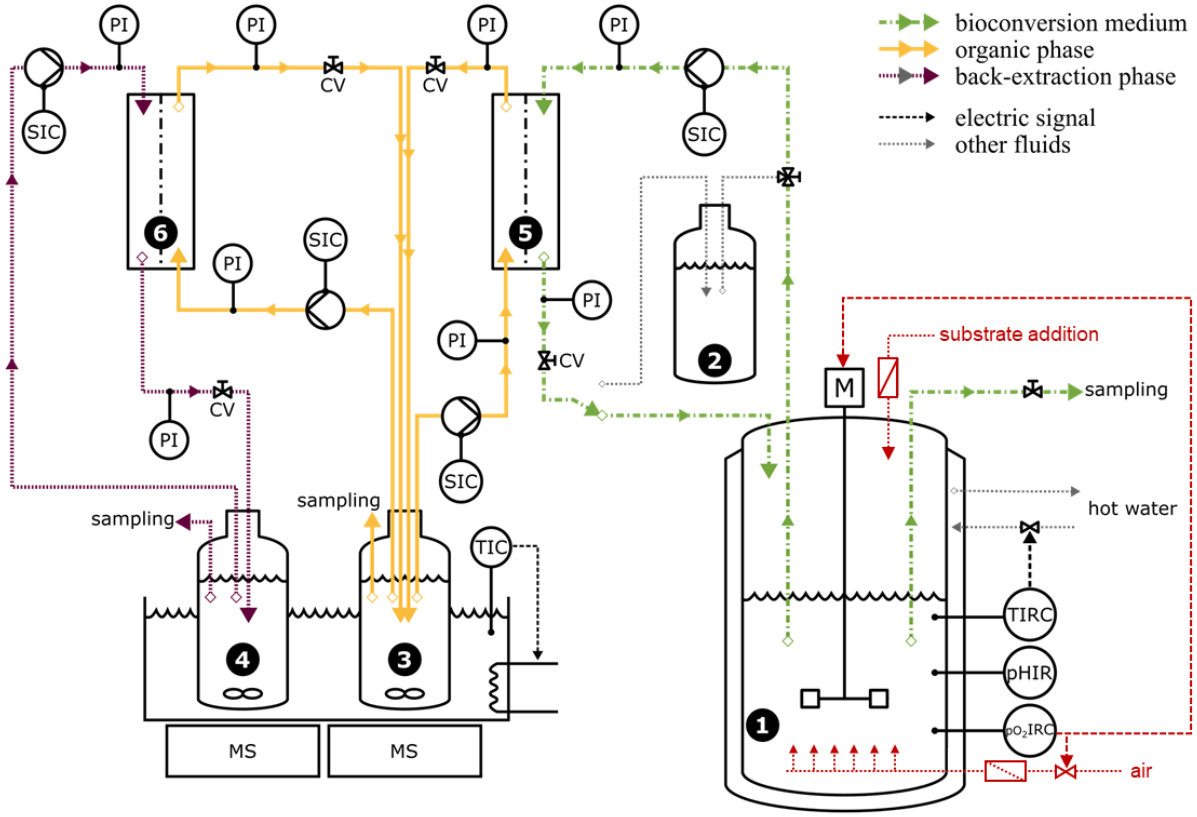


Figure 5.1 Process and Instrumentation Diagram of the extractive bioconversion (adapted from de Fouchécour (2019). 1) Bioreactor. Differences between configurations for bioconversion with *L. reuteri* DSM 17938 and *Acetobacter* sp. CIP 58.66 are indicated in red: glycerol was added to the bioconversion medium as substrate for *L. reuteri*, while 1,3-PDO was used for bioconversion with *Acetobacter* sp. Also, the latest bioconversion was performed at aerobic conditions, with air supply and agitation control. 2) Water for interface stabilization. 3) Organic phase. 4) Back-extraction phase. 5) Membrane contactor for 3-HP extraction from bioconversion medium into organic phase. 6) Membrane contactor for 3-HP back-extraction from organic phase into aqueous phase. Abbreviations: CV, control valve; M, motor; MS, magnetic stirrer; pHIR: pH indicator and recorder; PI: pressure indicator; pO_2IRC, pO_2 indicator, recorder and controller; SIC, speed indicator and controller; TIC, temperature indicator and controller; TIRC, temperature indicator, recorder and controller.

5.2.2. Preliminary experiment of extractive bioconversion with L. reuteri DSM 17938

5.2.2.1. Biomass production in bioreactor

An 8 h preculture performed in MRS broth, as described in Chapter 4 (Section 4.2.3.1), was used to inoculate a Biostat®-B Plus bioreactor (Sartorius, France). The culture medium consisted of 5 L of MRS broth supplemented with 20 g L^{-1} of glucose (initial pH = 6.0). Sterilisation was performed by autoclaving at 110 °C, during 20 min. Growth was carried out at 37 °C and 100 rpm during 16 h, when cells arrived to early stationary phase and no further base consumption was detected. pH value was maintained at 6 by addition of an NH_4OH solution 14.8 M. NH_4OH addition, temperature and pH were monitored and

controlled online with the software MFCS (Sartorius Stedim).

Harvesting of *L. reuteri* cells was performed by centrifugation at 10 000×g and 4 °C during 10 min. Then, cells were washed with sterilised RO water to decrease the residual amount of growth metabolites and used to inoculate bioreactor for fed-batch glycerol bioconversion into 3-HP.

Table 5.1 Initial operating conditions of the membrane contactor modules

Conditions		**Strains**	
		***L. reuteri* DSM 17938**	***Acetobacter* sp. CIP 58.66**
Temperature (°C)		37	30
Extracted aqueous phase	Composition	Glycerol bioconversion into 3-HP and 1,3-PDO	1,3-PDO bioconversion into 3-HP
	Inlet pressure (bar)	0.8	0.8
	Outlet pressure (bar)	0.7	0.7
	Flow rate (mL min^{-1})	250	450
	Volume (L)	1	1.2
Organic phase for extraction	Composition (% v/v)	20 DDMA 47 dodecanol 33 dodecane	20 DDMA 40 dodecanol 40 dodecane
	Inlet pressure (bar)	0.45	0.45
	Outlet pressure (bar)	0.45	0.45
	Flow rate (mL min^{-1})	430	430
	Volume (L)	1	1.5
Back-extraction phase	Composition	NaOH 1 M	NaOH 0.5 M
	Inlet pressure (bar)	0.8	0.8
	Outlet pressure (bar)	0.7	0.7
	Flow rate (mL min^{-1})	450	450
	Volume (L)	0.5	1

5.2.2.2. Extractive bioconversion by L. reuteri DSM 17938

L. reuteri cells were re-suspended in 1 L of sterilised RO water in a 2 L SGI bioreactor (Setric Génie Industriel, France), at a concentration of $1.2 \cdot 10^{10}$ cells mL^{-1}. The initial working volume was 1 L, then fed-batch bioconversion was carried out at 37 °C and 100 rpm, by addition of a glycerol solution at 100 g L^{-1} and a feeding rate of 0.5 g h^{-1}. This feeding rate was selected in order to avoid 3-HPA accumulation in the bioconversion medium, because of its high toxicity to the cells (Schaefer *et al.*, 2010). The pH was left uncontrolled during bioconversion, starting at pH 6.7. Then, medium acidification started because of 3-HP production. In reactive extraction, tertiary amines are able to react only

with the protonated form of the targeted acid (Kertes and King, 1986). Therefore, when pH is above the pK_A of the acid (4.51 for 3-HP (Haynes *et al.*, 2017)), extraction remains very limited. However, experiments of the ongoing PhD work of Phuong Nguyen (personal communication) showed that bioconversion with *L. reuteri* is very limited at pH below 5. It was thus established that extraction should be started when pH reaches 5. At this moment, the three-way valve installed at the fibres inlet (Figure 5.1) was switched in order to replace RO water in the extraction module with the bioconversion medium, to start 3-HP extraction. Then, after the residual RO water was removed from the circuit, the fibres outlet was connected to the bioreactor to close de loop. Samples were taken from the bioconversion medium, the organic phase and the back-extraction NaOH solution. Metabolites concentration in the three phases and biomass evolution in the bioconversion medium were analysed off-line, as described in Chapter 2 (Section 2.5). This extractive bioconversion was carried out once.

5.2.2.3. Calculations

5.2.2.3.1. Working volume correction for fed-batch bioconversion

The working volume in the bioreactor has a significant variation due to glycerol feeding and sampling. A glycerol solution at 100 g L^{-1} was supplied at a feed rate of 0.5 g h^{-1}, meaning that volume increased by 5 mL h^{-1}. Taking also into account the volume withdrawn by sampling, the total volume variation was determined as function of time. This was taken into account for the calculation of each metabolite amount during bioconversion.

5.2.2.3.2. Kinetic analysis

Glycerol consumption and 3-HP, 1,3-PDO and 3-HPA production values presented a linear variation during fed-batch bioconversion (Figure 5.2A). Therefore, consumption and production rates were calculated by linear regression of metabolites amount data over time, obtaining the slope value for each compound. The same method was applied for 3-HP extraction rate, for extracted 3-HP data during the first 2.5 h of extractive bioconversion.

5.2.3. Extractive bioconversion by Acetobacter sp. CIP 58.66

5.2.3.1. Acetobacter sp. CIP 58.66 cells production

Acetobacter sp. CIP 58.66 was purchased from the Biological Resource Centre of Pasteur Institute (Paris, France) in the form of a lyophilisate. It was activated by inoculation into 50 mL of a culture medium composed of 25 g L^{-1} of mannitol, 5 g L^{-1} of yeast extract and 3 g L^{-1} of peptone (initial pH = 6.5), in a 250 mL baffled shake-flask. The culture was put at 25 °C and 200 rpm during one week. After this time, 2 mL of this culture were used to inoculate 100 mL of fresh medium composed of 20 g L^{-1} of ethanol, 5 g L^{-1} of yeast extract and 3 g L^{-1} peptone (initial pH = 6.5), in a 500 mL baffled shake-flask. This second culture

was incubated one week in the same conditions. Then 20% (w/v) of glycerol was added to the medium for cryotubes preparation. One mL of the cell suspension was put in cryotubes and stored at -80 °C, with a cell density around $9.5 \cdot 10^8$ cell mL^{-1}.

Inoculum preparation was made by putting 1 mL of the stored cell suspension into 25 mL of sterile, basal medium (5 g L^{-1} of yeast extract, 3 g L^{-1} of peptone, and 8.7 g L^{-1} of K_2HPO_4) in a 250 mL baffled shake-flask. The culture was incubated at 30 °C and 200 rpm during 62 – 63 h, when cells arrived to an early stationary phase.

5.2.3.2. Biomass production in bioreactor

The obtained cells suspension was used to inoculate a 3.6 L Labfors 4 bioreactor (Infors), with an initial working volume of 1.2 L. The medium was initially composed of 10 g L^{-1} glycerol, 5 g L^{-1} yeast extract, 3 g L^{-1} peptone (initial pH = 5.0, adjusted with sulfuric acid 5.5 M). Sterilisation of the medium-containing bioreactor was performed by autoclaving at 120 °C, during 20 min. Medium was then inoculated with the preculture, in order to reach an initial cell dry weight (CDW) concentration of 0.02 g_{DW} L^{-1} (around $4.1 \cdot 10^7$ cell mL^{-1}). Due to inoculum addition, the initial pH rose up to 5.2. During growth on glycerol, pH was left uncontrolled, while partial pressure of dioxygen (pO_2) was automatically controlled at a minimal value of 40% of saturation. This was performed with a cascade of stirring rate (from 100 to 800 rotations per minute (rpm), Rushton turbine) and air flow rate (from 1 to 4 normal litre (NL) per minute) (Figure 5.1, indicated in red). Temperature was maintained at 30 °C. Biomass production on glycerol lasted for 32 h, until the late exponential phase was reached. The growth medium was sampled over time, in order to verify growth reproducibility among the replicates.

5.2.3.3. Extractive bioconversion by Acetobacter sp. CIP 58.66

After the late exponential phase was reached by *Acetobacter* sp. CIP 58.66 growing on glycerol, bioconversion was triggered by adding 6 mL of filter-sterilised 1,3-PDO (98% purity), corresponding to an initial concentration of 5.4 g L^{-1}. At the end of this primary growth on glycerol (32 h of culture), pH was close to 7. Because pH was left uncontrolled, medium acidification occurred after the 1,3-PDO addition, due to 3-HP production. As previously mentioned, reactive liquid-liquid extraction with amines remains very limited when pH is above the pK_a of the targeted acid. Consequently, the bacterial medium was brought in contact with the extracting phase only after pH reached 4.6, which is close to the pK_a of 3-HP (4.51). No base was added for pH control, and pH varied freely according to production and extraction rates. RO water was replaced in the extraction module with the bioconversion medium, by switching the three-way valve (Figure 5.1). Then the fibres' outlet was manually connected to the bioreactor. After 4.6 h of bioconversion, 6 mL of 1,3-PDO were added again to the medium. All along the bioconversion, pO_2 was controlled at a minimal value of 40 %, using the same control cascade as during the preliminary growth step (Section 5.2.3.2). Samples were taken from the bioconversion medium, the organic phase and the back-extraction NaOH solution for components quantification. Metabolites

concentration and biomass evolution were monitored and analysed off-line, as described in Section 2.9. The extractive bioconversion was carried out in triplicate.

5.2.3.4. Calculations

5.2.3.4.1. Working volume correction for bioconversion

A considerable volume loss in the bioreactor during bioconversion with *Acetobacter* sp. occurred due to air supply and long culture times. This was taken into account for the calculation of metabolites mass balance during bioconversion. The final working volume was measured and subtracted from the initial working volume. Taking also into account the volume withdrawn by sampling, the total volume loss due to evaporation was determined. Then, the overall air volume that had passed through the bioreactor was estimated from on-line measurements. With these values, the volume loss due to evaporation over time was estimated assuming that the evaporation rate was proportional to the aeration rate.

5.2.3.4.2. Kinetic analysis

The Gompertz model was used for kinetic analysis, with modifications from Zwietering *et al.* (1990) (Equation 5.1), as described by de Fouchécour (2019):

$$G(t) = a \cdot exp\left(-exp\left[\frac{b \cdot e}{a}(c - t) + 1\right]\right) \quad (5.1)$$

where e represents $exp(1)$, t is the time (h) and a, b and c are parameters to be fitted. This model was fitted to cell dry weight data (CDW) and to 3-HP concentrations, obtained as described in Chapter 2 (Section 2.5). When the model was fitted to CDW data, the parameter b corresponded to the maximal growth rate μ_{max} (h^{-1}). The time derivative of this model (Equation 5.2) against time is the derivative productivity r_{3-HP} ($g_{3\text{-HP}}\ L^{-1}\ h^{-1}$).

$$G'(t) = b \cdot exp\left(2 + \frac{b \cdot e}{a}(c - t) - exp\left[1 + \frac{b \cdot e}{a}(c - t)\right]\right) \quad (5.2)$$

Specific 3-HP productivities q_{3-HP} ($g_{3\text{-HP}}\ g_{CDW}^{-1}\ h^{-1}$) were also calculated using Gompertz models for 3-HP and CDW amounts, taking volume variations into account. Specific productivity was calculated with Equation 5.3, with a time step of 0.01 h:

$$q_{3-HP} = \frac{1}{CDW(t)} \cdot \frac{d[3-HP]}{dt} \quad (5.3)$$

From this equation, the highest value obtained was considered as the maximal specific productivity $q_{3-HP,max}$, and the average specific productivity q_{3-HP} was calculated as the mean of all values in the considered time span. Number of generations N_g of each growth phase were also calculated as $log_2(CDW_f/CDW_i)$, with CDW_f and CDW_i, being the final and initial cell dry weight respectively. These values were used to compare bioconversion with reactive pertraction and bioconversion alone. Parameter estimation was made with the Levenberg-Marquardt least-squares method, using the Phyton/Scipy software package

(Phyton Software Fundation, version 3.6).

5.2.3.4.3. Statistical analysis

Results are given as mean ± sample standard deviation, except for bacterial physiological state assessment by flow cytometry where n = 2. In this case, results are given as mean ± half the amplitude between replicates.

5.2.4. Model for 3-HP reactive pertraction simulation

In order to explore various optimisation strategies for the integrated process, a mathematical model previously developed by Chemarin (2017) was adapted to the properties of the organic phase and operating conditions used in extractive bioconversion with *Acetobacter* sp. CIP 58.66 (20% v/v DDMA, 40% dodecanol and 40% dodecane), then used as a simulation tool. This model takes into account chemical equilibrium reactions, combined with mass transfer fluxes and is capable to predict mass transfer dynamics during 3-HP reactive extraction. To this end, some physical and chemical parameters were determined experimentally at the same operating conditions as extractive bioconversion. The mathematical model was written in Matlab® 2018b (The MathWorks Inc., Natick, MA, U.S.).

Table 5.2 summarises all chemical reaction considered in the mathematical model. These are divided in three different locations: aqueous phase, interface and organic phase. The interface was supposed to be at chemical equilibrium, meaning that interfacial chemical reactions are much faster than species mass transfers (Chemarin *et al.*, 2017a).

Mass fluxes J in the HFMC configuration were calculated relying on the boundary layer model, considering mass transfer coefficients and driving forces at each layer (Chemarin, 2017): i) the aqueous bulk phase, ii) the aqueous boundary layer, iii) the membrane pores and organic boundary layer, and iv) the organic bulk phase. Pseudo steady-state regime was assumed and mass fluxes were thus expressed by Equation 5.11.

$$J_X = k_X S([X]-[X]_i) \tag{5.11}$$

where k is the mass transfer coefficient of the chemical species X, S is the surface available for mass transfer and the driving force is represented as the difference of the species concentration at the corresponding bulk $[X]$ and in the interface $[X]_i$.

Finally, chemical reactions and mass transfer fluxes can be related with mass conservation equations, assuming that accumulation of the chemical species occurs in the bulk phases only. The mass balance equations considered for the mathematical model are summarised in Table 5.3.

