Optimization of the glycerol bioconversion into 3-hydroxypropionic acid (3-HP) by Lactobacillus reuteri in bioreactor

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Résumé détaillé

Optimisation de la bioconversion du glycérol en acide 3-hydroxypropionique (3-HP) par *Lactobacillus reuteri* en bioréacteur

La mise en œuvre de microorganismes pour la production de molécules "plateformes" constitue un enjeu industriel de premier plan, dans un contexte d'accroissement de l'utilisation des ressources renouvelables en substitution aux ressources fossiles et de réduction de l'impact environnemental des productions. En particulier, l'acide 3-hydroxypropionique (3-HP) est reconnu comme une molécule plateforme d'intérêt car il est le précurseur d'une grande variété de produits chimiques impliqués dans de nombreuses applications. Sa production par voie biologique peut notamment être réalisée par la bactérie *Lactobacillus reuteri* selon un procédé en deux étapes : une phase de croissance en mode batch puis, après une étape de récolte et de concentration des cellules, une phase de bioconversion pour transformer le glycérol en 3-HP en mode fed-batch. Ce procédé permet d'activer séquentiellement les voies métaboliques d'intérêt de la bactérie. Cependant, ses performances sont limitées par l'effet inhibiteur du 3-HP et de son intermédiaire métabolique, le 3-hydroxypropionaldéhyde (3-HPA).

Dans le but d'améliorer la production de 3-HP, la thèse a pour objectifs (1) d'identifier les conditions nutritionnelles et environnementales mises en œuvre lors de la phase de croissance, capables d'améliorer la production ultérieure de la biomolécule par les bactéries, (2) de rechercher les conditions opératoires de la phase de bioconversion, permettant d'augmenter les performances de production et (3) d'évaluer certaines caractéristiques physiologiques des cellules au cours du bioprocédé pour mieux comprendre l'arrêt de la bioproduction.

Une étude bibliographique a tout d'abord permis de cerner les caractéristiques métaboliques et physiologiques de *Lactobacillus reuteri* et d'identifier les facteurs susceptibles d'affecter la bioproduction de 3-HP. Sur cette base, la démarche expérimentale retenue s'est appuyée sur deux plans d'expériences réalisés successivement. Le premier a cherché à identifier, parmi 11 conditions nutritionnelles et environnementales rencontrées par les bactéries au cours de leur croissance, celles permettant de maximiser les variables caractéristiques de la production de 3-HP (concentration, quantité, rendements, vitesses de production) au cours de la phase ultérieure de bioconversion. S'appuyant sur une sélection des meilleures conditions de croissance en vue de cette production, le second plan d'expériences a eu pour objectif de déterminer des conditions opératoires de l'étape de bioconversion (pH et vitesse spécifique d'apport de glycérol) permettant d'accroître les performances bactériennes de conversion du glycérol en 3-HP. Les méthodes employées se sont basées sur la conduite de fermentations et de bioconversions en bioréacteurs instrumentés, sur la quantification des concentrations en

substrats et métabolites produits par chromatographie liquide haute performance, sur des mesures des concentrations microbiennes, ainsi que sur le traitement statistique des résultats issus des plans d'expériences. Enfin, l'état physiologique des cellules bactériennes a été caractérisé par cytométrie en flux couplée à l'épifluorescence afin de mieux comprendre les raisons pour lesquelles la bioconversion s'arrête alors que le substrat est encore alimenté dans le bioréacteur.

Pour répondre à la première question scientifique de la thèse, les conditions nutritionnelles et environnementales, mises en œuvre pendant la phase de croissance et qui ont un impact sur la bioproduction de 3-HP par *L. reuteri* DSM 17938 lors de l'étape ultérieure de bioconversion, ont été déterminées grâce à un plan d'expériences de Plackett et Burman. Auparavant, des expériences préliminaires ont permis de déterminer les valeurs limites de température, de pH et de concentration en glucose pouvant être introduites dans le plan d'expériences, ainsi que le meilleur moment auquel les cellules doivent être récoltées en fin de croissance. Les conditions de la phase de croissance ont été définies par le plan d'expériences de Plackett et Burman alors que les conditions de la phase de bioconversion ont été fixées pour toutes les expériences (composition du milieu, vitesse d'agitation, température, pH et débit d'alimentation en glycérol). Chaque bioproduction a duré environ une semaine, au cours de laquelle des échantillons ont été prélevés et analysés. La méthode de quantification précise de la concentration en 3-HP par HPLC a été améliorée au cours de la thèse. Une analyse statistique a permis d'identifier les facteurs significativement influents sur les performances du bioprocédé et de quantifier leurs effets.