Table 5.2 Equations concerning chemical equilibria and kinetics (Chemarin *et al.*, 2017a)

	Reaction	Equation	
	Equilibrium at the interface		
Acid dissociation	$AH_{aq,i} + H_2O \overset{K_A}{\Leftrightarrow} A^-{}_{aq,i} + H_3O^+{}_{aq,i}$	$[A^-]_{aq,i}[H_3O^+]_{aq,i} - K_A[AH]_{aq,i} = 0$	(5.4)
Water autoprotolysis	$2H_2O \overset{K_W}{\Leftrightarrow} H_3O^+{}_{aq,i} + OH^-{}_{aq,i}$	$[H_3O^+]_{aq,i}[OH^-]_{aq,i} - K_w = 0$	(5.5)
Acid partitioning	$AH_{aq,i} \overset{m}{\Leftrightarrow} AH_{org,i}$	$[AH]_{org,i} - m[AH]_{aq,i} = 0$	(5.6)
Complex formation	$AH_{aq,i} + DDMA_{org,i} \overset{K_{11}}{\Leftrightarrow} CPX_{org,i}$	$[CPX]_{org,i} - K_{11}[AH]_{aq,i}[DDMA]_{org,i} = 0$	(5.7)
	Reaction kinetics in the aqueous phase		
Acid dissociation	$AH_{aq} + H_2O \overset{K_A}{\Leftrightarrow} A^-{}_{aq} + H_3O^+{}_{aq}$	$r_1 = R_{-1}(K_A[AH]_{aq} - [A^-]_{aq}[H_3O^+]_{aq})$	(5.8)
Water autoprotolysis	$2H_2O \overset{K_W}{\Leftrightarrow} H_3O^+{}_{aq} + OH^-{}_{aq}$	$r_2 = R_{-2}(K_w - [H_3O^+]_{aq}[OH^-]_{aq})$	(5.9)
	Reaction kinetics in the organic phase		
Complex dissociation	$CPX_{org} \overset{\frac{1}{K_{11}}}{\Leftrightarrow} \frac{AH_{org}}{m} + DDMA_{org}$	$r_3 = R_{-3}([\frac{m}{K_{11}}[CPX]_{org} - [AH]_{org}[DDMA]_{org})$	(5.10)

Table 5.3 Mass balance and fluxes conservation for 3-HP reactive extraction

Mass balance equations in the aqueous phase		
Protonated acid	$\frac{d[AH]_{aq}}{dt} = -\frac{JAH_{aq}}{V_{aq}} - r_1 + P$	(5.12)
Dissociated acid	$\frac{d[A^-]_{aq}}{dt} = -\frac{JA^-{}_{aq}}{V_{aq}} + r_1$	(5.13)
Hydronium ion	$\frac{d[H_3O^+]_{aq}}{dt} = -\frac{JH_3O^+{}_{aq}}{V_{aq}} + r_1 + r_2$	(5.14)
Hydroxide ion	$\frac{d[OH^-]_{aq}}{dt} = -\frac{JOH^-{}_{aq}}{V_{aq}} + r_2$	(5.15)
Mass balance equations in the organic phase		
Acid-amine complex	$\frac{d[CPX]_{org}}{dt} = -\frac{JCPX_{org}}{V_{org}} - r_3$	(5.16)
Amine	$\frac{d[TOA]_{org}}{dt} = -\frac{JTOA_{org}}{V_{org}} + r_3$	(5.17)
Protonated acid	$\frac{d[AH]_{org}}{dt} = -\frac{JAH_{org}}{V_{org}} + r_3$	(5.18)
Interfacial fluxes conservation		
Total acid conservation	$JAH_{aq,i} + JAH_{org,i} + JCPX_{org,i} + JA^-{}_{aq,i} = 0$	(5.19)
Charge conservation	$JA^-{}_{aq,i} + JOH^-{}_{aq,i} + JH_3O^+{}_{aq,i} = 0$	(5.20)
Total amine conservation	$JTOA_{org,i} + JCPX_{org,i} = 0$	(5.21)

5.2.4.1. Experiments for model calibration

5.2.4.1.1. Chemical equilibrium coefficient determination

Chemical equilibrium coefficient K_{11}, was determined according to Chemarin *et al.* (2017a), by fitting experimental data of the extraction yield at different 3-HP concentrations to Equation 5.22. Dispersive extraction was performed by putting 25 mL of 3-HP solutions (concentration range 0.5 - 25 g L^{-1}) in contact with 25 mL of the organic phase. After vigorous mixing during 3 min, solutions were left to achieve equilibrium at 30 °C for 48 h and subsequently separated by centrifugation. The aqueous phase was recovered for analysis in HPLC (Section 2.5.1.1). Experiments were made in triplicate.

$$Y = \frac{V_{org}}{V_{aq}} \cdot \frac{[AH]_{org,total}}{[AH]_{ini}} = \frac{V_{org}}{V_{aq}} \cdot \frac{\varphi m [AH]_{aq}^{eq} + K_{11}[AH]_{aq}^{eq}[DDMA]_{org}^{eq}}{[AH]_{ini}} \quad (5.22)$$

where φ is the volume fraction of the diluent in the organic phase and was considered as the mixture of dodecanol and dodecane in the organic phase used in this study (φ = 0.8). 3-HP physical partition in the diluent m, was determined experimentally by performing

dispersive extraction of 25 mL of 3-HP solutions at a concentration range of 0.5 - 25 g L^{-1}, using the organic phase composition without considering the amine (a mixture of 50% v/v dodecanol and 50% dodecane). $[AH]_{aq}^{eq}$ is the 3-HP concentration at the aqueous phase at equilibrium, and $[DDMA]_{org}^{eq}$ the amine concentration at the organic phase at equilibrium.

5.2.4.1.2. Increase of the organic phase viscosity with 3-HP concentration

The mathematical model takes into account the viscosity μ increase of the organic phase with 3-HP concentration, because μ was found to change significantly. The diffusion coefficient D is inversely proportional to μ, according to Wilke-Chang relation (Equation 5.23) (Bird *et al.*, 2007):

$$D = 7.4 \cdot 10^{-12} \frac{(\phi M)^{0.5} T}{\mu v_i^{0.6}} \quad (5.23)$$

Therefore, the viscosity increase of the used organic phase was also determined. For this, organic phase solutions containing different 3-HP concentrations were prepared. For this, an organic phase enriched in 3-HP was prepared by putting in contact 50 mL of the organic phase with 50 mL of a 3-HP solution at 50 g L^{-1}. Both phases were left to reach equilibrium at 25 °C during 48 h, and then separated by centrifugation at 8 228×g and 25 °C, during 30 min. In order to avoid the formation of a stable emulsion, the organic phase enriched with 3-HP was diluted with fresh organic phase at 25, 50, and 75%, resulting in organic solutions with different 3-HP concentrations. Then, the viscosity of each organic solution was determined with a cone and plate rheometer (Rheostress 600, Thermo Scientific), using a linear increase of shear rate from 0 to 100 s^{-1} at 30 °C. Experiments were made in triplicate.

In order to determine the exact 3-HP concentration in each organic phase, all solutions were back-extracted for analysis in HPLC as described in Section 2.5.1.1. Linear regression of viscosity values *vs.* 3-HP concentration was used to model the change of viscosity with acid concentration.

5.2.4.1.3. Mass transfer coefficient

Mass transfer coefficient k values are proportional to the diffusion coefficient D, as expressed in mathematical expressions like the Lévêque equation, which was used by Chemarin (2017) to estimate k values of all the chemical species in the aqueous phase. However, estimation of k values of the species in the organic phase inside the membrane pores could be less accurate because the porosity and tortuosity of the membrane are not precisely measured, adding more uncertainty. Because of that, the product of the mass transfer coefficient of the acid-amine complex k_{CPX} (m s^{-1}) and the available surface S (m^2) was determined by comparing model predictions with the results of a 3-HP extraction experiment in the HFMC. This product has the units of a volumetric mass transfer coefficient (m^3 s^{-1}). The product of the other species in the organic phase can be derived from this value using the relation based on the Wilke-Chang correlation (Equation 5.24).

$$k_{X,org}S = k_{CPX}S\left(\frac{v_{CPX}}{v_X}\right)^{0.6} \quad (5.24)$$

where v is the molar volume of the corresponding chemical species.

Experimental data was obtained by extracting a 3-HP solution at 1 g L^{-1} with the used organic phase in the HFMC. The 3-HP solution (500 mL) circulated through the lumen side of the fibres with an inlet pressure of 0.8 bar and a flow rate of 450 mL min^{-1}, while the organic phase (500 mL) circulated in the shell side at 0.45 bar and a flow rate of 430 mL min^{-1}. Samples from the 3-HP solution were taken periodically during 24 h. Experiments were performed at 30 °C and made in duplicate.

5.2.4.2. Experiment for model validation

In order to validate the mathematical model, an experiment coupling extraction and back-extraction was performed using a 3-HP solution at 10 g L^{-1}. For back-extraction, a NaOH 0.5 M solution was used. 3-HP and NaOH solutions (666 mL) where circulated in the fibres sides of the extraction and the back-extraction units respectively with an inlet pressure of 0.8 bar and a flow rate of 450 mL min^{-1}, while the organic phase (1 L) circulated in the shell side at 0.45 bar and a flow rate of 430 mL min^{-1}. Samples from the 3-HP, NaOH and organic phase solutions were taken periodically during 100 h. Experiments were performed at 30 °C in duplicate.

5.3. Results and discussion

5.3.1. Preliminary experiment of extractive bioconversion by L. reuteri DSM 17938

5.3.1.1. Biomass production for bioconversion

Glycerol bioconversion into 3-HP was performed with resting cells of *L. reuteri*. At the end of the growth phase, a final OD_{600nm} of 10.3 was achieved, which corresponds to around 4 g L^{-1} of CDW ($3.4 \cdot 10^9$ cells mL^{-1}). Ethanol, acetic acid and lactic acid were produced during this growth step, reaching a final concentration of 7.62, 1.11 and 16.4 g L^{-1} respectively. These results are consistent with previous experiments without extraction performed in the ongoing PhD work of Phuong Nguyen (personal communication), meaning that metabolite production performances during cell growth were reproducible. Since the presence of other acids in the bioconversion medium can compete with 3-HP extraction, cells were harvested and washed with RO water. This was done to decrease lactic acid concentration. Salts contained in the culture medium also has adverse effects on reactive extraction performance, their concentration was also decreased.

5.3.1.2. Bioconversion ability during in stream reactive pertraction

The obtained cell pellet was then re-suspended in 1 L of RO water in a second bioreactor,

at an initial concentration of $1.2 \cdot 10^{10}$ cells mL^{-1}. A lower concentration of ethanol, acetic acid and lactic acid were detected, being 0.29, 0.32 and 0.53 g L^{-1} respectively. Bioconversion was triggered by feeding the glycerol solution, at free pH. 3-HP production started immediately, and pH started to decrease. The pH of the medium was left to decrease freely from an initial value of 6.7 to 5. Extraction was started after 5.7 h of bioconversion when pH 5 was reached. Figure 5.2 shows the medium composition, pH and pO_2 evolution during bioconversion. Three different stages could be distinguished according to metabolites evolution (Figure 5.2A) – Stage 1: before starting extraction, Stage 2: metabolites production during extractive bioconversion, and Stage 3: glycerol consumption after metabolites production stopped – Table 5.4 summarises the production/consumption rate values at these stages.

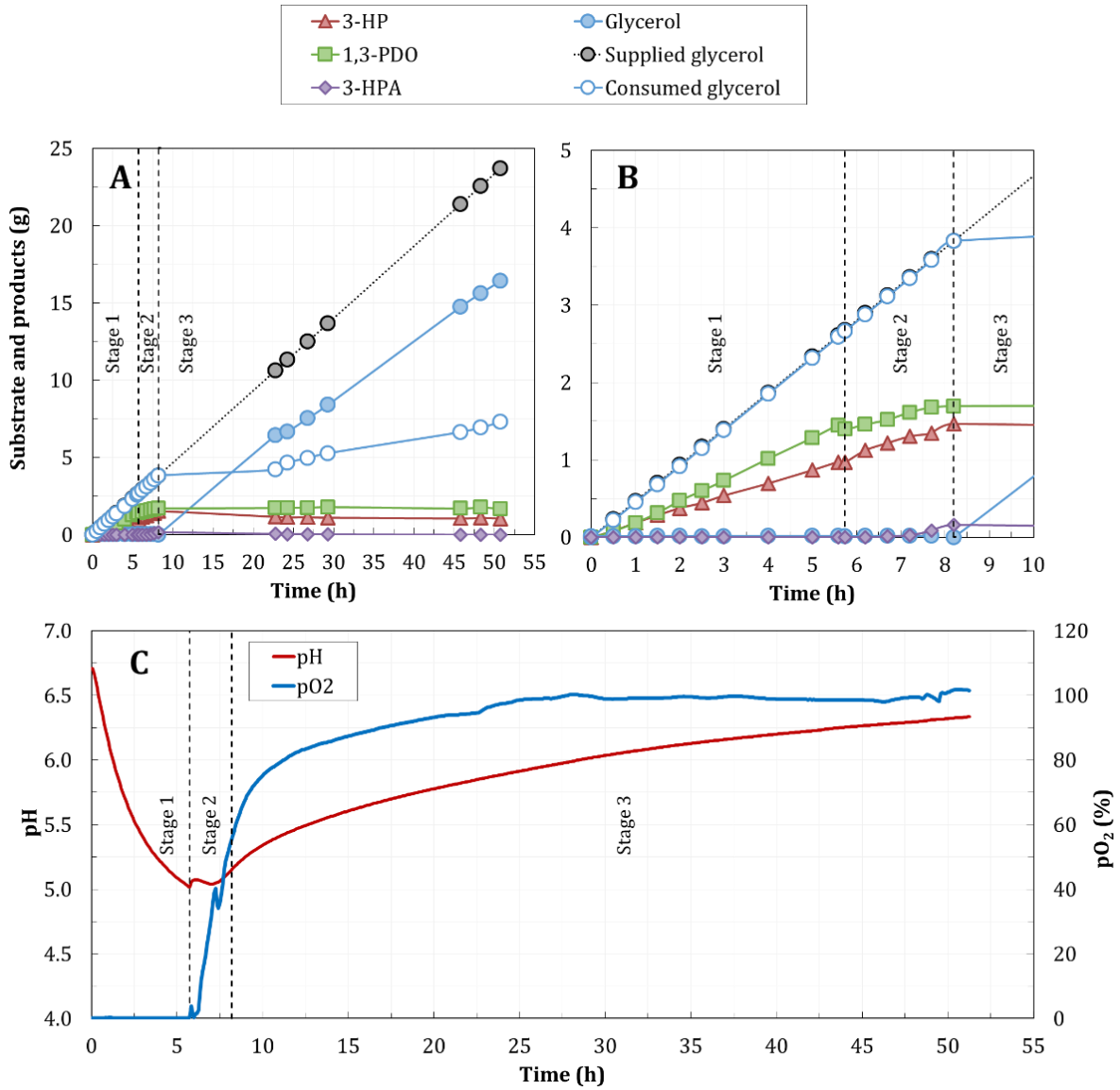


Figure 5.2 Evolution of the medium composition during reactive extraction. Substrate and products evolution: A. Total bioconversion time. B. Focus on the first 10 h of bioconversion. C. pH and pO2 evolution during bioconversion.

Table 5.4 Production/consumption rate evolution during bioconversion

Time span (h)	Stage 1 (0 - 5.7)	Stage 2 (5.7 - 8.2)	Stage 3 (8.2 - 50.7)	Stage 1 (0 - 5.7)	Stage 2 (5.7 - 8.2)	Stage 3 (8.2 - 50.7)
	Production/consumption rate (g h^{-1})			**Production/consumption rate (mol h^{-1})**		
Glycerol	0.46	0.46	0.09	0.0050	0.0050	0.0010
3-HP	0.17	0.20	0	0.0019	0.0022	0
1,3-PDO	0.26	0.13	0	0.0034	0.0017	0
3-HPA	0	0.04	0	0	0.0005	0

Before extraction, glycerol was consumed as it was supplied (0.46 g h^{-1}), and no accumulation was detected in the medium (Figure 5.2B). 3-HP was produced at a constant rate of 0.17 g h^{-1} (0.0019 mol h^{-1}), while 1,3-PDO was produced at 0.26 g h^{-1} (0.0034 mol h^{-1}). After reactive pertraction was started, glycerol consumption continued at the same rate. 3-HP production rate increased slightly to 0.20 g h^{-1}, and 1,3-PDO production rate decreased to 0.13 g h^{-1}. Production rates during the first 8.2 h of bioconversion (Table 5.5) are similar to the values observed during bioconversion without extraction at regulated pH in the ongoing PhD work of Phuong Nguyen (personal communication). This suggests that reactive pertraction has a limited effect on *L. reuteri*'s bioconversion ability, at least during the first 2.6 h of contact. However, metabolites production stops not long after 8.2 h of bioconversion, since 3-HP and 1,3-PDO amounts at time 8.2 and 22.7 h are very similar (Figure 5.2A). This behaviour was also observed in previous glycerol bioconversions at different pH. In bioconversions without extraction at pH 5 and 6, 3-HP production rates were similar to the one obtained in this experiment (Table 5.5). Indeed, glycerol feeding rate (0.5 g h^{-1}) is very slow compared to *L. reuteri*'s maximum consumption rate (Dishisha *et al.*, 2015), thus being the limiting factor for these bioconversions. Concerning 3-HPA, there was no significant accumulation during all bioconversion time, as expected through the fed-batch strategy.

Table 5.5 Comparison with bioconversions without extraction[a]

	3-HP production rate (g h^{-1})	**3-HP produced (g)**	**Bioconversion time (h)**
This experiment	0.18	1.5	8.2
pH regulated at 5	0.23	6.7	29.5
pH regulated at 6	0.21	12.4	57.7

[a] Ongoing PhD work of Phuong Nguyen

Dissolved O_2 had a particular behaviour during extractive bioconversion (Figure 5.2C). Before extraction, pO_2 was maintained at a value of 0. Once the bioconversion medium started circulating through the membrane contactor, pO_2 increased in a continuous way. The presence of a micro-leak at a connector or control valve was suspected. This can create a local vacuum and suction air from the outside, resulting in an unexpected supply of oxygen from the pertraction unit.

Reactive pertraction strongly inhibited bioconversion, given the short period of time where the strain was able to produce metabolites, compared to experiments without extraction (Table 5.5). Nevertheless, there was an improvement on 3-HP production compared to the previous attempt of extractive bioconversion with this strain. Indeed, Burgé *et al.* (2017), performed a batch bioconversion of 18 g L^{-1} of glycerol, coupled to reactive extraction using 20% v/v TOA diluted in decanol as the organic phase. There was no back-extraction unit on those experiments. Results showed that 3-HP production stopped after 1 h of extractive bioconversion, giving a 3-HP concentration of 0.4 g L^{-1}, followed by a slight concentration decrease in the bioconversion medium, suggesting slow 3-HP extraction in the organic phase. Several inhibitory factors were addressed with the configuration used in this study: i) 3-HPA accumulation was avoided by setting a fed-batch bioconversion strategy, ii) toxicity of the organic phase was diminished, and iii) cells contact with the reactive pertraction unit started after reaching a more adequate pH for 3-HP extraction, avoiding unnecessary stress for the cells. However, *L. reuteri* DSM 17938 appeared very sensitive to reactive pertraction.

5.3.1.3. Physiological state of the cells

Figure 5.3 shows the evolution of the physiological state of *L. reuteri* during bioconversion. Before extraction, cells viability remains high and the proportion of viable cells is kept constant. However, the total number of cells tends to decrease slightly. Once extraction was launched, the total number of cells started to decrease faster, as well as the proportion of viable cells. After 22.8 h of bioconversion (17 h of extraction), the total number of cells decreased by 75% compared to the initial amount. This suggests that an important cellular disruption occurred due to shear stress caused by circulation through the HFMC, which is in concordance with the experiments in Chapter 4 (Section 4.3.2), where cells integrity decreased after 24 h of circulation. Dead and altered cells proportion also increased by the end of the extractive bioconversion. This information confirms that *L. reuteri* cells are particularly sensitive to reactive pertraction. Nevertheless, there was an important alleviation of the inhibitory effect, compared to the previous attempt of extractive

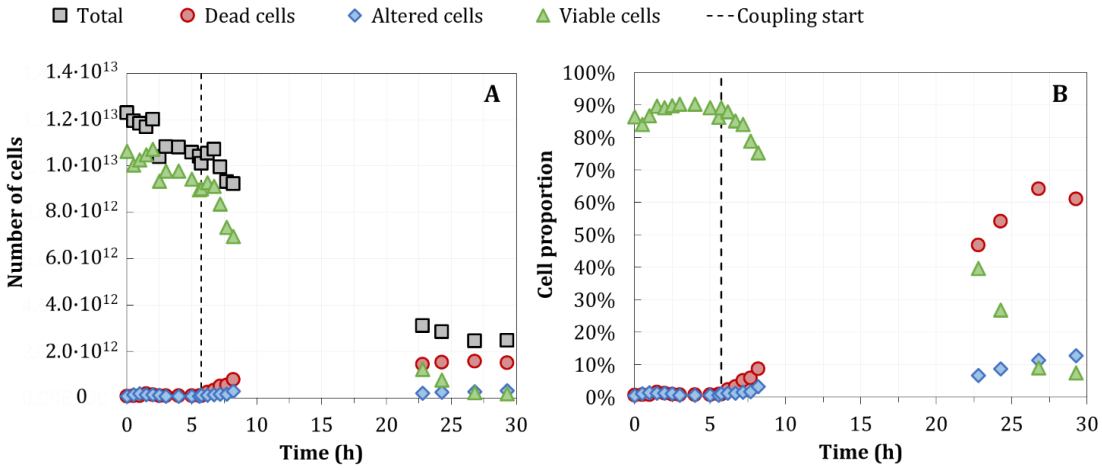


Figure 5.3 Phisyological state of cells during extractive bioconversion. Given as A. number of cells, and B. cell proportion.

bioconversion (Burgé *et al.*, 2017). In the previous experiments, a significant decrease of the viable cells proportion was observed after 30 min, while all cells lost its membrane integrity after 1.5 h of contact.

5.3.1.4. Extraction performance

A total amount of 1.5 g of 3-HP was produced during extractive bioconversion. Figure 5.4 shows the distribution among the three liquid phases over time. It can be observed that 3-HP extraction was very slow (extraction rate = 0.014 g h^{-1}, during the first 2.5 h of extractive bioconversion, corresponding to 0.039 g h^{-1} m^{-2} of available surface area in the membrane) and only 5.8% was extracted after 45 h of contact. 3-HP production rate (0.18 g h^{-1}) was 12.8 times higher than the extraction rate, and 4.6 times higher than the specific extraction capacity of the HFMC. This indicates that pH 5 is still a high value to ensure an effective and quick reactive extraction as soon as it was activated, even though it was selected to have a compromise with the bioconversion ability of the strain.

3-HP was first accumulated in the organic phase, while back-extraction started slowly. After 22.7 h, 3-HP accumulated in the back-extraction phase, while the organic phase was depleted. The pH of the bioconversion medium had an interesting behaviour during extraction (Figure 5.2C). When pH reached 5, extraction was launched and pH decreased slowly during 1.7 h. After that, because 3-HP production rate (0.20 g h^{-1}) was higher than the extraction rate (0.014 g h^{-1}), pH increased continuously. 3-HP extraction was very limited, so it cannot be related to the continuous increase of pH. A possible explanation is that the interface inside the membrane pores had a slow but continuous destabilisation, leading to the passage of a small amount of the NaOH solution to the organic phase, and subsequently to the bioconversion medium.

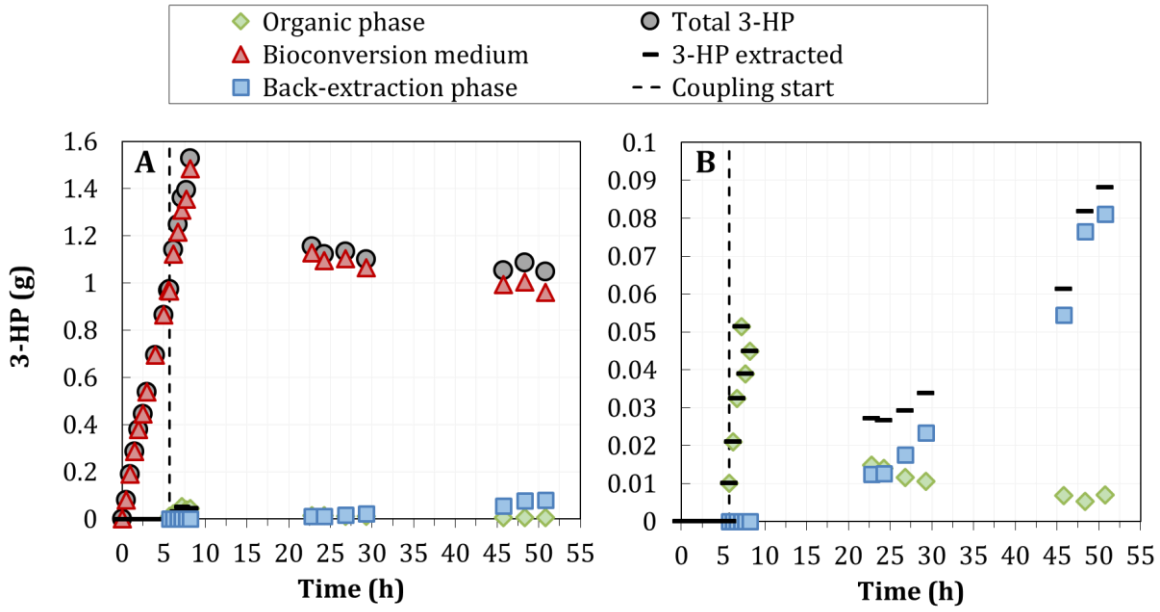


Figure 5.4 Total 3-HP and distribution among the three phases (bioconversion medium, organic phase and back-extraction phase). A) Distribution amog all phases. B) Focus on extracted 3-HP.

Because this was a preliminary experiment with no replicates, some details have been left without a satisfactory explanation. For example, Figure 5.4A shows a decrease of the total 3-HP present in the extractive bioconversion system after 8.2 h, while the extracted 3-HP showed a momentary decrease, to increase again at the end of extraction (Figure 5.4B). These inconsistencies on the results might be attributed to experimental errors due to the very low 3-HP concentrations detected, leading to low accuracy. However, in the case of the bioconversion medium, the chromatograms showed that when the area of the 3-HP peak (retention time = 20.1 min) decreased between 22.7 and 50.7 h of bioconversion, the area of a non-identified peak (retention time = 27 min) increased within time. This would suggest that 3-HP might be converted into another molecule in particular stress conditions.

5.3.1.5. Identified limiting factors for further optimisation

Given these preliminary results, some limiting factors were identified:

(i) The glycerol feeding rate used was slow compared with the maximal consumption capacity of the strain. This might represent a source of stress for the cells, rendering them more sensitive to the reactive pertraction operating conditions. A higher glycerol feeding rate needs to be set, which still avoids the accumulation of 3-HPA at toxic levels.

(ii) Extractive bioconversion initiated at pH 5 resulted in a very low 3-HP extraction performance. However, lower pH values are inhibitory for *L. reuteri* DSM 17938. Another method to improve the extraction performance is to further decrease the concentration of salts and other acids from the growth medium, which have significant competing effects on 3-HP extraction (Chemarin *et al.*, 2019a). To this end, an additional number of washes with RO water after growth are recommended.

(iii) An important supply of O_2 from the pertraction unit was detected, which might represent an additional source of stress for the cells. This could be reduced with lower flow rates through the fibres, together with N_2 addition to the bioconversion medium.

(iv) Shear stress caused by cells circulation through the pumps, valves and the membrane contactor had a detrimental effect on cells as observed in Chapter 4 (Section 4.3.4). Cells immobilisation or retention by filtration devices can prevent circulation, being a very promising strategy for cells protection, not only from shear stress, but also to solvent (Wu and Yang, 2003a) and metabolites toxicity (Dishisha *et al.*, 2012; Zamudio-Jaramillo *et al.*, 2009), resulting in increased lifetime and productivity (Krauter *et al.*, 2012).

Even with the implementation of these measures, *L. reuteri* DSM 17938 might be too sensitive to be used with this in stream reactive pertraction configuration. Therefore, extractive bioconversion was also performed with the strain *Acetobacter sp.* CIP 58.66, which is potentially more resistant to organic solvents and to acidic environments (Raspor and Goranovič, 2008; Vermuë *et al.*, 1993). Results are described in the next section.

5.3.2. Extractive bioconversion by Acetobacter sp. CIP 58.66

This section was co-written with Florence de Fouchécour (2019), with the following contributions: Bioreactor preparation, biomass production, bioconversion, sampling, biomass, substrates and metabolites concentration determination, data analysis, modelling and writing of the bioconversion sections was performed by Florence de Fouchécour. Reactive pertraction unit preparation, sampling, determination of the extracted 3-HP concentration, data analysis, modelling and writing of the sections concerning reactive pertraction was performed by Ana Karen Sánchez Castañeda.

5.3.2.1. Bacterial growth and physiological state during extractive bioconversion

5.3.2.1.1. Biomass production prior to extractive bioconversion

Bioconversion of 1,3-PDO into 3-HP was triggered after a preliminary growth phase on glycerol. Cell dry weight concentration reached 0.98 ± 0.12 g_{CDW} L^{-1} at the end of this preliminary growth step, with a corresponding yield of 0.42 ± 0.12 g_{CDW} $g^{-1}_{glycerol}$. Moreover, pH rose from 5.2 to 6.9 ± 0.1 during growth. This rise in pH is a likely cause for growth slowing down, since glycerol was not fully depleted: only 21.1 ± 3.3 % of the initial glycerol was consumed, which was consistent with previous results (de Fouchécour, 2019). Based on this, it can be considered that the biomass production step was reproducible enough to constitute a consistent basis for the subsequent extractive bioconversions.

A noteworthy feature of this preliminary growth phase is that no by-product of glycerol consumption (such as organic acids) could be detected in the medium. This is in accordance with previous results (de Fouchécour, 2019). Yet, this observation is in contradiction with an earlier study dedicated to the growth of *Acetobacter aceti* on glycerol (Kylmä *et al.*, 2004). In that study, succinate and lactate were found in concentrations (up to *ca.* 1.1 and 0.1 g L^{-1}, respectively) that would have been detected in the analytical conditions used here. In the present study, glycerol is therefore hypothesised to be fully oxidised into CO_2, which was the main product of glycerol oxidation in the study of Kylmä *et al.* (2004). Consequently, this preliminary growth phase was considered to be suitable for the subsequent in stream extraction, since no organic acid was accumulated, that could compete with 3-HP.

5.3.2.1.2. Secondary growth after 1,3-propanediol addition

After the 32 h-long preliminary growth phase, bioconversion was initiated by 1,3-PDO addition. As expected from previous results (de Fouchécour, 2019), both 1,3-PDO inputs (at 0 h and 4.6 h of bioconversion) could be associated with a new exponential growth phase, in all replicates (Figure 5.5A, Table 5.6). Numbers of generations (N_g) were similar for both growth phases, and overall, CDW increased from 1.01 ± 0.06 to 2.75 ± 0.07 g_{CDW} L^{-1}. However, it could be observed that the maximal growth rate (μ_{max}) following the first 1,3-PDO addition was higher than the one following the second addition (Table 5.6). During this first exponential phase, the pH decreased from 6.9 to 4.2, and it decreased even

further, until 3.9, after the second 1,3-PDO addition (Figure 5.6B). Therefore the difference in maximal growth rates was attributed to this difference in pH conditions, together with the physiological state of the cells at each 1,3-PDO addition: there was an 87% of viable cells at the first 1,3-PDO addition, and they decreased to 68% after the second addition (Figure 5.5C). Indeed, it was previously shown that the strain's growth on glycerol remained very limited at pH 4, in comparison to pH above 4.5 (de Fouchécour, 2019). This is also consistent with a previous study in which the optimal pH for *Acetobacter aceti*'s resistance to acetic acid was found around 6 (Steiner and Sauer, 2003). During these secondary growth phases, glycerol was further consumed, but never fully depleted: at 25 h

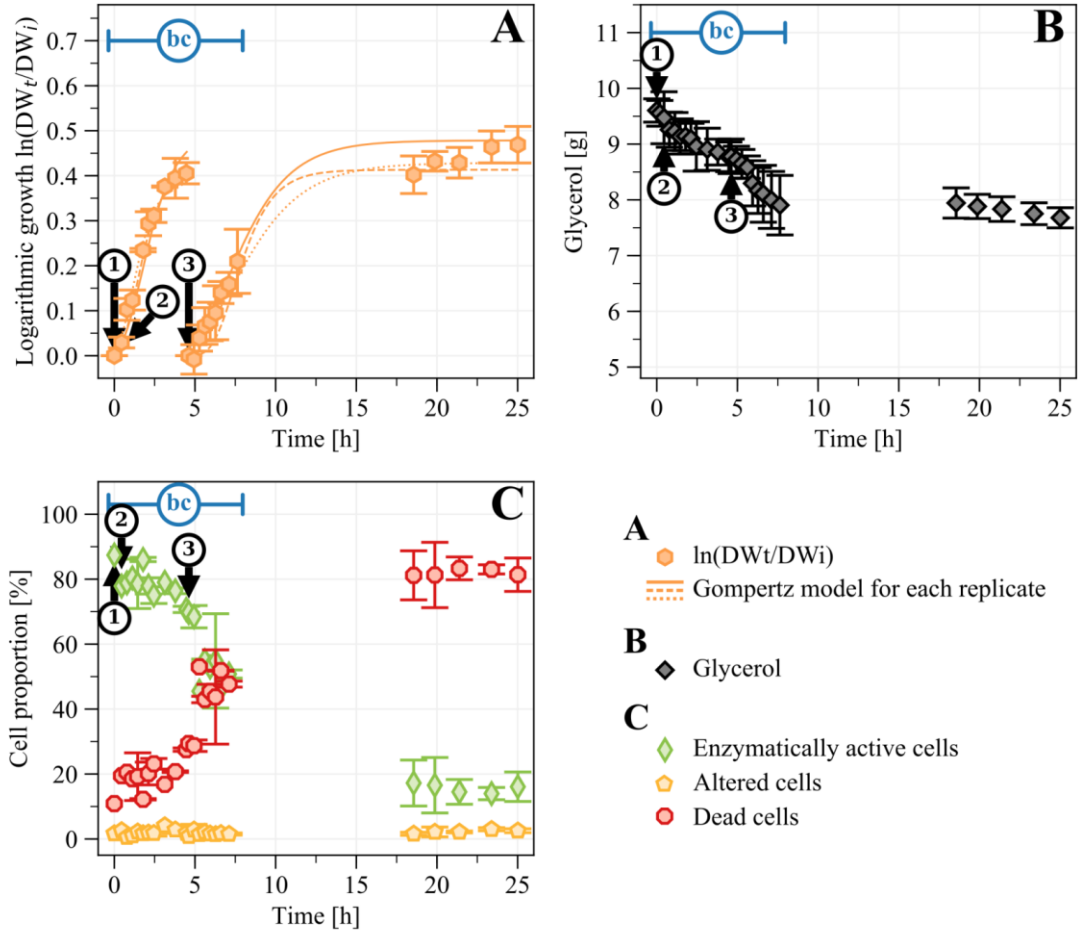


Figure 5.5 Growth characteristics of *Acetobacter* sp. CIP 58.66 during extractive bioconversion. A) Logarithmic growth of the successive growth phases following each 1,3-PDO addition (n = 3). For each new growth phase, logarithmic growth is calculated on the basis of the initial DW of the concerned phase. B) Glycerol evolution in the medium (n = 3). Values are given in grams to take volume variations into account (the working volume varied from 1.12 ± 0.01 to 0.96 ± 0.01 L). C. Flow cytometry analysis of the bacteria physiological state (n = 2, except for values with no error bars, where n = 1). ① Bioconversion is triggered by initial 1,3-PDO addition. ② The reactive pertraction unit is connected to the bioreactor when pH 5 is reached. ③ Second 1,3-PDO addition. The horizontal rules marked "bc" indicate the time span corresponding to the bioconversion: after that, 1,3-PDO was fully depleted.

of bioconversion, 37.9 ± 2.9 % of the glycerol initially supplied was consumed, compared to 21.1 ± 3.3% at the beginning of the bioconversion (Figure 5.5B). While glycerol is likely the main growth substrate, the presence of 1,3-PDO in concentrations under 10 g L^{-1} was also shown to enhance the growth of *Acetobacter* sp. CIP 58.66 on complex medium (de Fouchécour, 2019). The respective roles of glycerol and 1,3-PDO as growth substrates during the bioconversion phase remain unclear, and further studies would be required in order to better understand the strain's catabolism (for instance, through enzymatic activities assessment or metabolomic analysis). However, in contrast to the previous study where 1,3-PDO was continuously fed to *Acetobacter* sp. CIP 58.66 during the bioconversion, here, 1,3-PDO was added discontinuously. The fact that each 1,3-PDO addition was associated here with a new growth phase thus substantiates the hypothesis that 1,3-PDO plays a significant role in these secondary growth phases.

Table 5.6 Growth and bioconversion performances of *Acetobacter* sp. CIP 58.66 during extraction bioconversion

Parameter	**1st 1,3-PDO addition**		**2nd 1,3-PDO addition**		**End of growth**
Time	0 h	4.46 h	4.61 h	7.63 h	25.01 h
Growth					
Growth phase	1st exponential phase		2nd exponential phase		Late stationary phase
DW (g L^{-1})	1.01 ± 0.06	1.57 ± 0.07	1.56 ± 0.06	1.99 ± 0.05	2.75 ± 0.07
N_g[a] of the considered phase	0	0.57 ± 0.04	0	0.30 ± 0.11	0.68 ± 0.06[b]
μ_{max} (h^{-1})	0.17 ± 0.01		0.08 ± 0.03		-
Bioconversion					
Total 3-HP production (g)	0	6.05 ± 0.48	6.65 ± 0.66	13.09 ± 1.02	13.48 ± 1.07
3-HP yield from 1,3-PDO (mol mol^{-1})	-	0.83 ± 0.06	0.90 ± 0.07	0.91 ± 0.06	0.90 ± 0.07
Maximal specific productivity $q_{3\text{-HP,max}}$ ($g_{3\text{-HP}}$ g_{DW}^{-1} h^{-1})	2.36 ± 0.15		2.58 ± 0.21		-
Average specific productivity $q_{3\text{-HP}}$ ($g_{3\text{-HP}}$ g_{DW}^{-1} h^{-1})	1.11 ± 0.18		1.12 ± 0.07		-

[a] Number of generations

[b] Calculated from the beginning of the second 1,3-PDO addition

5.3.2.1.3. Biocompatibility of the in stream reactive pertraction system

The physiological state of *Acetobacter* sp. CIP 58.66 was monitored during the bioconversion, by dual fluorescent staining and flow cytometry analysis. For each sample, cells could be divided into three sub-populations: (i) enzymatically active cells, (ii) altered cells, that are still enzymatically active, but whose membranes are deteriorated, and (iii) dead cells. During the first four hours of bioconversion, the proportion of enzymatically active cells remained between 75 and 87 %, while dead cells represented between 11 and 23 % of the overall population (Figure 5.5C).