Les résultats montrent que la supplémentation du milieu de croissance avec du glucose (20 $g \cdot L^{-1}$), de la phytone peptone (25 $g \cdot L^{-1}$), du Tween 80 (4 $g \cdot L^{-1}$), du 1,2-propanediol (3 $g \cdot L^{-1}$) et de la bétaïne associée à du KCl (0,234 $g \cdot L^{-1}$ + 0,745 $g \cdot L^{-1}$), ainsi que le contrôle du pH à 6,0 avec de l'ammoniaque, améliorent la production de 3-HP (19.1 $g_{3\text{-HP}}$) et la durée de la bioconversion (88.6 h) par rapport à la condition initiale (13.4 $g_{3\text{-HP}}$, 55.8 h). En revanche, l'ajout d'extrait de levure et le type de neutralisant utilisé pour le contrôle du pH ne démontrent aucun effet significatif, tandis que l'ajout de vitamine B12 et de cystéine induit un effet négatif sur la bioproduction de 3-HP. Enfin, un effet positif d'une faible température de croissance (33 °C) est observé sur le rendement de production du 3-HP, mais il est lié à la valeur limitante du débit d'alimentation en glycérol (fixé à 0,5 $g \cdot h^{-1}$) qui ne permet pas de fournir suffisamment de glycérol à la quantité plus élevée de biomasse obtenue à l'issue d'une croissance menée à 37 °C. Une expérience de validation, réalisée dans les conditions identifiées grâce à cette étude, confirme que cet ensemble de conditions améliore la concentration en 3-HP (+ 25 %), la quantité de 3-HP produit (+ 46 %), la vitesse spécifique de production du 3-HP (+ 61 %) et son rendement de production (+ 150 %), par rapport aux conditions initiales. Si la concentration en 3-HP obtenue est supérieure à celles trouvées dans la littérature, la productivité volumique reste néanmoins inférieure aux valeurs précédemment rapportées, du fait d'une vitesse spécifique de production de 3-HP plus faible dans nos conditions. Enfin, bien que certaines performances du bioprocédé aient été augmentées par rapport à celles

obtenues dans les conditions initiales de la thèse, cette amélioration reste limitée du fait des conditions expérimentales utilisées lors de la phase de bioconversion. Sur la base de ces travaux, les étapes suivantes de l'étude ont porté sur les conditions de bioconversion dans le but de poursuivre l'amélioration du bioprocédé.

Pour répondre à la deuxième question scientifique de la thèse, les conditions pertinentes permettant d'améliorer les performances de bioproduction du 3-HP par *L. reuteri* DSM 17938 lors de l'étape de bioconversion ont été définies et optimisées. Afin de contourner la limitation de la disponibilité du substrat observée dans la première partie de l'étude et grâce à des expériences préliminaires, la vitesse spécifique d'alimentation en glycérol et le pH de bioconversion ont été identifiés comme deux facteurs clés de cette deuxième phase. Ils ont été introduits dans un plan d'expériences centré isovariant par rotation afin de définir leurs valeurs optimales, maximisant les performances de la bioproduction du 3-HP. Ce deuxième plan expérimental a bénéficié des résultats du premier, car les conditions de croissance ont été fixées, pour tous les essais, comme lors de l'expérience de validation décrite ci-dessus, à l'exception de la température fixée à 37 °C. Il a également profité des résultats de plusieurs expériences préliminaires qui ont fourni des informations sur le nombre de centrifugations nécessaire lors de la récolte des bactéries, sur la nécessité d'utiliser des cellules non congelées, sur la température lors de l'étape de bioconversion et sur la gamme de valeurs de pH pouvant être testées. Il a également été établi que la bioconversion peut être suivie en ligne en se référant à la consommation de base permettant de maintenir le pH, qui s'arrête avec la bioproduction de 3-HP, et à la variation de la pression partielle en oxygène dissous, qui augmente lorsque la bioconversion du glycérol s'arrête.

Grâce à l'établissement de surfaces de réponse, les conditions optimales de pH et de vitesse spécifique d'alimentation en glycérol ont été identifiées pour maximiser la concentration en 3-HP, la quantité finale de 3-HP et le rendement de production du 3-HP. Ils correspondent à un pH de 6,0 et à une vitesse spécifique d'alimentation en glycérol de 60 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. Cependant, les valeurs maximales de la vitesse de production de 3-HP, de la vitesse spécifique de production du 3-HP et de la productivité volumique en 3-HP ont été obtenues à une vitesse spécifique d'alimentation en glycérol plus élevée de 80 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, quelle que soit la valeur de pH entre pH 4,8 et pH 7,2. Il est à noter que la vitesse spécifique d'alimentation en glycérol optimale n'a pas pu être identifiée dans la plage de valeurs testées, car l'optimum se situe au-dessus de la valeur maximale. Néanmoins, l'accumulation du 3-HPA augmentant également avec la vitesse spécifique d'alimentation en glycérol, l'optimum est probablement proche de 80 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ pour *L. reuteri* DSM 17938. Sur la base de ces résultats, deux expériences de validation ont été réalisées pour confirmer l'amélioration de la bioproduction de 3-HP. Elles ont été conduites à pH 6,0 et à 60 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ ainsi qu'à 80 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

Les résultats expérimentaux de ces expériences de validation s'accordent bien avec les résultats prédits par les modèles. La concentration en 3-HP et la quantité finale en 3-HP sont similaires à ceux obtenus dans les conditions de référence (point central), ce qui était attendu

car le pH optimal (pH 6,0) et la vitesse spécifique d'alimentation en glycérol (60 au lieu de 50 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) sont également proches. Le rendement de production en 3-HP n'est cependant pas amélioré par rapport à l'expérience de référence. La vitesse et la vitesse spécifique de production du 3-HP sont respectivement augmentées de 80 % et 34 % pour une vitesse spécifique d'alimentation en glycérol de 80 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ par rapport au point central. La productivité volumique en 3-HP est améliorée de 39 % par rapport à ce point central. De plus, la vitesse spécifique de production de 3-HP et la productivité volumique en 3-HP sont respectivement 1,6 et 2 fois supérieures à celles obtenues dans la première partie de l'étude réalisée à un débit d'alimentation en glycérol fixé. Au global, la vitesse spécifique de production et la productivité volumique en 3-HP sont augmentées de 78 % et de 138 % par rapport aux conditions initiales utilisées au tout début de la thèse. Enfin, l'accumulation faible de 3-HPA observée pour une vitesse spécifique d'alimentation en glycérol de 80 $mg_{glycérol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ montre que cette condition est proche de l'optimum pour *L. reuteri* DSM 17938. Ainsi, la combinaison des conditions de croissance qui améliorent la capacité des bactéries à produire le 3-HP et des conditions optimisées pendant l'étape de bioconversion ont conduit à une amélioration significative des performances de bioproduction de la molécule, tout en limitant la formation de 3-HPA délétère pendant le bioprocédé.

Cependant, malgré l'amélioration obtenue des performances de production, celles-ci restent plafonnée par l'arrêt brutal de la bioconversion du glycérol en 3-HP, alors même que le substrat est continuellement apporté dans le milieu. En améliorant les conditions pendant les étapes de croissance et de bioconversion, cet arrêt a été retardé ou il s'est produit à une concentration en 3-HP plus élevée, mais il survient toujours. Afin de mieux comprendre les causes de ce phénomène, des expériences complémentaires ont été réalisées pour explorer ce qui se passe à l'intérieur des bactéries, en évaluant certaines de leurs caractéristiques physiologiques. Une première hypothèse a porté sur les effets inhibiteurs du 3-HP et du 3-HPA qui affectent la physiologie et le métabolisme de *L. reuteri*. Cependant, la concentration en 3-HPA est restée inférieure à sa concentration inhibitrice. D'autre part, le pH étant contrôlé à 6,0, la concentration de la forme non dissociée du 3-HP (de pKa égal à 4,51), qui est la plus inhibitrice, est restée faible (3 % du 3-HP produit). Une seconde hypothèse a attribué cet arrêt de la production à un manque soudain d'énergie intracellulaire. Tant que le 3-HP est produit, de l'ATP est également produit ce qui permet aux cellules bactériennes d'assurer leur maintenance en milieu pauvre. Mais lorsque la bioproduction s'arrête, la maintenance cellulaire ne peut plus être assurée. Pour valider cette hypothèse, certaines caractéristiques physiologiques des cellules ont été étudiées. En adaptant des méthodes basées sur l'utilisation de fluorochromes couplée à la cytométrie en flux, le pH intracellulaire (pHi) et le niveau d'énergie intracellulaire (DeltaIF20) ont été mesurés lors de l'étape de bioconversion de deux expériences. Il s'est avéré que ces caractéristiques physiologiques sont affectées au moment où la production de 3 HP cesse. Ainsi, après 24 h, lorsque la bioconversion est en cours et juste avant son arrêt, le pHi diminue de 0,05 unité pH et le DeltaIF20 d'une valeur de points de 3.1 %. Plus tard, après 44 h, la réduction est plus forte avec une diminution totale du pHi de 0,1

unité pH et l'obtention d'une valeur finale d'énergie intracellulaire proche de 0 %. Ces observations devront être confirmées en évaluant la reproductibilité des résultats, pour contribuer à comprendre les phénomènes physiologiques se produisant au cours du processus métabolique de la bioconversion du glycérol en 3-HP.