After 4 h of bioconversion, the proportion of enzymatically active cells decreased during approximately two hours, before reaching values between 46 and 55 % at the end of the bioconversion (approx. 8 h). In the meantime, the proportion of dead cells followed the opposite trend, before stabilising between 43 and 53 % (Figure 5.5C). This evolution of the ratio of dead cells to active cells (from approximately 1:5 to 1:1) begins around 0.6 h before the second 1,3-PDO addition, which is consistent with the lower μ_{max} of the last growth phase (Table 5.6). Overall, the biocompatibility of the system is still satisfactory, since half of the bacterial population was still active at the end of bioconversion, when 1,3-PDO was almost fully depleted. Interestingly, between 18 and 25 h after the bioconversion started, the proportion of enzymatically active cells remained between 14 and 17 %. This suggests that this process setup could be suitable for continuous extractive bioconversion, for at least 25 h.

Several factors might be involved in the gradual decline of the basal enzymatic activity of cells. First of all, 3-HP accumulation in the medium (up to 10.59 ± 1.18 g L^{-1}, Figure 5.6A) and low pH (close to 4.0, Figure 5.6B) might affect the cells. In particular, pH 4.0 was shown to hinder growth of *Acetobacter* sp. CIP 58.66 on glycerol in shake-flasks (de Fouchécour, 2019). In addition to these factors, more specific stresses due to the process integration are described in the literature:

(i) Prolonged exposure to solvent. Solvent toxicity is a common concern for ISPR processes (Yabannavar and Wang, 1991a). By minimising direct contact between cells and organic solvents, the use of HFMC was shown to limit toxicity (Yang *et al.*, 2007), even though it cannot always prevent detrimental effects on bacteria (Burgé *et al.*, 2017). Yet, the extracting phase used in this study was formulated based on the trade-off determined between extraction performances and biocompatibility with the 3-HP-producing strain *Lactobacillus reuteri* DSM 17938 (Chapter 4). Therefore, a limited solvent toxicity was expected in the present study.

(ii) Nutrient depletion. Indeed, after the extraction was initiated, the initially yellowish medium progressively faded while the organic phase became a bit coloured, suggesting that some coloured nutrients (such as some vitamins) might also have been extracted from the bioconversion medium, thus possibly hindering bacterial growth. This phenomenon was also observed by Jin and Yang (Jin and Yang, 1998), while performing extractive fermentation by

Propionibacterium acidipropionici for propionic acid production. However, their results suggested that their strain's growth was not impacted.

(iii) Oxygen limitation. Acetic acid bacteria are obligate aerobes and even short (less than 1 min) oxygen deprivation was shown fatal to vinegar production processes with *Acetobacter* spp. (Hitschmann and Stockinger, 1985; Murakoa *et al.*, 1982). Here, the bioconversion medium was supplied with air inside the bioreactor, but not in the extraction unit (pipes and membrane contactor). The overall volume in the extraction unit was 200 mL (one sixth of the total working volume), so the average medium residence time in the extraction circuit was 33 s. Under the assumption that the bioreactor is perfectly stirred, this means that bacteria would experience a 33 s-long aeration limitation every 3.3 min, which could lead to oxygen deprivation stress.

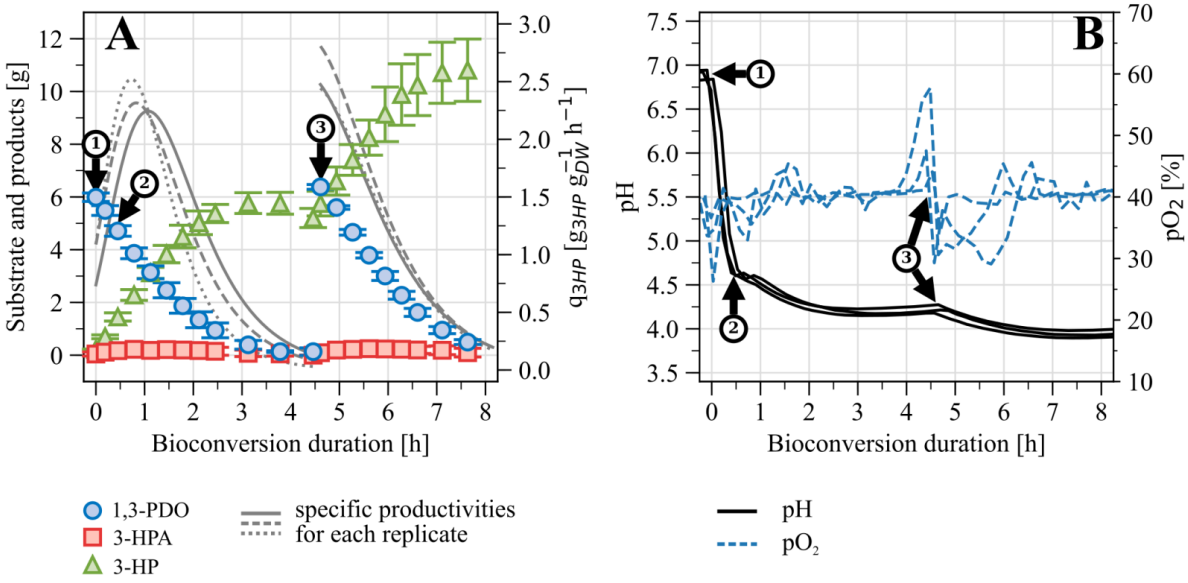


Figure 5.6 Evolution of the medium composition during extractive bioconversion. A. Substrate and product amounts in the medium. Values are given in grams to take into account volume variations (the working volume varied from 1.12 ± 0.01 to 1.02 ± 0.01 L). Each grey line style (*i.e.* solid, dashed, dotted) represents the calculated specific productivities (q3-HP) of one of the three replicates. B. Acidification kinetics (solid line, left scale) and partial O2 pressure (dashed line, right scale) during bioconversion. ① Bioconversion is triggered by initial 1,3-PDO addition. ② The reactive pertraction unit is connected to the bioreactor when pH 5 is reached. ③ Second 1,3-PDO addition.

In a previous study, the strain was similarly grown on glycerol, then bioconversion was performed with a continuous 1,3-PDO feed and at a controlled pH, but no reactive pertraction was implemented (de Fouchécour, 2019). In terms of number of generations (N_g) and maximal growth rates (μ_{max}), the values presented in this study (μ_{max} = 0.17 ± 0.01 h^{-1} and N_g = 0.57 ± 0.04, Table 5.6) are in the same range than those obtained during those fed-batch bioconversions: μ_{max} was 0.12 ± 0.05 $h^{-1}$and N_g was 0.54 ± 0.09 when pH was controlled at 4.0, while μ_{max} was 0.13 ± 0.02 h^{-1} and N_g was 0.70 ± 0.03 when pH was controlled at 4.5. Results of the present study cannot be fully compared with those of the previous study, due to the differences in experimental setup. This still suggests, however,

that the strain is tolerant to the overall impact of reactive pertraction. The observed decline of the basal enzymatic activity of bacteria was more likely caused by a combination of several of the stress factors described above.

5.3.2.2. Bacterial production of 3-HP during extractive bioconversion

5.3.2.2.1. Bioconversion performances

Following the growth of *Acetobacter* sp. CIP 58.66 on glycerol, the bioconversion was triggered by 1,3-PDO addition (reaching 5.36 g L^{-1} in the medium). Its consumption began immediately, without any observable latency (Figure 5.6A). This first substrate input was fully depleted after 3.8 h, and additional 1,3-PDO (reaching 5.99 g L^{-1} in the medium) was therefore added at 4.6 h. Again, 1,3-PDO was immediately consumed and almost completely depleted within 3.0 h. This shows that, even though glycerol and 1,3-PDO are assumed to be metabolised through distinct pathways by *Acetobacter* sp. CIP 58.66 (de Fouchécour, 2019), cells grown on glycerol are well suited for 1,3-PDO oxidation. For both 1,3-PDO additions, 3-HP was the main product, with an overall yield of 0.91 ± 0.06 $mol_{3\text{-}HP}$ $mol^{-1}_{1,3\text{-}PDO}$. The maximal specific productivities ($q_{3\text{-}HP,max}$) that were reached for each successive phase were similar (Table 5.6). 3-HP was mainly accumulated in the bioconversion medium, even though a slight decrease is noticeable prior to the second substrate input, due to extraction (Figure 5.6A). Moreover, a slight and transient 3-HPA accumulation was detected around 1 h after each substrate input, without exceeding 0.20 ± 0.11 and 0.24 ± 0.14 g L^{-1}, respectively. This could be the consequence of a slight kinetic imbalance between the two successive enzymatic steps of the metabolic pathway. Indeed, Zhu *et al.* (Zhu *et al.*, 2018) showed that the aldehyde dehydrogenase was the rate limiting enzyme for 3-HP production by another acetic acid bacterium, *Gluconobacter oxydans*. As 3-HPA is highly toxic to bacteria, its level has to be carefully monitored during the process. In the present study, its maximum values remained below the minimal inhibitory concentrations estimated for another Gram-negative bacterium, *Escherichia coli*: from 0.56 to 1.11 g L^{-1} (Cleusix *et al.*, 2007). Therefore, 3-HPA was not considered as an inhibiting factor of the designed process.

These results bring out two key features of the implemented extractive bioconversion. **Firstly**, in spite of the cumulated potential stresses, no substantial difference in bacterial performances (yield and specific productivities) could be observed between the conversion of the first and second 1,3-PDO input (Table 5.6). Similar specific productivities, but higher DW could explain why the production rate was higher after the second 1,3-PDO addition (Figure 5.6C). So it appears that the bacterial population is able to maintain its high capabilities over at least 8 h, which might come as a surprise since a decline in the enzymatic activity of cells was observed from 4 h of bioconversion (Figure 5.5C). An explanation to this might be the absence of correlation between the 1,3-PDO oxidation ability of acetic acid bacteria, and their basal enzymatic activity. Indeed, it was previously reported that acetic acid bacteria could still oxidise ethanol, even when they had lost their ability to grow (Park *et al.*, 1991). In particular, the basal enzymatic activity was assessed

here using the esterase activity as a proxy, while the enzymes for 1,3-PDO oxidation into 3-HP are dehydrogenases (Zhu *et al.*, 2018). **Secondly**, the bacterial performances achieved in this study are comparable with the ones obtained during non-extractive, fed-batch bioconversions (de Fouchécour, 2019). During these previous experiments, cells were also grown on glycerol before bioconversion, but 1,3-PDO was continuously fed to bacteria, according to a pH-based strategy, and pH was controlled at 5.0, 4.5, or 4.0. Similarly to the present study, 3-HP yields were always close to the theoretical maximum (1.00 mol mol^{-1}). However, the lower the pH was, the higher the $q_{3\text{-}HP,max}$ was: at pH 4.0, $q_{3\text{-}HP,max}$ was 2.31 ± 0.06 $g_{3\text{-}HP}$ g_{DW}^{-1} h^{-1}. This is similar to the values from the extractive bioconversion (Table 5.6) during which pH was left uncontrolled, but remained between 4.6 and 3.9 after a rapid initial decrease (Figure 5.6B). All these results show that, as implemented, reactive pertraction did not substantially affect the oxidation activity *Acetobacter* sp. CIP 58.66. This integrated process therefore shows a promising potential for extractive bioconversion of 3-HP with a continuous 1,3-PDO feeding. However, continuous 3-HP pertraction did not enhance the bioconversion performances for the considered process design and feeding strategy. The main reasons are that (i) acetic acid bacteria are naturally resistant to organic acids – most notably to acetic acid – (Wang *et al.*, 2015a), while 3-HP concentrations remained relatively low; and (ii) the extracting rate was much slower than the production rate, so 3-HP accumulation in the medium was not significantly different from a non-extractive bioconversion.

5.3.2.2.2. Acidification and aeration of the bioconversion medium

In order to limit the changes in medium composition, which affect the extraction yield (Chemarin *et al.*, 2019a), the pH was left uncontrolled and no base solution was used. Therefore, the pH rose from 5.0 to 6.9 during growth on glycerol and, after 1,3-PDO was added, it sharply decreased due to 3-HP production (Figure 5.6B). The bioconversion medium was put in contact with the organic phase in the HFMC only when pH had reached 4.6, which is close to the pK_a of 3-HP. This pH threshold was reached 28 min after the initial 1,3-PDO supply. After reactive pertraction was initiated, the pH further decreased but at a slower rate, until it stabilised at 4.2 (Figure 5.6B). When the second 1,3-PDO addition was performed, pH decreased again and stabilised at 3.9 at the end of bioconversion. After that, pH increased again slowly, due to acid extraction (data not shown). These successive decreases in pH are due to the imbalance between 3-HP production and extraction rates, the latter being much slower than the former. pH values between 4.5 and 4.0 are still compatible with high oxidative activity of *Acetobacter* sp. CIP 58.66 (see section 5.3.2.2.1), and are a good compromise for guaranteeing a satisfactory extraction yield.

Dissolved oxygen is also a key parameter for the process, since acetic acid bacteria are obligate aerobes. Overall, pO_2 was successfully kept at its setpoint (40% of the saturation value) all along the bioconversion, meaning O_2 availability was not limiting (Figure 5.6B). The setpoint was however maintained at the expense of high stirring and air flow rates (800 rpm and 4 NL min^{-1}, respectively). After each 1,3-PDO addition, a transient pO_2 decrease

was recorded, before it stabilised at its setpoint again. A brief increase could also be observed between 3.8 and 4.6 h, corresponding to 1,3-PDO depletion. After 8 h of bioconversion, 1,3-PDO was fully depleted, so oxygen uptake decreased and pO_2 rose again, up to 100%. This suggests that oxygen uptake during the bioconversion was mainly due to 1,3-PDO rather than glycerol oxidation, since the pO_2 rose as soon as 1,3-PDO was depleted while glycerol was still abundant in the medium (Figure 5.5B). Therefore, O_2 uptake appears as a convenient indicator of 1,3-PDO availability in the medium for future process control: using online acquisitions of pO_2, stirring rate, and air flow rate, the O_2 uptake rate may be estimated in real-time, and used for automatic control of the 1,3-PDO feeding rate.

5.3.2.3. Extraction performance of the in stream reactive pertraction system

3-HP distribution among the bioconversion medium, the organic phase and the back-extraction phase is shown in Figure 5.7. During bioconversion (Figure 5.7A), extraction started slowly, leading to a progressive 3-HP accumulation in the organic phase. The extraction rate increased progressively with 3-HP production, and back-extraction in the NaOH solution started later: 3-HP was detected in the back-extraction phase only after 1.7 h. Back-extraction rate increased as acid accumulated in the organic phase, eventually exceeding the extraction rate, as shown by the decrease in 3-HP in the organic phase (Figure 5.7B). After 8 hours of bioconversion, all the added 1,3-PDO was consumed and 3-HP production stopped. From then on, 3-HP concentration decreased both in the bioreactor and in the organic phase, while the concentration in the back-extraction phase increased. No further 1,3-PDO was added and the system was left running in order to recover all the produced 3-HP. After 114 h, nearly all 3-HP was recovered in the NaOH solution, thus achieving a final concentration of 12.63 ± 0.50 g L^{-1}, while almost no 3-HP was detected in the two other phases (Figure 5.7B).

The 3-HP production rate by *Acetobacter* sp. CIP 58.66 was remarkably higher than the extraction (Figure 5.7C). Consequently 3-HP was mainly accumulated in the bacterial medium during its production. Similar studies for other carboxylic acids have reported an adequate extraction performance for maintaining a low acid concentration in the bioreactor, while controlling pH at constant values between 5.2 and 6.3 all along bioconversion (Jin and Yang, 1998; Nelson *et al.*, 2017; Wu and Yang, 2003b). In these cases, pH control was achieved at these relatively high values because extraction and production rates were balanced in most cases: the strains used in these studies performed at production rates between 0.26 and 1.2 g h^{-1}. The present strain displayed a higher production rate (Figure 5.7C), so pH stabilised at lower values (3.9 to 4.2, Figure 5.7B) using the present reactive pertraction system. However, this had a limited impact on the strain's production capacity.

A further important specificity of this study is the obligate aerobic nature of *Acetobacter* sp. CIP 58.66. Oxygen requirement implies the presence of air bubbles in the bioconversion medium, which circulate inside the contactor fibres. Air bubbles decrease the surface of direct contact between the bioconversion medium and the organic phase and slow down the mass transfer rate of 3-HP to the interface. This could contribute to the lower extraction

performance observed in this study, as compared to the extractive fermentation studies cited above, where the used strains were anaerobic.

An increase in the variability of the 3-HP concentration in each of the three phases can be observed at the end of bioconversion, and it further increases toward the end of extraction (Figure 5.7B). This was attributed to the clogging of the fibres inlet, which was revealed during the membrane cleaning step, thus resulting in a decrease in extraction capacity over the successive replicates. This clogging was due to several factors, such as solid particles accumulation and emulsion formation.

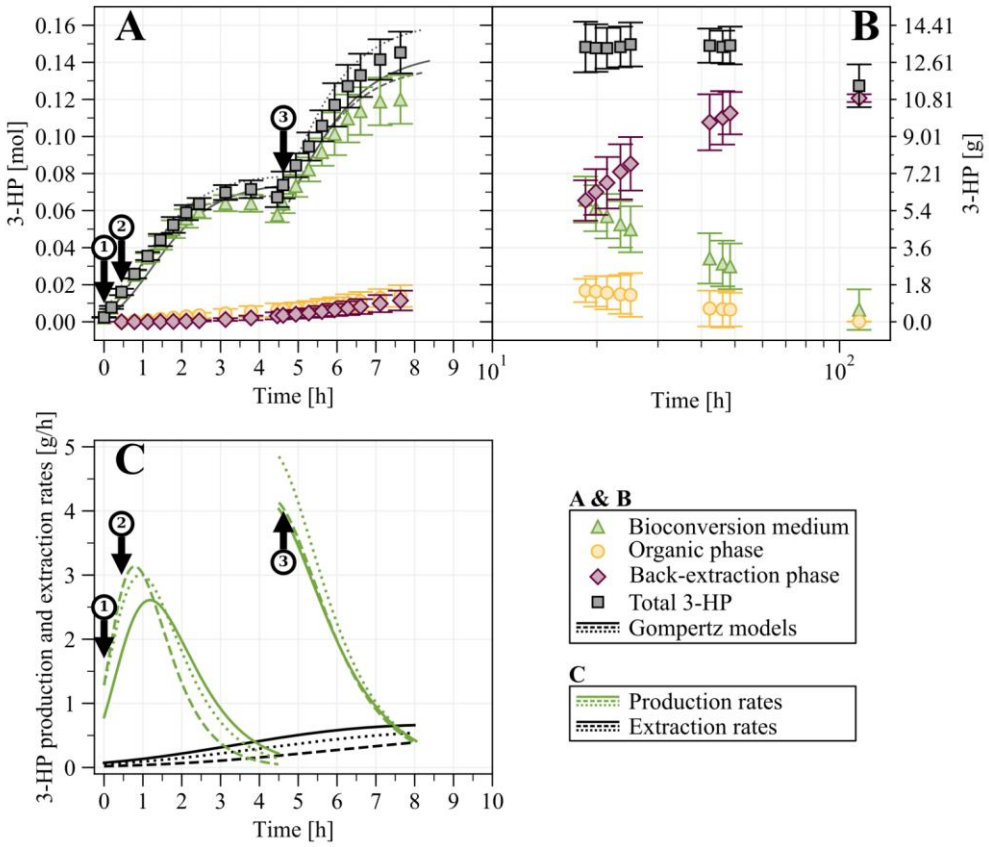


Figure 5.7 Total 3-HP and its distribution among the three phases (bioconversion medium, organic phase and back-extraction phase). A) Focus on the first 8 hours, corresponding to bioconversion duration. B) Evolution of the 3-HP distribution, after bioconversion is finished (time is represented in logarithmic scale, due to the longer time scale considered, compared to bioconversion). C) Production and extraction rates during bioconversion. Each line style (i.e. solid, dashed, dotted) represents one of the three replicates. ① Bioconversion is triggered by initial 1,3-PDO addition. ② The reactive pertraction unit is connected to the bioreactor when pH 5 is reached. ③ Second 1,3-PDO addition.