Finalement, cette thèse a permis de montrer que les conditions de la phase de croissance de *L. reuteri* impactent significativement les performances de la phase ultérieure de production du 3-HP. Les conditions à mettre en œuvre lors de la phase de croissance ont été identifiées et l'amélioration du procédé a été confirmée expérimentalement. Les conditions relatives à l'étape de bioconversion ont également été optimisées, ce qui a permis d'obtenir des résultats supérieurs à ceux décrits dans la littérature. L'amélioration a cependant plafonné du fait d'une dégradation brutale de l'état physiologique des bactéries, comme démontré par la diminution du pH intracellulaire et du niveau d'énergie intracellulaire.

Le travail de thèse ouvre plusieurs perspectives. Il sera tout d'abord nécessaire d'approfondir les recherches afin de mieux expliquer le lien entre la dégradation de l'état cellulaire et l'arrêt de la bioproduction du 3-HP, en s'appuyant notamment sur des analyses de l'état cellulaire, ainsi que des analyses protéomiques et transcriptomiques. Le travail sur les conditions de l'étape de bioconversion pourra également être poursuivi, en particulier au regard de la vitesse spécifique d'alimentation en glycérol et de la température. D'autres modes de conduite du bioprocédé pourront être étudiés, comme le couplage de la bioconversion avec l'extraction en ligne du 3-HP, afin de réduire l'inhibition des bactéries et de récupérer la molécule d'intérêt. Enfin, une approche par modélisation des étapes de bioconversion et d'extraction pourra être considérée.

Contents

Publication and communications

Article published in an international peer-reviewed journal

Thi-Lan-Phuong Nguyen, Claire Saulou-Bérion, Jérôme Delettre, Catherine Béal (2021). Culture conditions affect *Lactobacillus reuteri* DSM 17938 ability to perform glycerol bioconversion into 3-hydroxypropionic acid, *Journal of Bioscience and Bioengineering*, https://doi.org/10.1016/j.jbiosc.2020.12.011, available online 15th Feb 2021

Oral presentation

Thi-Lan-Phuong Nguyen, Claire Saulou-Bérion, Catherine Béal (2019). Influences of environmental factors on *Lactobacillus reuteri* growth and 3-hydroxypropionic acid production in the context of coupling with extraction. 5th European Congress of Applied Biotechnology (ECAB), Florence, Italy (15/09/2019 – 19/09/2019)

Poster presentations

Thi-Lan-Phuong Nguyen, Claire Saulou-Bérion, Catherine Béal (2019). Culture conditions during growth of *Lactobacillus reuteri* DSM 17938 influence the ability of cells to produce 3-hydroxypropionic acid by glycerol bioconversion. 22ème colloque du Club des Bactéries Lactiques, Caen, France (12/06/2019 – 14/06/2019)

Thi-Lan-Phuong Nguyen, Ana-Karen Sanchez-Castañeda, Claire Saulou-Bérion, Catherine Béal (2019). The growth conditions of *Lactobacillus reuteri* in bioreactor influence further 3-hydroxypropionic acid bioproduction. 17ème congrès SFGP, Nantes, France (15/10/2019 – 17/10/2019)

List of abbreviations

Abbreviations

BLAST	Basic local alignment search tool
BNICE	Biochemical Network Integrated Computational Explorer
CMB_B	Carbon mass balance during bioconversion
CMB_G	Carbon mass balance during growth
cF	Carboxyfluorescein
cFDA	Carboxyfluorescein diacetate
cFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CDW	Cell dry weight
CCRD	Central composite rotatable design
MRS	de Man, Rogosa and Sharpe medium
DeltaIF20	Difference of intracellular fluorescence intensity after a 20 min incubation
Dha	Dihydroxyacetone pathway
DOE	US Department of Energy
pO_2	Dissolved oxygen pressure
EMP	Embden Meyerhof Parnas pathway
FCM	Flow cytometry
FSC	Forward-angle light scatter
G.R.A.S	Generally Recognized as Safe
GMO	Genetically modified organism
GlpF	Glycerol facilitator
HPLC	High-performance liquid chromatography
pHi	Intracellular pH
MI	McIlvaine buffer
MIC	Minimum inhibitory concentration
MB_G	Molecular balance during growth
NCBI	National Center for Biotechnology Information
OD	Optical density
PEP	Phosphoenolpyruvate
PK	Phosphoketolase pathway
Pdu	Propanediol utilization pathway

PI	Propidium iodide
Q.P.S.	Qualified Presumption of Safety
RI	Refractive index
SSC	Side-angle light scatter

Molecules

1,3-PDO	1,3-Propanediol
3-HP	3-Hydroxypropionic acid
3-HPA	3-Hydroxypropionaldehyde
AA	Acetic acid
CO_2	Carbon dioxide
EtOH	Ethanol
LA	Lactic acid