5.3.3. Mathematical model for simulation-based process optimisation

The mathematical model developed by Chemarin (2017) was used as a simulation tool to explore different strategies to optimise the extractive bioconversion with *Acetobacter* sp. Parameters determined for the organic phase composed of 20% DDMA v/v, 40% dodecanol and 40% dodecane are given below.

5.3.3.1. Parameters for model calibration

5.3.3.1.1. Chemical equilibrium constant

For chemical equilibrium constant K_{11} determination, 3-HP physical partition coefficient was measured, giving a value of 0.004 ± 0.0006 for the 50% v/v dodecanol and 50% dodecane solution. This value was used in Equation 5.19, together with the 3-HP reactive extraction yield results at different initial concentrations. Experimental data showed a different behaviour from the values obtained by the model (Figure 5.8). Indeed, extraction yield increased with 3-HP concentration from 0.5 to 10 g L^{-1}, while the model predicts an opposite trend. This could be explained by the fact that the model was initially developed for a binary mixture amine-alcohol and applied here for a ternary mixture amine-alcohol-alkane with the implicit assumption than the alkane plays strictly no role in complex formation, except dilution. Another explanation is possible presence of remaining impurities from the DDMA, even after purification. Impurities from DDMA could be more difficult to eliminate with the applied protocol than those contained in TOA. These impurities show a more important effect at low acid concentrations, resulting in lower extraction yields than using the pure tertiary amine (Chemarin *et al.*, 2017a). In addition, dispersive extraction with DDMA in yield determination experiments leads to the formation of a very stable emulsion, even after centrifugation, affecting the accuracy of the method. The obtained value of K_{11} using this method was 1.72 ± 0.05 L mol^{-1} and was used as an initial guess for parameter determination by fitting the mathematical model to experimental data in a HFMC, as described in the next section.

A more detailed study of the stoichiometry of the complex formed during the reactive extraction of 3-HP using this ternary organic phase is needed. Also, further purification of the amine must be performed to eliminate the effect of impurities and determine the effect of inert diluent addition to the extraction phase.

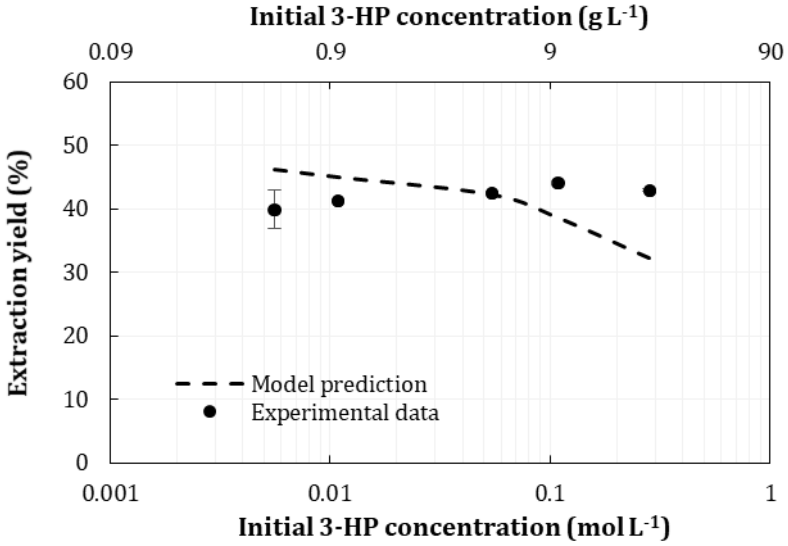


Figure 5.8 3-HP extraction yield as a function of initial acid concentration

5.3.3.1.2. Increase of the organic phase viscosity

Figure 5.9 shows the viscosity evolution for the organic phase composition used in extractive bioconversion by *Acetobacter* sp. CIP 58.66 (20% v/v DDMA, 40% dodecanol, 40% dodecane). The viscosity of this organic phase displays the same linear increase with 3-HP concentration than the mixture of 20% v/v TOA diluted in decanol, but the initial value is lower (4.69 ± 0.03 *vs.* 12.06 mPa·s (Chemarin, 2017)), as well as the viscosity increasing factor (11.42 *vs.* 19.51 mPa·s L mol^{-1} (Chemarin, 2017)). A lower viscosity is advantageous, resulting in faster mass transfer (Coelhoso *et al.*, 1997). This linear behaviour is represented by Equation 5.24, which was used in the mathematical model.

$$\mu_{org} = 11.42\,[AH]_{org,total} + 4.55 \qquad (5.24)$$

where μ_{org} is the viscosity of the organic phase in mPa·s and $[AH]_{org,total}$ is the total acid concentration in the organic phase in mol L^{-1}.

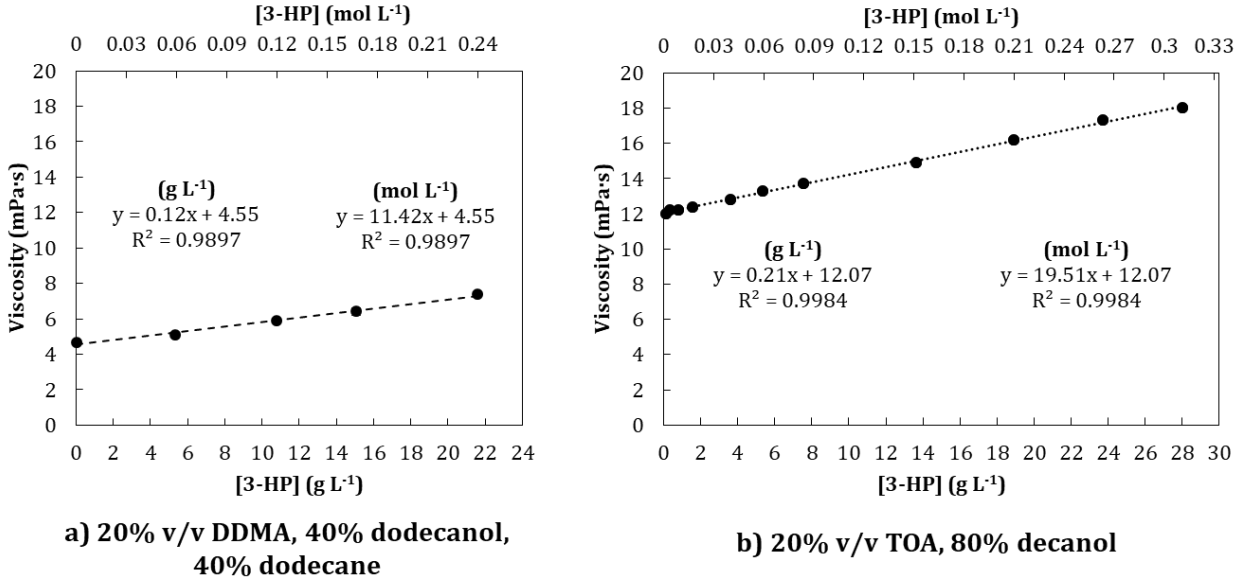


Figure 5.9 Organic phase viscosity increase with 3-HP concentration. a) 20% v/v DDMA, 40% dodecanol, 40% dodecane at 30°C. b) 20% v/v TOA 80% decanol at 25 °C.

5.3.3.1.3. Mass transfer coefficients

The mass transfer coefficient in HFMC value (k) of the different chemical species in the aqueous phase were estimated using the Lévêques equation, as described by Chemarin (2017). Experimental data of the reactive extraction of a 3-HP solution at 1 g L^{-1} was then used to determine the value of the product $k_{CPX}S$ (m^3 s^{-1}), while the values for the other chemicals species were derived from it, using the Wilke-Chang correlation. Figure 5.10 shows the relation of the obtained experimental data with model predictions, $k_{CPX}S$ was found to be 9.64·10^{-8} m^3 s^{-1}, which is higher than the value obtained by Chemarin (2017) for 20% v/v TOA in decanol (5.4·10^{-8} m^3 s^{-1}). This is consistent with the fact that a lower viscosity of the organic phase leads to faster mass transfer. The K_{11}value was determined

simultaneously using the experimental data, giving a value of 2.65 L mol^{-1}. Model predictions follow experimental results very well (R^2 = 0.998, Figure 5.10B), so the obtained values were used for model validation.

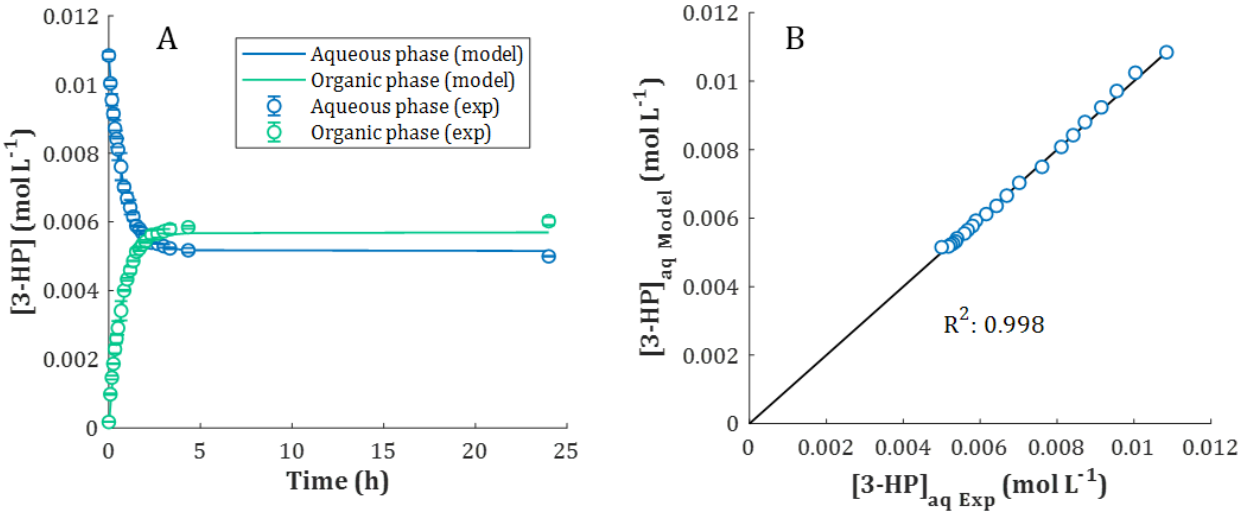


Figure 5.10 Experimental and model results for mass transfer and chemical equilibrium parameters determination. A. Reactive extraction in HFMC of a 3-HP solution at 1 g L^{-1} with 20% v/v DDMA, 40% dodecanol and 40% dodecane. B. Comparison of the experimental data with model simulations after parameter calibration (K_{11} and $k_{CPX}S$) predictions.

5.3.3.1.4. Model validation

The obtained parameter values were used to predict the experimental results of the reactive pertraction (*i.e.* simultaneous extraction and back-extraction using two HFMC) of a 3-HP solution at 10 g L^{-1}. Figure 5.11 shows that experimental data is well predicted by the model, especially for the 3-HP aqueous solution and the back-extraction phase (NaOH 0.5 M). The 3-HP concentration in the organic phase shows a small time lag between experimental data and model predictions, indicating that the model predicts a slightly faster 3-HP extraction and back-extraction than the observed experimentally. As discussed above, this difference might be caused by the presence of impurities from the amine and by inert diluent addition in the organic phase. Dodecane modified the organic phase properties, which are included in the model as a dilution factor only. Nevertheless, model predictions can be considered adequate to be used as simulation tool for the extractive bioconversion values (R^2 > 0.928, Figure 5.11B).

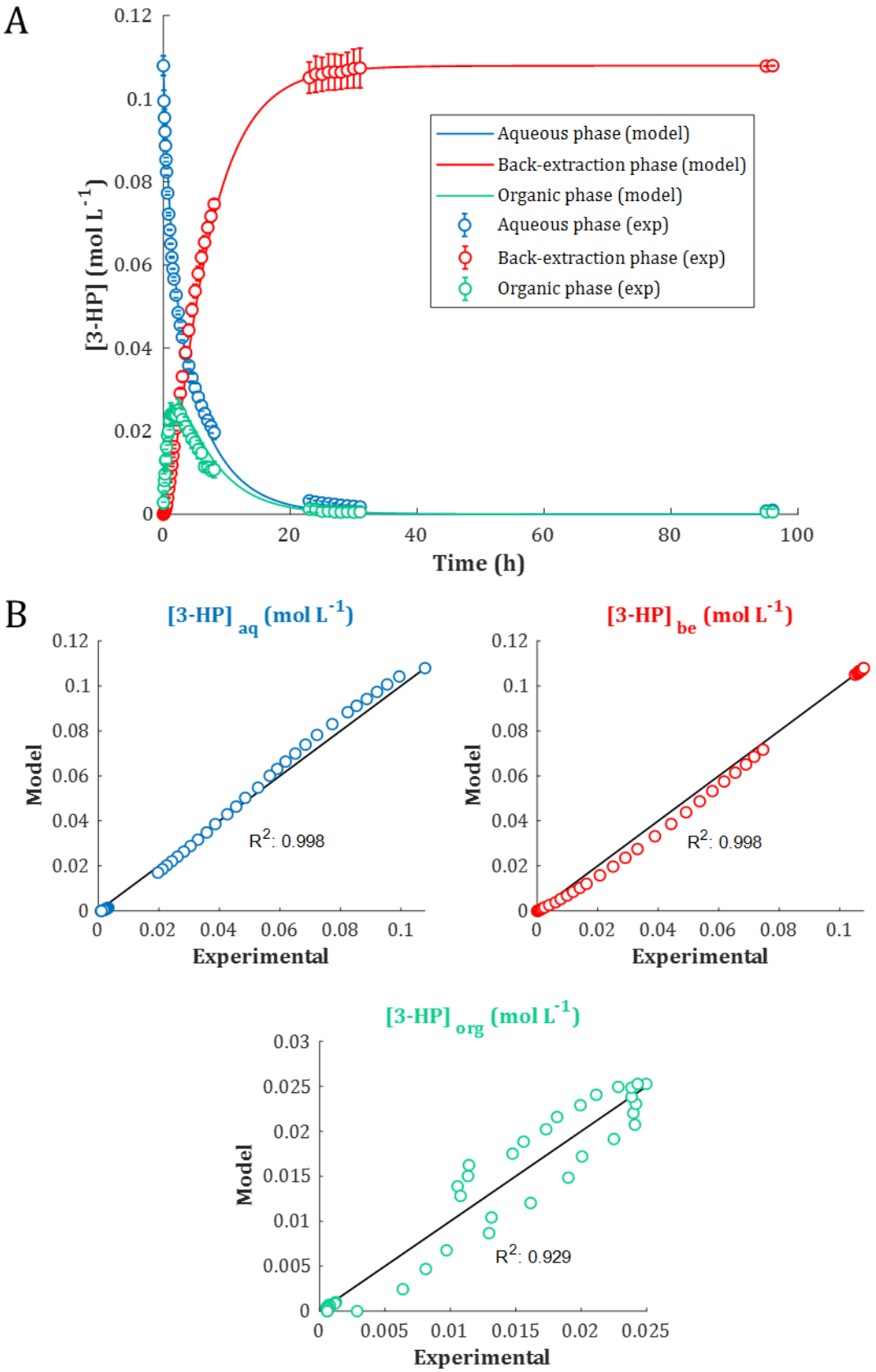


Figure 5.11 Experimental and model results for model validation. A. Reactive pertraction of a 3-HP solution at 10 g L^{-1}. B. Comparison of the experimental data with model predictions.

5.3.3.1.5. Model prediction of extractive bioconversion

In the case of 3-HP reactive pertraction from a real bioconversion medium, the parameter values of the organic phase where assumed to be the same as those obtained in model solution. However, the bioconversion medium composition can have an important effect on chemical equilibrium and affect the global mass transfer dynamics (Chemarin *et al.*, 2019a). In order to adapt the mathematical model to real bioconversion conditions, a 3-HP production rate *P* was calculated from experimental data at each measured time, and used in Equation 5.12. Also, the K_{11} was assumed to decrease because of the ion salts contained in the bioconversion medium (Chemarin *et al.*, 2019a) and was determined by comparing the model results with the experimental data, the obtained value was 0.695 L mol^{-1}.

Figure 5.12 shows the obtained model predictions for 3-HP concentrations in the bioreactor, the organic phase and the back-extraction phase during extractive bioconversion. Predictions are compared with the experimental results obtained in the extractive bioconversion with *Acetobacter* sp. CIP 58.66. For simplicity's sake, the volume of the three liquid phases was considered to remain constant all along extractive bioconversion. It can be observed that model predictions follow the same trends than the experimental results. However, the curve of 3-HP concentration in the bioreactor describe a faster 3-HP extraction from the bioconversion medium than observed with the experimental results. The same tendency is observed in the curve of 3-HP concentration in the back-extraction phase, where model predictions describe a faster 3-HP back-extraction. Nevertheless, the model describes reasonably well the 3-HP distribution in the three liquid phases during extractive bioconversion. Therefore, it was used for simulation of different experimental conditions in order to explore some strategies for process optimisation.

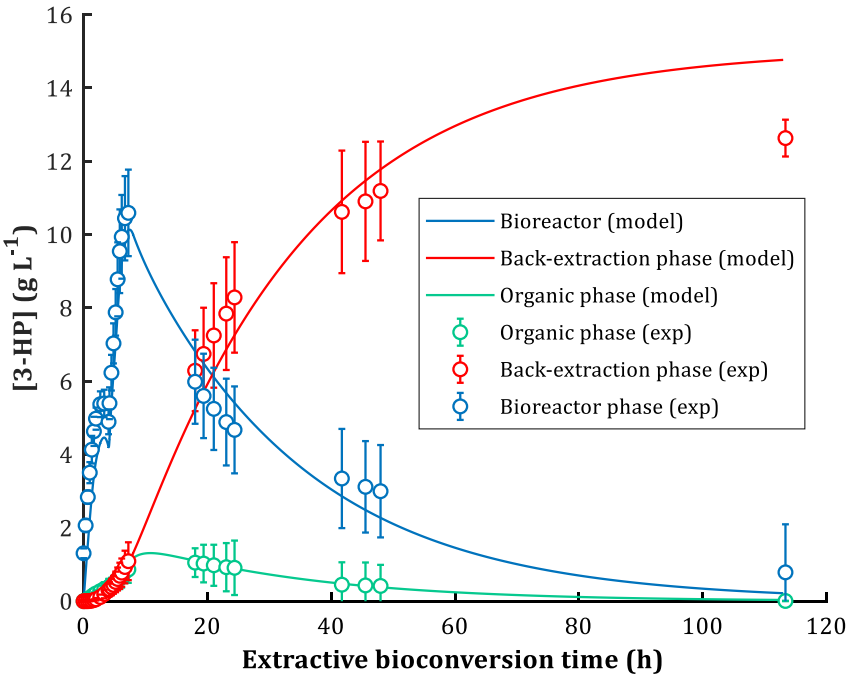


Figure 5.12 Model predictions and experimental results of the 3-HP concentration at the bioconversion medium in the bioreactor, the organic phase and back-extraction phase during extractive bioconversion with *Acetobacter* sp. CIP 58.66.