Microorganisms

C. testosteroni	*Comamonas testosteroni*
E. coli	*Escherichia coli*
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
L. buchneri	*Lactobacillus buchneri*
L. casei	*Lactobacillus casei*
L. helveticus	*Lactobacillus helveticus*
L. plantarum	*Lactobacillus plantarum*
L. reuteri	*Lactobacillus reuteri*
L. delbrueckii subsp. *bulgaricus*	*Lactobacillus. delbrueckii* subsp. *bulgaricus*
S. cerevisiae	*Saccharomyces cerevisiae*

Genes coding for enzymes

dhaB	Glycerol dehydratase
Kgsadh	α-Ketoglutaric semialdehyde dehydrogenase
gabd4	Aldehyde dehydrogenase
dhaT	Glycerol dehydratase

ldhA	Lactate dehydrogenase
Pta	Phosphate acetyltransferase
pduP	Propionaldehyde dehydrogenase
pduCDE	B12-dependent Glycerol dehydratase
pduL	Phosphotransacylase
pduW	Propionate kinase
pduQ	1,3-Propanediol oxidoreductase
glpF	Glycerol facilitator
LreuNox	NADH oxidase

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Introduction

The societal demand for products coming from renewable resources combined to the climate change issues and the medium-term depletion of fossil fuel reserves are strong drivers for the development of bioeconomy. The transition from the actual petroleum-based economy to a biomass-based economy requires new strategies and researches, in order to transform molecules derived from biomass into a wide variety of chemicals and "building-block" molecules displaying higher-value potential. These building-block molecules, also called "platform" molecules display a chemical structure allowing them to be further transformed for generating many chemical derivatives that display new properties and functionalities. To become competitive compared to fossil-based chemicals, the bio-based resources have to be available in sufficient amount without creating competition with other applications (notably food and feed), the production scale shall meet the needs of the society and the production cost shall be comparable to that of current products (Bomtempo *et al.*, 2017).

In that context, the research about renewable and sustainable platform molecules is of crucial importance, in order to forecast their future industrial production. This was promoted by the US department of Energy (DOE, 2010) that published a list of 10 "building-block" molecules of interest (Bozell and Petersen, 2010). Among them, the bifunctional 3-hydroxypropionic acid (3-HP) is the precursor of various chemicals, such as acrylic acid or biobased polymers.

The biotechnological production of 3-HP from biomass is an alternative to the chemical production that meets the requirements of this transition towards a green economy. Being performed by combining an upstream process that involves microorganisms (and/or enzymes) and downstream processes to separate and purify the biomolecule, these biotechnological approaches are cleaner, less energy consuming and generate less greenhouse gas (Tang and Zhao, 2009). However, the biotechnological production of 3-HP is not yet industrialized as a lot of barriers hampers its development. The biosynthesis of this molecule, which is a position isomer of the well-known and already commercialized lactic acid, requires specific metabolic pathways that lead to low yields and productivity, as a consequence of the production of co-products together with 3-HP and of low rates of metabolic fluxes (Dishisha *et al.*, 2014). In addition, this molecule acts as an inhibitor of its own production and one metabolic intermediate of the pathway is highly toxic. To improve the performances of the bioprocesses for 3-HP production, research is ongoing according to two main approaches. The development of new strains, either by modifying natural producers or by creating new strains as "cell factories" is under progress and many research labs are now working on these approaches. As the second approach, the improvement of the process is mainly investigated by searching efficient and low-cost bio-based substrates and by

developing integrated processes that combine the bioproduction with downstream processes in order to reduce the inhibitory effect during the production.

By considering natural producers, the bacterium *Lactobacillus reuteri* is of great interest. First, it possesses the G.R.A.S. (FDA, 2008) and Q.P.S status (EFSA, 2009), which allows to use it as a probiotic without any safety issue. It is able to produce 3-HP from glycerol that is an available substrate derived from biodiesel manufacturing industry. It is also anaerobic or microaerobic that helps reducing the production costs due to agitation and aeration (Kandler *et al.*, 1980). Within a whole-biocatalyst approach, the production of 3-HP by *L. reuteri* requires two successive steps, in order to sequentially activate the relevant metabolic pathways: a first step of bacterial growth on glucose (or other catabolic substrates) in batch mode, then, after harvesting and eventually concentrating the cells, a step of glycerol bioconversion into 3-HP performed in fed-batch mode with resting cells. The performances of this biotechnological process have been studied by only one author that focused on few parameters. These performances have to combine simultaneously high 3-HP titers (or quantities) to facilitate the further separation processes, high yields to efficiently convert the substrate and avoid co-products, together with high productivities to achieve high efficiency of the process. The main levers to improve the bioprocess consist in the determination of optimized conditions to favor firstly, the growth of the microorganism with the aim of improving the subsequent bioconversion and secondly, the bioconversion of glycerol into 3-HP as such. By considering the growth conditions, most of the published works focused on the improvement of the *L. reuteri* growth to use it as a probiotic without aiming at enhancing its bioconversion performance. By considering the bioconversion conditions, some environmental factors were already studied but not optimized.