5.3.3.2. Prospects for improving the extraction performance

Optimisation of reactive pertraction is required in order to achieve continuous 3-HP production at high productivity, and to obtain a final 3-HP solution at higher concentration and purity than in the bioconversion medium. It was observed that the overall effect of reactive pertraction was well tolerated by *Acetobacter* sp. CIP 58.66 during bioconversion. The selected organic phase already showed the best compromise between extraction performance and biocompatibility with a 3-HP producing strain (Chapter 4). It is thus necessary to explore alternate strategies other than further optimisation of the organic phase composition.

A first possible approach is to improve pertraction performance by increasing the specific contact area (contact area per unit of bioconversion medium) of one of the extraction or back-extraction modules, or both. For example, Jin and Yang (Jin and Yang, 1998) maximised the specific extraction rate of their system by reducing the volume of the bioconversion medium from 2.2 L to 0.3 L, corresponding to a 7.3-fold increase in the specific contact area. They also increased the pH from 4.8 to 5.2, thus improving the global process productivity. In order to assess this effect in the present study, simulations with a working volume in the bioreactor of 1.2 L (this study) and 0.3 L were compared, corresponding to 4-fold increase of the specific contact area. A 3-HP productivity of 2.1 g L^{-1} h^{-1} was set, which corresponds to the average productivity observed between 4.5 to 6.6 h of bioconversion in this study. Simulation with 1.2 L showed a 3-HP accumulation in the bioconversion medium up to 71 g L^{-1} after 300 h. This confirms that the extraction

performance of the present reactive pertraction system is not capable of maintaining a low 3-HP concentration in the bioconversion medium during a continuous production by *Acetobacter* sp. Simulation with 0.3 L, however, reached a stationary concentration of 13 g L^{-1} after 30 h of bioconversion. Even though this strain is resistant to 3-HP concentrations of this order of magnitude (de Fouchécour, 2019), pH in the bioconversion medium would be slightly lower than 4, which will affect the production performance with time. Alternately, the contact area for extraction and back-extraction can be increased by using several HFMC modules, instead of reducing the volume in the bioreactor. In the present example, in order to maintain a concentration close to 13 g L^{-1} with a 1.2 L working volume, four extraction/back-extraction units should be used. In practice, both these strategies are limited by the volume of medium required to fill all the extraction circuit. For the bioreactor, this means instrumentation has to be functional with a 1.2 L working volume (during growth phase on glycerol), as well as with 0.4 L (during extractive bioconversion), since 0.8 L are needed to fill the 4 modules. Moreover, this implies a longer residence time without oxygen for the bacterial strain, which may affect its production performance. For the organic and back-extraction phases, this means that higher volumes are required, and that the back-extraction phase will be more diluted in 3-HP. To avoid dilution, the number of modules for back-extraction could be reduced compared to the number of modules for extraction, at the price of running the back-extraction loop for a longer time, possibly after bioconversion is completed. Technical solutions to increase specific contact area thus constitute another point to take into account when searching for compromises in the integrated process.

5.3.3.3. Integrated approach: the art of compromise

The process described in the present study shows a promising potential, but further optimisation is still required for scaling-up. When designing such an integrated process, the optimal operating conditions are not necessarily the optimum of each process unit. Results presented here highlight some key parameters that have to be considered in future works, for determining an adequate trade-off between production and extraction performances.

To start with, the primary growth phase on glycerol can be further optimised, in order to be shortened. Yet, this has to be done in accordance with constraints from the reactive pertraction system, both in terms of medium composition (*e.g.* no addition of pH control solution) and working volume. In particular, pH is a crucial aspect for extractive bioconversion, impacting both the upstream and downstream performances. Here, pH stabilised around 4.0 during bioconversion, which ensured adequate microbial performances, but lower extraction performances than at even lower pH. However, as pH 4.0 is already below the pKa of 3-HP, only limited improvement of extraction can be expected from lower pH. Another crucial aspect is oxygen availability in the medium: on the one hand oxygen deprivation (for less than 1 min) can be fatal to acetic acid bacteria, which have high oxygen demand, and on the other hand, high aeration rates hindered extraction due to interface instability and possible effective contact area decrease due to air bubbles. Furthermore, critical thresholds have to be determined both for cell residence time

in the bioreactor and in the reactive pertraction unit. This will impact both the bacterial activity and the reactive pertraction system design: in particular, more insights are needed on the impact of successive oxygen deprivations on the strain's ability to produce 3-HP.

A further key aspect is substrate feeding: adapting the feeding rate is an easy-to-implement strategy, in order to balance the 3-HP production and extraction rates. The feeding rate also needs to guarantee a constant and low (below 5 g L^{-1}) 1,3-PDO concentration in the bioconversion medium, notably in order to prevent deleterious 3-HPA accumulation.

Finally, bacteria immobilisation in the bioreactor would be an interesting strategy to protect them from the stresses associated with their circulation in the reactive pertraction unit (Jin and Yang, 1998), and it could also help limiting the clogging in the fibres inlet. Interestingly, immobilisation of *Acetobacter* sp. CGMCC 8142 was investigated for 3-HP production from 1,3-PDO, and notably showed promising stability and storability properties (Li *et al.*, 2016a). All things considered, extractive bioconversion appears as a complex optimisation problem. As illustrated in section 5.3.2.3.2, implementing an accurate model for the overall process will constitute a valuable tool for further process development, so that promising designs and operating conditions can be selected prior to their experimental testing.

5.4. Conclusion

The present chapter explored the feasibility of extractive bioconversion with two different 3-HP producing strains, providing valuable insights for decision-making in the design of an integrated process for bio-based 3-HP. First, extractive bioconversion with *L. reuteri* DSM 17938 revealed the great sensitivity of the strain to reactive pertraction conditions, even with the biocompatibility improvement of the organic phase. Moreover, 3-HP extraction was very limited at pH 5. On the contrary, extractive bioconversion using *Acetobacter* sp. CIP 58.66 had a more promising outcome. During bioconversion, 3-HP was continuously removed from the bioconversion medium. The high 3-HP production capacity of the strain was maintained during the integrated process. Its resistance to cumulative stresses makes it a great candidate for extractive bioconversion. Yet, a significant imbalance between production and extraction rates was observed, leading to pH decrease during 3-HP production. However, this had a limited impact on *Acetobacter* sp. CIP 58.66 bioconversion ability. These results pave the way towards continuous extractive bioconversion of 3-HP or other short-chain organic acids, and towards its scaling-up.

General conclusion and prospects

Production of carboxylic acids by microbial processes, and 3-hydroxypropionic acid (3-HP) in particular, has made remarkable advances, achieving production performances that are getting close to those necessary for the process to be economically viable. The best titres (between 72 – 102 g L^{-1}) and productivities (up to 0.86 g L^{-1} h^{-1}) have been obtained by genetically modified bacterial strains, *i.e. Klebsiella pneumoniae* and *E. coli* (Chu *et al.*, 2015; Zhao *et al.*, 2019), that exhibit low tolerance to very acidic pH (lower than pH 5, or even 6). In addition, the inherent toxicity of 3-HP at certain concentrations causes most bio-production processes to be subjected to product inhibition. The use of genetically modified yeast strains is probably the most promising approach that can solve such problems. However, most of their production performances are currently lower than those of bacterial strains. Only one production process using a modified yeast with titres as high as those obtained by bacterial strains (80 – 100 g L^{-1}) has been reported and it was achieved by Cargill, Inc. However, the utilisation of genetically modified strains present restrictions such as the unknown stability of the introduced gen and that are subjected to strict regulations. More recently, acid acetic bacteria (AAB) have entered the scene of 3-HP production. Strains like *Gluconobacter oxydans* and *Acetobacter* sp. have been used to oxide 1,3-propanediol (1,3-PDO) into 3-HP with encouraging results (titres of 60 – 67 g L^{-1} and productivities of 0.9 – 2.5 g L^{-1} h^{-1}) (Li *et al.*, 2016a; Zhao *et al.*, 2015), without using genetically modified strains. In fact, 1,3-PDO can be produced from glucose or glycerol by several microorganisms and be further oxidised into 3-HP. In addition, these strains are more tolerant to acidic pH than the other bacterial strains used for 3-HP production, which makes them an attractive option for economically viable 3-HP production. In fact, this approach was recently explored in our laboratory, resulting in the best 1,3-PDO oxidation into 3-HP performance found in literature (a titre of 70 g L^{-1} and a productivity of 2.8 g L^{-1} h^{-1}) (de Fouchécour, 2019).

Another key issue in the bio-production of chemical compounds is their recovery from the fermentation or bioconversion medium. 3-HP recovery from aqueous media is particularly challenging because of its hydrophilic nature, and its tendency to form oligomers at high concentrations and to decompose at high temperatures. Reactive extraction assisted by a hollow fibre membrane contactor (HFMC) has been evaluated for 3-HP recovery in our laboratory and resulted to be compatible with the characteristics of this organic acid. In addition, this separation method could be integrated to 3-HP bioconversion for its continuous removal from the bioconversion medium. Such an integrated process appears attractive because of its advantages:

i) selective and continuous 3-HP removal can alleviate product inhibition and improve the overall performance of the process,

ii) bioconversion pH can be regulated without the addition of base solutions which represents a reduction in process consumables and subsequent waste production,

iii) a more purified and concentrated 3-HP solution (in comparison with the bioconversion medium) can be obtained in one step,

iv) the number of post-bioconversion steps for further concentration and purification is significantly reduced.

The present work focused on the limiting aspects that have to be addressed for the implementation of 3-HP extractive bioconversion using reactive extraction assisted by HFMC. First, 3-HP reactive extraction mechanism was studied through the observation of the dynamic interfacial tension, then the selection of a biocompatible organic phase with an adequate extraction performance was carried out. Finally, experimental demonstration of the integrated process was achieved and further optimisation strategies were explored using a mathematical model as a simulation tool.

The mechanisms of 3-HP reactive extraction performed in HFMC have been studied in previous works. Tertiary amines and long-chain alcohols showed to be the best extraction agents for 3-HP. The tertiary amine reacts with 3-HP at the liquid-liquid interface, forming an acid-base complex which is solubilised in the organic phase with the help of the long-chain alcohol by interaction through H-bonds with the complex. The mixture of 20% v/v tri-octylamine (TOA) and n-decanol was initially considered to be a good candidate for 3-HP extraction, considering essentially extraction performance, and used as reference to study the physicochemical mechanisms of 3-HP reactive extraction. The generated information was used to develop a mathematical model able to predict with good accuracy 3-HP extraction in a HFMC at different experimental conditions. This mathematical model considers chemical equilibrium and mass transfer dynamics of the species in the extraction system. In Chapter 3, the model was modified to account for the dynamic interfacial tension change during 3-HP extraction. Results confirmed that the acid-amine complex has surface-active properties. Further analysis revealed that mass transfer near the interface is not governed by diffusion only. Local convection should be taken into account to better describe local mass transfer mechanism. However, further investigation is needed in order to identify the nature of this phenomenon. Concentration-induced convection was suspected, which can be caused by density gradients and associated buoyancy forces or by interfacial tension gradients and associated Marangoni convection. Additional experimental configurations should also be explored in order to observe the effect of other surface-active compounds that could be found in the bioconversion medium. For example, proteins and anions from salts might contribute to a further decrease on interfacial tension or stronger natural convection phenomena. In consequence, interface stabilisation inside the membrane pores of the HFMC might become more difficult and emulsions might be formed.

Although the organic phase consisting of 20% v/v TOA in n-decanol presented a good extraction performance in HFMC, its inherent toxicity highly inhibited 3-HP bioconversion with the strain *Lactobacillus reuteri* DSM 17938. Therefore, a more biocompatible composition was sought in Chapter 4 through the evaluation of different mixtures containing 6 different long-chain alcohols (active diluents), 2 alkanes (inert diluents) and 2

tertiary amines (extractants). As expected, inert diluent addition decreased the toxicity and viscosity of the organic phase but also decreased its extraction performance. The use of alcohols with longer carbon-chains also increased the biocompatibility of the organic phase, but resulted in a lower extraction yield and higher viscosity. It was thus necessary to find a compromise between extraction performance (high extraction yield and low viscosity) and biocompatibility (low solvent toxicity). The biocompatible solutions that showed the best extraction yields were 20% v/v TOA in oleyl alcohol (18 C), and 20% v/v TOA in 47% dodecanol (12 C) and 33% dodecane. However, 20% TOA in oleyl decanol presented a very high viscosity, which will slow down mass transfer in the organic phase. Interestingly, the replacement of TOA with di-dodecylmethylamine (DDMA) at the same concentration, increased the extraction yield of the organic phase and maintained a similar viscosity and biocompatibility. This was attributed to a lesser steric hindrance in the DDMA molecule that favours the reaction. In consequence, the organic phase composition of 20% v/v DDMA, 47% dodecanol and 33% dodecane was selected.

3-HP extractive bioconversion was then implemented using the selected organic phase and two identical HFMC modules. This permitted simultaneous 3-HP extraction and back-extraction using a NaOH solution (reactive pertraction). Extractive bioconversion experiments with *L. reuteri* DSM 17938, presented in Chapter 5, showed that the strain was highly inhibited by reactive pertraction, despite the lower toxicity of the organic phase. Experiments in HFMC showed that *L. reuteri* DSM 17938 is particularly sensitive to shear stress caused by recirculation through the fibres. Moreover, 3-HP extraction was very limited during bioconversion at pH 5, not optimal for reactive pertraction. In contrast, studies performed in our laboratory revealed that 1,3-PDO bioconversion into 3-HP by *Acetobacter* sp. CIP 58.66 was subjected to product inhibition, while being little affected by pH values slightly lower than the pKa of 3-HP (4.51). This made the strain an interesting candidate for extractive bioconversion with reactive pertraction.

The experimental demonstration provided in Chapter 5 revealed that reactive pertraction did not have a significant effect of the 3-HP performance production of *Acetobacter* sp. CIP 58.66, validating its biocompatibility. Moreover, 3-HP was almost completely removed from the bioconversion medium and fully recovered in the NaOH solution used for back-extraction. However, an imbalance between the extraction and production rate remained during bioconversion. The extraction rate was lower than the production rate, leading to accumulation of 3-HP in the bioconversion medium and a pH decrease from 4.5 to 4. The complete 3-HP removal was achieved only many hours after the stop of bioconversion due to 1,3-PDO depletion. Further optimisation of the integrated process is needed in order to balance the extraction and production rates. Exploration of some optimisation strategies, using simulation, suggests that the most promising approach is to increase the specific interfacial area available for mass transfer between the bioconversion medium and the organic phase. This can be verified either by decreasing the working volume in the bioreactor or by adding more HFMC modules. With a higher specific interfacial area, it will be possible to evaluate the effect of 3-HP removal from the bioconversion medium and determine whether reactive pertraction can improve the overall

3-HP production performance. A continuous 1,3-PDO supply needs also to be tested to avoid excessive product accumulation. Control of the 1,3-PDO feeding rate could be based to pH or oxygen uptake variations.

Although *Acetobacter* sp. CIP 58.66 resulted to be a good candidate for extractive bioconversion, the economic viability of an integrated process for bio-based 3-HP production depends on many other aspects. Obtaining added value from 1,3-PDO by its conversion into 3-HP is not straightforward, since 1,3-PDO is itself a promising platform molecule. This strategy would be advantageous in cases where 1,3-PDO and 3-HP are present in the bioconversion medium at important concentrations after a first bioconversion step. Such is the case of a glycerol bioconversion by strains of *Lactobacillus reuteri* or *Klebsiella pneumoniae*. In fact, converting 1,3-PDO into 3-HP could have an economical advantage over having to use two separate downstream processes to recover both molecules separately. Another advantage is the remarkable oxidation capacity of *Acetobacter* sp. CIP 58.66. This indicates that any upstream integrated approach will not be limited by this strain.

Cell immobilisation could be an interesting approach that has already been implemented for another *Acetobacter* sp. strain (Li *et al.*, 2016a). Several process limitations can be addressed by this method. For example, since *Acetobacter* sp. is a strict aerobe, intermittent subjection to anoxic conditions during its circulation through the HFMC could affect its production performance. Although no significant effect was observed in this study, the implementation of additional HFMC modules to increase the interfacial area for extraction will increase the residence time without an oxygen supply, which could limit the scaling-up of the process. Keeping cells immobilised in the bioreactor would not only avoid this limitation, but also remove the shear stress induced by circulation though the HFMC modules observed with *L. reuteri* DSM 17938.

In this study, back-extraction was only performed by chemical swing using a NaOH solution because of its simplicity and direct regeneration of the organic phase. In addition, almost all 3-HP was recovered in the stripping solution by this method. However, 3-HP sodium salt is recovered instead of the free acid, which will need further purification steps depending on subsequent applications. Although other strategies can be applied for 3-HP back-extraction such as diluent and temperature swing, the chemical swing approach is one of the most interesting for the reactive pertraction approach, especially for the direct and continuous regeneration of the organic phase An approach that would be interesting to implement is the use of a volatile water-soluble amine, such as tri-methylamine (TMA), instead of NaOH. This method has been used for lactic acid and citric acid with good results (Jarvinen *et al.*, 2000; King and Tung, 1992). In fact, it is probable that this method will work better in the case of 3-HP, because it is more hydrophilic than the other acids. This permits to recover the totality of the acid and then the volatile base can be removed by thermal cracking at temperatures around 130°C, yielding 3-HP in its free form.

It has also been demonstrated that the viscosity of the organic phase increases with 3-HP concentration, slowing down mass transfer inside the membrane pores. An attempt to

address this limitation was performed by testing HFMC modules with hydrophilic membranes. This approach was based on the hypothesis that the aqueous phase would fill the membrane pores instead of the organic phase, reducing significantly the resistance of the mass transfer inside the pores. In practice, membrane materials that can be used with organic phases, *e.g.* polyethersulfone (PES), are not inherently hydrophilic and are subjected to unpredictable swelling behaviour. In fact, the organic phase can also wet the membrane pores as much as the aqueous phase. This made interface stabilisation very difficult, even impossible, with our reactive extraction system.

More specifically regarding hydrophobic HFMC, it will be interesting to get an easier interfacial stabilisation at larger pore size and get higher membrane porosity. For that purpose, other hydrophobic membrane materials and HFMC configurations can also be explored. For example, membranes made of polytetrafluoroethylene (PTFE) are much more hydrophobic than polypropylene (material used in this work). This could lead to lower membrane resistance for larger pore size PTFE membrane with a good interfacial stabilisation, which is a key aspect for the successful implementation of reactive pertraction.

Further investigation is also needed concerning modelling of the integrated process. The dynamic model for 3-HP reactive pertraction takes into account the chemical reaction and mass transfer mechanisms, using the binary composition of 20% v/v TOA and decanol. However, during model calibration and validation with the organic phase composed of 20% v/v DDMA, 40% dodecanol and 40% dodecane, experimental data was predicted with lower accuracy. Discrepancies with model predictions can be caused by several factors. For example, the presence of a significant amount of impurities from DDMA that remained even after purification, or that the inert diluent addition changes the stoichiometry of the complex formed in the organic phase. A more meticulous study of the chemical equilibrium of the ternary organic phase composition (extractant – active diluent – inert diluent) is needed for better understanding of the reaction mechanisms. Also, it would allow the model to predict results of a wider range of organic compositions. The mathematical model can be further improved to take into account competing reactions with the compounds from the bioconversion medium, such as ions from salts and the presence of other acids. A preliminary mathematical model for 1,3-PDO oxidation into 3-HP by *Acetobacter* sp. CIP 58.66 was developed in our laboratory. This model includes1,3-PDO and 3-HPA limitations, along with 3-HP inhibition on both bioconversion and growth. The integration of this model with the one of reactive pertraction will represent a valuable simulation tool to explore different process optimisation strategies.

The obtained results of 3-HP extractive bioconversion using reactive pertraction are very promising and further optimisation works will make it possible to achieve continuous 3-HP removal from the bioconversion medium. Recently, a PhD work has been started with the aim of further improving the predictive mathematical model and its utilisation for exploration and experimental demonstration of different optimisation strategies. This integrated approach can also be applied to other organic acids and strains, the obtained insights could be extended to such processes.

Appendix

Résumé: Bioconversion extractive d'acide 3-hydroxypropionique : mécanismes limitants et optimisation du procédé intégré

Les ressources fossiles sont aujourd'hui la principale matière première des industries chimique et énergétique, qui jouent un rôle dominant dans l'activité économique mondiale. Bien que de grands progrès technologiques aient été réalisés grâce à ces ressources non renouvelables, ils présentent des inconvénients inquiétants, tels que leur épuisement inévitable et leurs effets négatifs sur l'environnement. Leurs émissions de gaz à effet de serre (GES) sont particulièrement préoccupantes, en raison de leur contribution au réchauffement climatique (Cherubini, 2010). Tout cela a conduit à une forte volatilité du prix du pétrole, qui a un impact direct sur l'activité économique mondiale (van Eyden *et al.*, 2019). Cela a éveillé la préoccupation mondiale de diminuer notre dépendance aux ressources fossiles.

L'industrie chimique a apporté une contribution inestimable à la qualité de vie actuelle, mais c'est l'un des secteurs qui subissent le plus de pressions pour réduire sa dépendance excessive aux ressources fossiles (Gavrilescu et Chisti, 2005). La pétrochimie est le plus grand consommateur de pétrole et de gaz, avec respectivement 14% et 8% de la demande primaire totale. Cela est responsable du 18% des émissions de CO_2 du secteur industriel et de 5% des émissions totales de CO_2 liées à la combustion (International Energy Agency, 2018). Parmi les actions nécessaires pour aborder ce problème, on a le défi de se tourner vers l'exploitation des ressources renouvelables, comme la biomasse, tout en répondant à la demande toujours croissante de biens et de services. Ces préoccupations sont le moteur du développement d'une large gamme de technologies permettant de désassembler la biomasse, en tant que ressource primaire, en leurs éléments constitutifs et de les utiliser à la place des ressources fossiles pour produire des biocarburants et des produits chimiques. Dans ce contexte, des concepts tels que la chimie verte et le bioraffinage sont apparus comme une mesure prometteuse pour relever ces défis (Anastas et Eghbali, 2010 ; Cherubini, 2010). Les gouvernements ont encouragé la transition vers une bioéconomie depuis le début des années 1990 avec différentes politiques publiques qui ont fourni des lignes directrices bien définies pour des conceptions durables, par exemple l'Accord de Paris (United Nations, 2015), et le Plan d'action européen pour la bioéconomie 2018 - 2030 (European Commission, 2018). Les grandes économies comme l'Union européenne (UE) sont technologiquement prêtes à atteindre des objectifs ambitieux en matière de développement de la bioéconomie, tels que la production de 30 % des produits chimiques à partir de la biomasse d'ici à 2030 (Bio-based Industries Consortium, 2012), à partir de 6,8 % en 2015 (Piotrowski *et al.*, 2016). En termes d'impact environnemental, l'objectif de l'UE est de réduire les émissions de GES d'au moins 40 % par rapport aux niveaux de 1990, afin de limiter le réchauffement climatique à moins de 2 °C par rapport aux niveaux

préindustriels (Ohliger, 2019).

L'industrie des biocarburants a eu une croissance particulière depuis que certaines études d'analyse du cycle de vie (ACV) ont constaté une réduction nette des émissions de GES lorsque le bioéthanol et/ou le biodiesel sont utilisés pour remplacer le diesel et l'essence classiques (ADEME, 2010 ; Cherubini, 2010). Cela génère une quantité importante de résidus et de produits secondaires qui pourraient être utilisés pour donner une valeur ajoutée aux bioraffineries. Dans le cadre d'une initiative visant à soutenir le développement d'installations de traitement intégrées pour inclure la valorisation de ces résidus et produits secondaires, le Département Américain de l'Énergie (DoE) a publié, en 2004 et 2010, une liste de produits chimiques prometteurs dérivés de la biomasse qui pourraient servir de moteur économique pour une bioraffinerie (Bozell et Petersen, 2010 ; Werpy et Petersen, 2004). Ces rapports ont orienté la recherche tant de l'initiative privée que de la communauté scientifique, et le marché industriel a connu une croissance remarquable pour plusieurs composés mentionnés dans la liste. Un cas de réussite est celui des bioraffineries d'acide succinique, qui sont maintenant parvenues à maturité économique et produisent entre 13 600 et 20 000 t par an d'acide succinique biosourcé (Ghayur et al., 2019). En fait, ce composé appartient à un type de produits chimiques attractifs, les acides carboxyliques, principalement utilisés dans les domaines alimentaire, pharmaceutique et chimique. Jusqu'à récemment, ce type de composants étaient presque exclusivement produits par des moyens pétrochimiques (Zacharof et Lovitt, 2013) et ont une large gamme d'applications, ce qui en fait un groupe clé qui représente la majorité des composés de la liste originale du DoE (Werpy et Petersen, 2004). Toutefois, des efforts supplémentaires sont encore nécessaires pour produire ces composés au niveau industriel.

L'industrie des plastiques est un secteur qui a suscité de remarquables préoccupations environnementales au cours des dernières décennies. La grande versatilité et les propriétés pratiques de ces polymères synthétiques en ont fait des matériaux indispensables utilisés par pratiquement toutes les industries du monde. Cependant, 90 % de ces produits ne sont utilisés qu'une seule fois et sont ensuite mis au rebut, étant une source de pollution inquiétante qui peut prendre des centaines d'années à se dégrader ("L'avenir du plastique", 2018). La production de polymères biosourcés pourrait être une mesure clé pour atténuer ce problème. L'acide polylactique (PLA), qui est le composé le plus largement utilisé pour la fabrication de polymères grâce à ses propriétés uniques telles que la facilité de traitement, la biocompatibilité et la biodégradabilité, est un exemple de réussite (Luckachan et Pillai, 2011). Des exemples comme celui-ci ont motivé les chercheurs à explorer d'autres molécules plateforme, afin de produire des polymères biosourcés avec des propriétés diversifiées.

L'acide 3-hydroxypropionique (3-HP) est une molécule plateforme de la liste originale du DoE (Werpy et Petersen, 2004) qui a attiré l'attention de l'industrie chimique. C'est un isomère positionnel de l'acide lactique (2-HP) et il peut également être utilisé pour la fabrication de polymères biosourcés (Sabet-Azad, 2015), bien qu'à l'heure actuelle, sa ligne de valorisation la plus intéressante soit la production d'acide acrylique biosourcé. Sa production par voie microbiologique a fait des progrès remarquables ces dernières années,

mais sa commercialisation industrielle est encore limitée par les faibles productivités dues à l'inhibition par produit final (Gopal Ramakrishnan *et al.*, 2015 ; Vidra et Németh, 2017 ; Zhao *et al.*, 2015). Plusieurs microorganismes capables de produire du 3-HP ont été découverts et étudiés. Une meilleure compréhension de leurs voies métaboliques a permis d'obtenir des informations précieuses pour la conception de procédés de production de 3-HP et le développement d'agents microbiens plus efficaces par le biais du génie génétique (de Fouchécour, 2019). De meilleures performances de production des 3-HP sont atteintes dans le temps, se rapprochant de celles nécessaires à la commercialisation à l'échelle industrielle.

La récupération et la purification des produits est un aspect très important à considérer pour la commercialisation d'un produit chimique. La contribution des procédés en aval au coût total du produit final se situe entre 20 et 60% (Hoppe *et al.*, 2015 ; Straathof, 2011). De plus, la récupération du 3-HP de milieux de bioconversion à haute concentration et pureté est particulièrement difficile. Cet acide a une nature hautement hydrophile, a tendance à former des oligomères à des concentrations élevées et se décompose à des températures élevées (Hoppe *et al.*, 2015). Ces caractéristiques font que les procédés traditionnels de récupération soient moins efficaces et plus coûteux que pour d'autres acides carboxyliques commercialisés industriellement, comme l'acide lactique et l'acide citrique. En plus, le problème de l'inhibition du produit final doit être atténué et une approche prometteuse est l'élimination continue de l'acide du milieu de bioconversion, également connue sous le nom de récupération *in situ* du produit (ISPR) (López-Garzón et Straathof, 2014). Parmi les différentes méthodes d'ISPR, l'extraction liquide-liquide réactive a été largement étudiée pour la récupération d'acides carboxyliques de milieux aqueux. Une phase organique qui contient une molécule capable de réagir avec l'acide est utilisée pour former un complexe acide-amine soluble dans la phase organique (Tamada *et al.*, 1990).En outre, des dispositifs appelés contacteurs à membrane à fibres creuses (HFMC) peuvent être utilisés pour mettre les deux phases liquides en contact, en évitant leur dispersion mutuelle. L'extraction et la désextraction simultanées de l'acide peuvent être réalisées en utilisant deux modules HFMC : les deux modules sont alimentés avec la même phase organique qui est mise en contact avec l'acide à extraire et avec une phase aqueuse de stripping en même temps, ce qui permet une élimination continue de l'acide, une régénération de la phase organique et d'obtenir une seconde phase aqueuse enrichie avec l'acide récupéré (Gabelman et Hwang, 1999). Cette configuration est également connue sous le nom de pertraction réactive (Schlosser *et al.*, 2005). Malgré ses nombreux avantages, cette stratégie n'a pas encore été mise en œuvre avec succès pour l'ISPR du 3-HP. Cependant, elle a été mise en œuvre pour son isomère positionnel, l'acide lactique (acide 2-hydroxypropionique) (Chen et Lee, 1997).

Dans ce cadre, l'unité mixte de recherche Microbiologie et génie des procédés alimentaires (UMR 782 GMPA, AgroParisTech, INRA, Thiverval-Grignon, France), s'est intéressée aux 3-HP. En 2012, un projet a débuté en collaboration avec l'unité de recherche et développement Agro-biotechnologies Industrielles (URD ABI, AgroParisTech, Pomacle, France) pour développer un procédé intégré de production microbienne 3-HP et de récupération simultanée à partir du milieu de bioconversion. Des avancées significatives ont été réalisées, à partir de la bioconversion du glycérol en 3-HP par la souche

Lactobacillus reuteri DSM 17938 (Burgé *et al.*, 2015b), au niveau du bioréacteur. Une approche ISPR consistant en une extraction réactive assistée par un contacteur à membrane à fibres creuses (HFMC) a été développée, qui permet une élimination sélective et continue du 3-HP d'une phase aqueuse modèle (Burgé *et al.*, 2016 ; Moussa *et al.*, 2016). L'équilibre chimique et les mécanismes de transfert de masse pendant l'extraction réactive des 3-HP ont été méticuleusement étudiés, ce qui a permis d'obtenir un modèle mathématique prédictif qui pourrait être utilisé comme outil d'optimisation (Chemarin, 2017 ; Chemarin *et al.*, 2017b, 2017a, 2019b). Cependant, les premières tentatives de couplage de la bioconversion avec l'extraction réactive ont révélé quelques difficultés à surmonter pour démontrer la faisabilité du procédé intégré :

i) la phase organique utilisée pour l'extraction réactive présentait une toxicité élevée vis-à-vis de *L. reuteri* DSM 17938.

ii) le 1,3-propanediol (1,3-PDO) est un produit secondaire obligatoire de la bioconversion du glycérol, qui limite le rendement final à un maximum de 0,5.

iii) le pH optimal de la bioconversion est supérieur au pKa du 3-HP (4,51 (Haynes *et al.*, 2017)), ce qui limite les performances d'extraction.

En plus, une meilleure compréhension des mécanismes physico-chimiques de l'extraction réactive des 3-HP des milieux de bioconversion est nécessaire pour améliorer le modèle mathématique développé précédemment et l'utiliser comme outil de simulation pour explorer d'autres stratégies d'optimisation du procédé intégré.

En 2016, trois thèses de doctorat ont été lancées pour poursuivre le développement d'un procédé intégré pour la production de 3-HP biosourcé. Un tel procédé peut intégrer trois opérations unitaires différentes : i) la bioconversion du glycérol en 3-HP et 1,3-PDO par L. reuteri DSM 17938, ii) l'oxydation du 1,3-PDO en 3-HP par la souche de la bactérie acétique Acetobacter sp. CIP 58.66, et iii) la récupération en continu de 3-HP du milieu de bioconversion par extraction réactive assistée par un HFMC. Le présent travail de doctorat se concentre sur ce dernier défi.

L'objectif général est de démontrer la faisabilité d'un procédé intégré comprenant l'extraction réactive du 3-HP assistée par des contacteurs membranaires, qui pourrait être directement couplé à la bioconversion et permettre de retirer le 3-HP en continu. À cette fin, les objectifs spécifiques suivants ont été définis :

- ✓ Effectuer une analyse basée sur un modèle des mécanismes d'extraction réactive du 3-HP à l'interface liquide-liquide, par l'observation de la tension interfaciale dynamique pendant la formation du complexe acide-base et le transfert de matière.
- ✓ Optimiser la composition de la phase organique pour l'extraction réactive du 3-HP, en termes de performance d'extraction et de biocompatibilité avec la souche productrice du 3-HP *Lactobacillus reuteri* DSM 17938.
- ✓ Évaluer la faisabilité expérimentale de la bioconversion extractive avec deux souches productrices de 3-HP différentes : *Lactobacillus reuteri* DSM 17938 et *Acetobacter* sp. CIP 58.66, en utilisant la pertraction réactive pour l'ISPR.

- ✓ Identifier les mécanismes limitant la mise en œuvre de la bioconversion extractive en utilisant la pertraction réactive, et explorer d'autres stratégies d'optimisation en utilisant un modèle mathématique prédictif comme outil de simulation.

Ce manuscrit est divisé en 5 chapitres. Le **Chapitre 1** commence par une revue de la littérature qui décrit la production et la récupération de certains acides carboxyliques biosourcés qui sont produits à grande échelle dans l'industrie chimique. La section suivante souligne le potentiel du 3-HP en tant que molécule plateforme prometteuse. Les avancées de la production microbienne de 3-HP sont également présentées dans ce chapitre, la discussion se concentrant sur les défis à relever pour parvenir à une production à l'échelle industrielle. Les procédés en aval sont également décrits comme une étape clé pour une production économiquement viable et la pertraction réactive est mise en évidence comme une technique prometteuse pour la récupération du 3-HP de milieux de bioconversion. Le chapitre se termine par quelques exemples de fermentations extractives réussies d'acides carboxyliques et les stratégies qui pourraient être appliquées dans le cas du 3-HP. Ensuite, une description de l'approche expérimentale globale est donnée au **Chapitre 2**. La méthodologie suivie pour atteindre les différents objectifs spécifiques et les résultats obtenus sont présentés dans les chapitres 3, 4 et 5. Tout d'abord, le **Chapitre 3** décrit l'analyse basée sur un modèle des mécanismes d'extraction réactive du 3-HP par l'observation de la tension interfaciale dynamique. Les connaissances obtenues sur le transfert de masse à proximité de l'interface sont présentées. Le **Chapitre 4** illustre la stratégie suivie pour la sélection d'une composition d'une phase organique qui présente un compromis entre une bonne performance d'extraction et la biocompatibilité vis-à-vis avec la souche *Lactobacillus reuteri* DSM 17938. Des aperçus obtenus sur l'impact de l'extraction réactive assistée par un HFMC sur l'état physiologique et la capacité de bioconversion des cellules sont également détaillés. La démonstration expérimentale de la bioconversion extractive avec les souches *L. reuteri* DSM 17938 et *Acetobacter* sp. CIP 58.66, en utilisant la phase organique sélectionnée, est décrite au **Chapitre 5**. Ce chapitre se termine par l'identification des mécanismes de limitation du processus et une exploration préliminaire des stratégies d'optimisation ultérieures. À cette fin, un modèle mathématique prédictif a été utilisé comme outil de simulation. Enfin, une conclusion générale intègre les principaux résultats et remarques obtenus dans le cadre de ce travail de thèse, ainsi que les perspectives de recherches ultérieures.

La production d'acides carboxyliques par des procédés microbiens, et en particulier de l'acide 3-hydroxypropionique (3-HP), a fait des progrès remarquables, atteignant des performances de production qui se rapprochent de celles nécessaires pour que le procédé soit économiquement viable. Les meilleurs titres (entre 72 et 102 g L^{-1}) et productivités (jusqu'à 0,86 g L^{-1} h^{-1}) ont été obtenus par des souches bactériennes génétiquement modifiées, c'est-à-dire *Klebsiella pneumoniae* et *E. coli* (Chu *et al.*, 2015 ; Zhao *et al.*, 2019), qui présentent une faible tolérance à un pH très acide (inférieur à pH 5, voire 6). En outre, la toxicité inhérente du 3-HP à certaines concentrations fait que la plupart des processus de bioproduction sont soumis à l'inhibition du produit. L'utilisation de souches de levures génétiquement modifiées est probablement l'approche la plus prometteuse qui puisse résoudre ces problèmes. Cependant, la plupart de leurs performances de production

sont actuellement inférieures à celles des souches bactériennes. Un seul procédé de production utilisant une levure modifiée avec des titres aussi élevés que ceux obtenus par les souches bactériennes (80 - 100 g L^{-1}) a été signalé et il a été réalisé par Cargill, Inc. Cependant, l'utilisation de souches génétiquement modifiées présente des restrictions telles que la stabilité inconnue du gène introduit et qui sont soumises à des réglementations strictes. Plus récemment, les bactéries acétiques (AAB) sont entrées en scène dans la production de 3-HP. Des souches comme *Gluconobacter oxydans* et *Acetobacter* sp. ont été utilisées pour oxyder le 1,3-propanediol (1,3-PDO) en 3-HP avec des résultats encourageants (titres de 60 - 67 g L^{-1} et productivités de 0,9 - 2,5 g L^{-1} h^{-1}) (Li *et al.*, 2016a ; Zhao *et al.*, 2015), sans utiliser de souches génétiquement modifiées. En fait, le 1,3-PDO peut être produit à partir du glucose ou du glycérol par plusieurs microorganismes et être ensuite oxydé en 3-HP. En plus, ces souches sont plus tolérantes au pH acide que les autres souches bactériennes utilisées pour la production de 3-HP, ce qui en fait une option intéressante pour une production de 3-HP économiquement viable. En fait, cette approche a été récemment explorée dans notre laboratoire, ce qui a permis d'obtenir la meilleure performance d'oxydation de 1,3-PDO en 3-HP trouvée dans la littérature (un titre de 70 g L^{-1} et une productivité de 2,8 g L^{-1} h^{-1}) (de Fouchécour, 2019).