In that context, the objective of the thesis was to define the conditions that affect the 3-HP bioproduction in order to increase the bioprocess performance. This work aimed at answering three research questions:

1. **What are the nutritional and environmental conditions during the growth phase that affect the 3-HP bioproduction during the further bioconversion step?**
2. **What are the relevant conditions during the bioconversion step that may improve its performance?**
3. **What are the possible effectors at the intracellular level that drive the bioconversion process?**

To answer these questions, the thesis is structured in four main chapters. In the first chapter, a literature review presents the biomolecule 3-HP, the biotechnological processes for its production, with a focus on *L. reuteri* as a natural producer. The factors that affect the 3-HP bioproduction are reviewed, by successively considering the growth phase and the bioconversion phase.

The second chapter describes the materials and methods used for the preparation and operation of the bacterial cultures in bioreactors, the experimental designs and the analytical and statistical methods.

The third chapter is devoted to the study of the effect of 11 nutritional and environmental factors encountered during the growth phase on the growth and the subsequent bioconversion performances, using an experimental design. The corresponding results have been published in the *Journal of Bioscience and Bioengineering* (manuscript accepted).

In the fourth chapter, the effect of two environmental conditions, namely pH and specific glycerol feeding rate during the bioconversion in fed-batch mode, were established in order to optimize the 3-HP bioproduction. In addition, some physiological characteristics of the cells were studied, namely intracellular pH and energy level, in order to understand why the bioconversion process ceased.

Finally, the general conclusions of the thesis are presented and some prospects for future works are proposed.

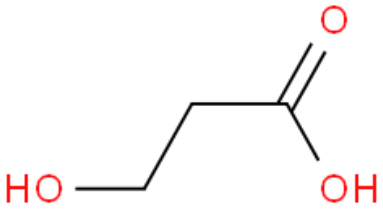
Chapter 1. Bibliographic study

1.1 Biobased 3-hydroxypropionic acid: a building block molecule for versatile industrial applications

1.1.1 Definition and physic-chemical properties of 3-HP

3-hydroxypropanoic acid (3-HP) is a three-carbon monocarboxylic acid in which one of the hydrogens linked to the terminal carbon is replaced by a hydroxyl group (Figure 1.1). Chemically, 3-HP is an optically inactive (non-chiral) organic compound and a structural isomer of lactic acid (2-hydroxypropanoic acid). This molecule is also known as hydroacrylic acid or ethylene lactic acid (Kumar *et al.*, 2013).

The bifunctionality of 3-HP, owing to the presence of a carboxyl group and a β-hydroxyl group, makes it become a versatile agent for several reactions such as dehydration, oxidation, reduction, or cyclization (Tingirikari *et al.*, 2016). Regarding its physical properties, it is an acidic viscous liquid with a pKa of 4.51. 3-HP is soluble in water and many oxygenated organic solvents like ethanol and it is miscible with diethyl ether (Wishart *et al.*, 2018). This property is an advantage in the context of its extraction using organic solvents (Burgé *et al.*, 2017).



Chemical formula	$C_3H_6O_3$
Molecular mass	90.078 g·mol^{-1}
pKa	4.51

Figure 1.1. Chemical structure of 3-hydroxypropionic acid

1.1.2 The increasing interest of biobased 3-HP and its applications

In the framework of growing bioeconomy, 3-HP is gaining more and more interest due to its applications. Firstly, 3-HP can be employed as a preservative in food or feed industry (Gokarn Ravi *et al.*, 2014). Secondly, as a platform chemical, 3-HP can be converted to a great number of derivatives through chemical modifications (Bomtempo *et al.*, 2017) such as acrylic acid, 1,3-propanediol (1,3-PDO), 3-hydroxypropionaldehyde (3-HPA), malonic acid, or biodegradable polymers and polyesters (Matsakas *et al.*, 2014). As most of these compounds derive from petrochemical resources at the moment, the bioproduction of 3-HP could help diminishing the use of oil reserves that will no longer fulfill the increasing demand of modern

society in the near future. The economic interest of biobased 3-HP is confirmed since it has been classified as one of the top value-added chemical produced from biomass by the US Department of Energy (DOE) (Bozell and Petersen, 2010) (Figure 1.2).

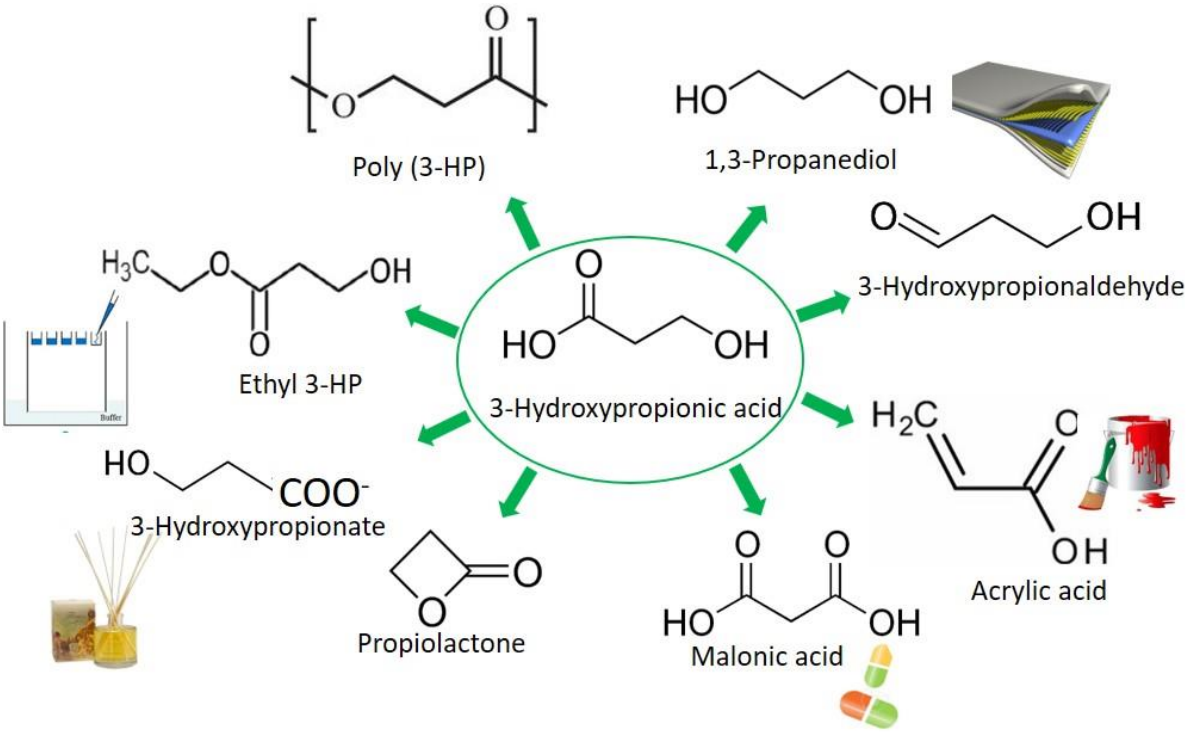


Figure 1.2. Scheme of 3-HP applications as a platform molecule

The reduction of 3-HP leads to 1,3-propanediol (1,3-PDO) that can be utilized as a solvent or for the production of polymers to form a variety of industrial products including composites, laminates, adhesives, coatings, moldings, cosmetics, polyesters, and polytrimethylene terephthalates used in carpets and textiles (Saxena *et al.*, 2009). The market size of 1,3-PDO is evaluated at 490 million USD in 2019 and forecasted to reach 870 million USD in 2024 (MarketandMarkets data) (Sittijunda and Reungsang, 2020).

3-HP can also be reduced into 3-HPA that is useful in food and feed additives, where it can be employed as a preservative (Jiang *et al.*, 2009). In combination with HPA-hydrate and HPA-dimer, it forms a multi-component system (called "HPA system") that can serve as a precursor for interesting chemicals such as acrylic acid, acrolein and 1,3-PDO, and for polymer production (Vollenweider and Lacroix, 2004). The global market of this aldehyde has been estimated up to 3.63 million tons per year (Neto *et al.*, 2017).

Dehydration of 3-HP results directly in the formation of acrylic acid that is used for the manufacturing of paints, papers, textiles, adhesives, specialty coatings, inks and super-absorbent polyacrylates (Matsakas *et al.*, 2014). The global acrylic acid market size was estimated up to 19 billion USD in 2022 (Allied-Market-Research, 2020). In addition, the esterification of acrylic acid with methanol under acid catalysis leads to methyl acrylate that

is a raw material for the production of acrylic fibers, adhesives, molding resins, coatings and emulsions (Ajekwene, 2020).

In another application, oxidation of 3-HP leads to the formation of malonic acid that can be used as a natural preservative to control acidity in food products, as an excipient in pharmaceutical industry. Malonic acid is also used for the production of surgical adhesives or to cross-link potato and corn starches to produce biodegradable thermoplastics (Anil and Trina, 2014).

3-HP, when cyclized, forms propiolactone that is a four-membered ring compound. It is commonly used as a disinfectant for the sterilization of different kinds of materials in hospital and as a vapor-phase disinfectant in enclosed chambers. β-propiolactone is also applied to inactivate viruses in the production of vaccines for animals and humans (Scheidler *et al.*, 1998).