Une autre question clé dans la bioproduction de composés chimiques est leur récupération à partir du milieu de fermentation ou de bioconversion. La récupération du 3-HP de milieux aqueux est particulièrement difficile en raison de leur nature hydrophile et de leur tendance à former des oligomères à des concentrations élevées et à se décomposer à des températures élevées. L'extraction réactive assistée par un contacteur à membrane à fibres creuses (HFMC) a été évaluée pour la récupération des 3-HP dans notre laboratoire et a permis de déterminer qu'elle était compatible avec les caractéristiques de cet acide organique. De plus, cette méthode de séparation pourrait être intégrée à la bioconversion des 3-HP pour leur élimination continue du milieu de bioconversion. Un tel procédé intégré semble intéressant en raison de ses avantages :

i) La récupération sélective et continue du 3-HP peut atténuer l'inhibition du produit et améliorer le rendement global du procédé,

ii) le pH de la bioconversion peut être régulé sans ajout de solutions de base, ce qui représente une réduction des consommables du procédé et de la production ultérieure de déchets,

iii) une solution de 3-HP plus purifiée et plus concentrée (par rapport au milieu de bioconversion) peut être obtenue en une seule étape,

iv) le nombre d'étapes après la bioconversion pour une concentration et une purification supplémentaires est considérablement réduit.

Les mécanismes de l'extraction réactive 3-HP réalisée dans le HFMC ont été étudiés dans des travaux précédents. Les amines tertiaires et les alcools à longue chaîne se sont avérés être les meilleurs agents d'extraction pour le 3-HP. L'amine tertiaire réagit avec les 3-HP à l'interface liquide-liquide, formant un complexe acide-base qui est solubilisé dans la phase organique à l'aide de l'alcool à longue chaîne par interaction avec le complexe par

l'intermédiaire de liaisons H. Le mélange de 20 % v/v de tri-octylamine (TOA) et de n-décanol a été initialement considéré comme un bon candidat pour l'extraction du 3-HP, compte tenu essentiellement des performances d'extraction, et utilisé comme référence pour étudier les mécanismes physico-chimiques de l'extraction réactive du 3-HP. Les informations générées ont été utilisées pour développer un modèle mathématique capable de prédire avec une bonne précision l'extraction 3-HP dans un HFMC dans différentes conditions expérimentales. Ce modèle mathématique tient compte de l'équilibre chimique et de la dynamique du transfert de matière des espèces dans le système d'extraction. Au chapitre 3, le modèle a été modifié pour tenir compte du changement de tension interfaciale dynamique pendant l'extraction du 3-HP. Les résultats ont confirmé que le complexe acide-amine a des propriétés tensioactives. Une analyse plus poussée a révélé que le transfert de matière près de l'interface n'est pas régi uniquement par la diffusion. La convection locale doit être prise en compte pour mieux décrire le mécanisme de transfert de matière local. Cependant, des recherches supplémentaires sont nécessaires afin d'identifier la nature de ce phénomène. On a suspecté une convection induite par la concentration, qui peut être causée par des gradients de densité et les forces de flottabilité associées ou par des gradients de tension interfaciale et la convection de Marangoni associée. Des configurations expérimentales supplémentaires devraient également être explorées afin d'observer l'effet d'autres composés tensioactifs qui pourraient être trouvés dans le milieu de bioconversion. Par exemple, les protéines et les anions provenant des sels pourraient contribuer à une diminution supplémentaire de la tension interfaciale ou à des phénomènes de convection naturelle plus forts. En conséquence, la stabilisation de l'interface à l'intérieur des pores de la membrane du HFMC pourrait devenir plus difficile et des émulsions pourraient se former.

Bien que la phase organique constituée de 20% v/v TOA dans le n-décanol ait présenté une bonne performance d'extraction dans le HFMC, sa toxicité inhérente a fortement inhibé la bioconversion du 3-HP avec la souche *Lactobacillus reuteri* DSM 17938. Par conséquent, une composition plus biocompatible a été recherchée au chapitre 4 par l'évaluation de différents mélanges contenant 6 alcools à longue chaîne différents (diluants actifs), 2 alcanes (diluants inertes) et 2 amines tertiaires (extractants). Comme prévu, l'ajout de diluants inertes a diminué la toxicité et la viscosité de la phase organique, mais a également réduit sa performance d'extraction. L'utilisation d'alcools à chaînes de carbone plus longues a également augmenté la biocompatibilité de la phase organique, mais a entraîné une diminution du rendement d'extraction et une augmentation de la viscosité. Il a donc fallu trouver un compromis entre les performances d'extraction (rendement d'extraction élevé et faible viscosité) et la biocompatibilité (faible toxicité du solvant). Les solutions biocompatibles qui ont montré les meilleurs rendements d'extraction étaient 20 % v/v TOA dans l'alcool oléique (18 C), et 20 % v/v TOA dans 47 % de dodécanol (12 C) et 33 % de dodécane. Cependant, le mélange de 20 % TOA dans l'alcool oléique a présenté une très forte viscosité, ce qui ralentit le transfert de matière dans la phase organique. Il est intéressant de noter que le remplacement de l'TOA par la di-dodécylméthylamine (DDMA) à la même concentration, a augmenté le rendement d'extraction de la phase organique et a maintenu une viscosité et une biocompatibilité similaires. Cela a été attribué à un moindre encombrement stérique dans la molécule de DDMA qui favorise la réaction. En

conséquence, la composition de la phase organique de 20 % v/v de DDMA, 47 % de dodécanol et 33 % de dodécane a été choisie.

La bioconversion extractive 3-HP a ensuite été mise en œuvre en utilisant la phase organique sélectionnée et deux modules HFMC identiques. Cela a permis l'extraction et la désextraction simultanées de 3-HP à l'aide d'une solution de NaOH (pertraction réactive). Les expériences de bioconversion extractive avec *L. reuteri* DSM 17938, présentées au chapitre 5, ont montré que la souche était fortement inhibée par la pertraction réactive, malgré la toxicité moindre de la phase organique. Les expériences menées dans le HFMC ont montré que *L. reuteri* DSM 17938 est particulièrement sensible aux contraintes de cisaillement causées par la recirculation à travers les fibres. De plus, l'extraction du 3-HP était très limitée lors de la bioconversion à pH 5, ce qui n'est pas optimal pour la pertraction réactive. En revanche, des études réalisées dans notre laboratoire ont révélé que la bioconversion du 1,3-PDO en 3-HP par *Acetobacter* sp. CIP 58.66 était soumise à une inhibition du produit, tout en étant peu affectée par des valeurs de pH légèrement inférieures au pKa du 3-HP (4.51). Cela a fait de cette souche un candidat intéressant pour la bioconversion extractive avec pertraction réactive.

La démonstration expérimentale présentée au chapitre 5 a révélé que la pertraction réactive n'a pas eu d'effet significatif sur la performance de production du 3-HP d'*Acetobacter* sp. CIP 58.66, validant ainsi sa biocompatibilité. De plus, le 3-HP a été presque entièrement éliminé du milieu de bioconversion et entièrement récupéré dans la solution de NaOH utilisée pour la rétro-extraction. Cependant, un déséquilibre entre le taux d'extraction et le taux de production est resté pendant la bioconversion. Le taux d'extraction était inférieur au taux de production, ce qui a entraîné une accumulation de 3-HP dans le milieu de bioconversion et une diminution du pH de 4,5 à 4. L'élimination complète du 3-HP n'a été obtenue que plus de 110 heures après l'arrêt de la bioconversion en raison de l'épuisement du 1,3-PDO. Une optimisation supplémentaire du processus intégré est nécessaire afin d'équilibrer les taux d'extraction et de production. L'exploration de certaines stratégies d'optimisation, à l'aide de la simulation, suggère que l'approche la plus prometteuse est d'augmenter l’aire interfaciale spécifique disponible pour le transfert de matière entre le milieu de bioconversion et la phase organique. Cela peut être vérifié soit en diminuant le volume de travail dans le bioréacteur, soit en ajoutant des modules HFMC supplémentaires. Avec une zone interfaciale spécifique plus élevée, il sera possible d'évaluer l'effet du retrait du 3-HP du milieu de bioconversion et de déterminer si la pertraction réactive peut améliorer la performance globale de production de 3-HP. Un approvisionnement continu en 1,3-PDO doit également être testé pour éviter une accumulation excessive de produit. Le contrôle du taux d'alimentation en 1,3-PDO pourrait être basé sur les variations du pH ou de l'absorption d'oxygène.

Bien qu'*Acetobacter* sp. CIP 58.66 se soit révélé être un bon candidat pour la bioconversion extractive, la viabilité économique d'un procédé intégré de production de 3-HP d'origine biologique dépend de nombreux autres aspects. Obtenir une valeur ajoutée du 1,3-PDO par sa conversion en 3-HP n'est pas chose aisée, car le 1,3-PDO est lui-même une molécule plateforme prometteuse. Cette stratégie serait avantageuse dans les cas où le 1,3-PDO et le

3-HP sont présents dans le milieu de bioconversion à des concentrations importantes après une première étape de bioconversion. C'est le cas d'une bioconversion du glycérol par des souches de *Lactobacillus reuteri* ou *Klebsiella pneumoniae*. En fait, la conversion du 1,3-PDO en 3-HP pourrait présenter un avantage économique par rapport à l'utilisation de deux procédés en aval distincts pour récupérer les deux molécules séparément. Un autre avantage est la remarquable capacité d'oxydation de l'*Acetobacter* sp. CIP 58.66. Cela indique que toute approche intégrée en amont ne sera pas limitée par cette souche.

L'immobilisation des cellules pourrait être une approche intéressante qui a déjà été mise en œuvre pour une autre souche d'*Acetobacter* sp. (Li *et al.*, 2016a). Cette méthode permet de remédier à plusieurs limitations du procédé. Par exemple, comme *Acetobacter* sp. est un aérobe strict, une soumission intermittente à des conditions anoxiques pendant sa circulation dans le HFMC pourrait affecter sa performance de production. Bien qu'aucun effet significatif n'ait été observé dans cette étude, la mise en œuvre de modules HFMC supplémentaires pour augmenter la zone interfaciale d'extraction augmentera le temps de séjour sans apport d'oxygène, ce qui pourrait limiter la mise à l'échelle du procédé. Le maintien des cellules immobilisées dans le bioréacteur permettrait non seulement d'éviter cette limitation, mais aussi de supprimer la contrainte de cisaillement induite par la circulation à travers les modules HFMC observés avec *L. reuteri* DSM 17938.

Dans cette étude, la désextraction a été effectuée uniquement par balancement chimique à l'aide d'une solution de NaOH en raison de sa simplicité et de la régénération directe de la phase organique. En plus, la quasi-totalité du 3-HP a été récupérée dans la solution de stripping par cette méthode. Toutefois, le sel de sodium 3-HP est récupéré à la place de l'acide libre, qui devra subir d'autres étapes de purification en fonction des applications ultérieures. Bien que d'autres stratégies puissent être appliquées pour la réextraction des 3-HP, telles que le diluant et l'oscillation de la température, l'approche de l'oscillation chimique est l'une des plus intéressantes pour l'approche de la pertraction réactive, en particulier pour la régénération directe et continue de la phase organique. Une approche qu'il serait intéressant de mettre en œuvre est l'utilisation d'une amine volatile soluble dans l'eau, telle que la tri-méthylamine (TMA), au lieu du NaOH. Cette méthode a été utilisée pour l'acide lactique et l'acide citrique avec de bons résultats (Jarvinen *et al.*, 2000 ; King et Tung, 1992). En fait, il est probable que cette méthode fonctionnera mieux dans le cas du 3-HP, car il est plus hydrophile que les autres acides. Cela permet de récupérer la totalité de l'acide, puis la base volatile peut être éliminée par craquage thermique à des températures d'environ 130°C, ce qui donne du 3-HP sous sa forme libre.

Il a également été démontré que la viscosité de la phase organique augmente avec la concentration en 3-HP, ce qui ralentit le transfert de matière à l'intérieur des pores de la membrane. Une tentative de remédier à cette limitation a été réalisée en testant des modules HFMC avec des membranes hydrophiles. Cette approche était basée sur l'hypothèse que la phase aqueuse remplirait les pores de la membrane au lieu de la phase organique, réduisant ainsi de manière significative la résistance du transfert de matière à l'intérieur des pores. En pratique, les matériaux de membrane qui peuvent être utilisés avec des phases organiques, par exemple le polyéthersulfone (PES), ne sont pas intrinsèquement

hydrophiles et sont soumis à un comportement de gonflement imprévisible. En fait, la phase organique peut également mouiller les pores de la membrane autant que la phase aqueuse. Cela rend la stabilisation de l'interface très difficile, voire impossible, avec notre système d'extraction réactif.

En ce qui concerne plus spécifiquement les HFMC hydrophobes, il sera intéressant d'obtenir une stabilisation interfaciale plus facile à une taille de pores plus importante et d'obtenir une porosité membranaire plus élevée. À cette fin, d'autres matériaux de membrane hydrophobe et d'autres configurations HFMC peuvent également être explorés. Par exemple, les membranes en polytétrafluoroéthylène (PTFE) sont beaucoup plus hydrophobes que le polypropylène (matériau utilisé dans ce travail). Cela pourrait conduire à une résistance membranaire plus faible pour une membrane en PTFE de plus grande taille de pore avec une bonne stabilisation interfaciale, ce qui est un aspect clé pour la mise en œuvre réussie de la pertraction réactive.

Des recherches supplémentaires sont également nécessaires concernant la modélisation du processus intégré. Le modèle dynamique de la pertraction réactive des 3-HP prend en compte les mécanismes de réaction chimique et de transfert de masse, en utilisant la composition binaire de 20 % v/v TOA et de décanol. Cependant, lors de la calibration et de la validation du modèle avec la phase organique composée de 20 % v/v DDMA, 40 % dodécanol et 40 % dodécane, les données expérimentales ont été prévues avec une précision moindre. Les divergences avec les prédictions du modèle peuvent être causées par plusieurs facteurs. Par exemple, la présence d'une quantité importante d'impuretés provenant du DDMA qui sont restées même après la purification, ou le fait que l'ajout de diluant inerte modifie la stoechiométrie du complexe formé dans la phase organique. Une étude plus minutieuse de l'équilibre chimique de la composition de la phase organique ternaire (extractant - diluant actif - diluant inerte) est nécessaire pour mieux comprendre les mécanismes de réaction. De plus, cela permettrait au modèle de prédire les résultats d'un plus large éventail de compositions organiques. Le modèle mathématique peut encore être amélioré pour prendre en compte les réactions concurrentes avec les composés du milieu de bioconversion, comme les ions des sels et la présence d'autres acides. Un modèle mathématique préliminaire pour l'oxydation du 1,3-PDO en 3-HP par *Acetobacter* sp. CIP 58.66 a été développé dans notre laboratoire. Ce modèle comprend les limitations du 1,3-PDO et du 3-HPA, ainsi que l'inhibition du 3-HP à la fois sur la bioconversion et la croissance. L'intégration de ce modèle avec celui de la pertraction réactive représentera un outil de simulation précieux pour explorer différentes stratégies d'optimisation des processus.

Les résultats obtenus de la bioconversion extractive des 3-HP par pertraction réactive sont très prometteurs et d'autres travaux d'optimisation permettront d'obtenir une élimination continue des 3-HP du milieu de bioconversion. Récemment, un travail de doctorat a été entamé dans le but d'améliorer encore le modèle mathématique prédictif et son utilisation pour l'exploration et la démonstration expérimentale de différentes stratégies d'optimisation. Cette approche intégrée peut également être appliquée à d'autres acides organiques et souches, les connaissances obtenues pouvant être étendues à ces procédés.

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ÉCOLE DOCTORALE
Agriculture, alimentation, biologie, environnement, santé (ABIES)

Titre : Bioconversion extractive d'acide 3-hydroxypropionique : mécanismes limitants et optimisation du procédé intégré

Mots clés : Extraction réactive, Modélisation, Bioconversion, Acide organique, Pertraction réactive.

Résumé : L'acide 3 hydroxypropionique (3-HP) est une molécule plateforme qui peut être produite à partir de la biomasse et peut être convertie en une large gamme de composés chimiques utilisés dans l'industrie des matériaux bio-sourcés. L'obtention de 3-HP par voie microbiologique a connu des progrès remarquables, mais sa production commerciale est encore limitée par les faibles productivités dues à la retro-inhibition des microorganismes par leurs produits et des difficultés de récupération et de purification. Couplée en ligne à la bioconversion, la pertraction réactive, est une stratégie prometteuse pour lever les phénomènes d'inhibition et intensifier la bioconversion du 3-HP. Ce travail vise à élucider certains aspects clés pour la conception et la conduite d'un tel procédé intégré. Le système d'extraction réactive a été étudié à l'échelle de l'interface liquide-liquide. Ensuite, une méthodologie complète pour la sélection d'une composition de phase organique, basée à la fois sur la performance d'extraction et la biocompatibilité avec une souche productrice de 3-HP a également été développée. Enfin, l'intégration de la pertraction réactive avec la bioconversion a été réalisée. La bioconversion extractive avec une souche d'*Acetobacter* sp. a permis d'obtenir les meilleures performances. Cependant, un déséquilibre important entre les taux de production et d'extraction a été mis en évidence. D'autres stratégies d'optimisation ont été explorées en utilisant un modèle mathématique comme outil de simulation. Les informations obtenues tout au long de ce travail ouvrent la voie à la conception d'un procédé intégré de production de 3-HP bio-sourcé intensifié.

Title : Extractive bioconversion of 3-hydroxypropionic acid: limiting mechanisms and integrated process optimisation

Keywords : Reactive extraction, Modelling, Bioconversion, Organic acid, Reactive pertraction.

Abstract : 3-hydroxypropionic acid (3-HP) is a platform molecule that can be produced from biomass and can be converted into a wide range of commercially valuable bio-based chemicals. 3-HP production by microbial processes has made remarkable advances, but its industrial commercialization is still limited by low productivities caused by product inhibition and challenging recovery and purification. Reactive pertraction coupled with bioconversion, is a promising strategy to intensify 3-HP bioconversion and reduce downstream process costs. This work aims to elucidate some key aspects for the proper design of such an integrated process. 3-HP reactive extraction was studied at the liquid-liquid interface scale. Then, a comprehensive methodology for the selection of an organic phase composition, based on both extraction performance and biocompatibility with a 3-HP producing strain was also developed. Finally, the integration of reactive pertraction with bioconversion was performed. Extractive bioconversion with an *Acetobacter* sp. strain resulted in the best performances. A significant imbalance between production and extraction rates was observed, however. Further optimisation strategies where explored using a mathematical model as a simulation tool. The information obtained throughout this work paves the route to the design of an integrated and intensified bio-based 3-HP production process.

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