The esterification of 3-HP creates 3-hydroxypropionate esters that can be used as fragrance ingredients (Pazicky *et al.*, 2018).

The primary alcohol of 3-HP (ethyl 3-HP) is involved in the renewable acrylonitrile production via dehydration and nitrilation with ammonia, using titanium dioxide as a catalyst (Karp *et al.*, 2017). Ethyl 3-HP can be used for the synthesis of acrylics, resins, carbon fibers and polymers (Karp *et al.*, 2017). Moreover, acrylonitrile can be hydrolyzed to form acrylamide that is engaged in the manufacture of polyacrylamide, which is used as a thickening agent and in water treatment (Kurenkov *et al.*, 2002).

The polymerization of 3-HP leads to the synthesis of a valuable homopolymer (poly 3-HP) or of copolymers (containing 3-HP) (Andreessen *et al.*, 2014). These 3-HP polymers are very flexible and ductile. Hence, they could be used for thermoforming and blow molding as well as for breathable cast films, coatings, and lamination. They act as plasticizers to soften brittle materials. Owing to its biodegradability and biocompatibility properties, poly 3-HP can be applied in pharmaceutical industry to manufacture slow-release drug capsules (Andreessen *et al.*, 2014).

1.1.3 Summary of information

As a building block molecule, 3-HP can be involved into a great number of chemical reactions including reduction, oxidation, dehydrogenation, cyclization, esterification, alcoholization or polymerization to form 1,3-PDO, acrylic acid, malonic acid, 3-HPA, propiolactone, 3-hydroxypropionate esters and ethyl 3-HP, respectively. These derivatives show a great interest for versatile applications, intended for various sectors of human's life.

1.2 Bioproduction of 3-HP by using microorganisms

1.2.1 Biotechnological production of 3-HP: a challenge in the context of bioeconomy

Various chemical synthesis routes are proposed to synthesize 3-HP from acrylic acid, 3-propiolactone, 3-hydroxypropinitrile, allyl alcohol, vinyl acetate or 1,3-propanediol (Matsumoto *et al.*, 2017) and poly 3-HP (Andreessen *et al.*, 2014). However, none of them are promising due to expensive starting materials, high costs for the operating processes and environmental issues (Tingirikari *et al.*, 2016). Moreover, there is no real market for fossil-based 3-HP as its derivatives can be directly obtained from petroleum resources.

Due to the issues of climate change, there is a growing pressure to reduce the use of non-renewable resources and a real urgency to make our production and consumption models more sustainable. The shift towards the use of renewable biomass instead of fossil-based resources can help to reduce waste, environmental contamination and climate change. This shift implies a whole set of changes in production as well as in industrial and economical processes and is recognized as bioeconomy (Scarlat *et al.*, 2015).

Within such context, bioproduction of 3-HP has been intensively studied, by using either naturally producing microorganisms or genetically modified strains, in order to reach high titers, yields and productivities at industrial scale.

Among the renewable resources used for 3-HP bioproduction, glucose and glycerol are the most-studied raw materials owing to their abundance and competitive price (Reddy *et al.*, 2019). The next paragraphs will focus on these biotechnological processes, by using these renewable substrates.

1.2.2 Bioproduction of 3-HP from glucose

Glucose is known as one of the main carbon sources for the cultivation of microorganisms, and can also be used as a renewable material to produce 3-HP. The glucose used at industrial scale is commonly obtained from plants, including corn (Jabasingh and Wolde, 2019), wheat (Salim *et al.*, 2019) and potatoes (Tunde, 2020) that contain high levels of starch that has to be hydrolyzed before being used by microorganisms. Glucose syrups can also be obtained from rice, barley, beet and cassava (Hull, 2010). However, due to the argument that these agricultural resources should be dedicated to food or animal feed better than serving for industrial production, the use of sugars derived from lignocellulose is a most promising trend. The lignocellulosic biomass is available from forest biomass, energetic crops and agricultural byproducts (Matsakas *et al.*, 2018). However, its utilization is more complicated as a preliminary treatment is needed to remove the lignin from the cellulose and hemicellulose

parts, as well as an enzymatic hydrolysis using cellulases that is a very costly step for the bioprocess (Hardy *et al.*, 2017).

Regarding the use of glucose as the starting material for 3-HP production, the US-based agricultural company Cargill proposed and patented seven interesting biochemical pathways to produce 3-HP from glucose (Gokarn Ravi *et al.*, 2014; Liao *et al.*, 2007; Liao *et al.*, 2010; Marx *et al.*, 2009). Figure 1.3 shows a synthetic scheme that represents all the possible metabolic pathways to transform glucose into 3-HP.

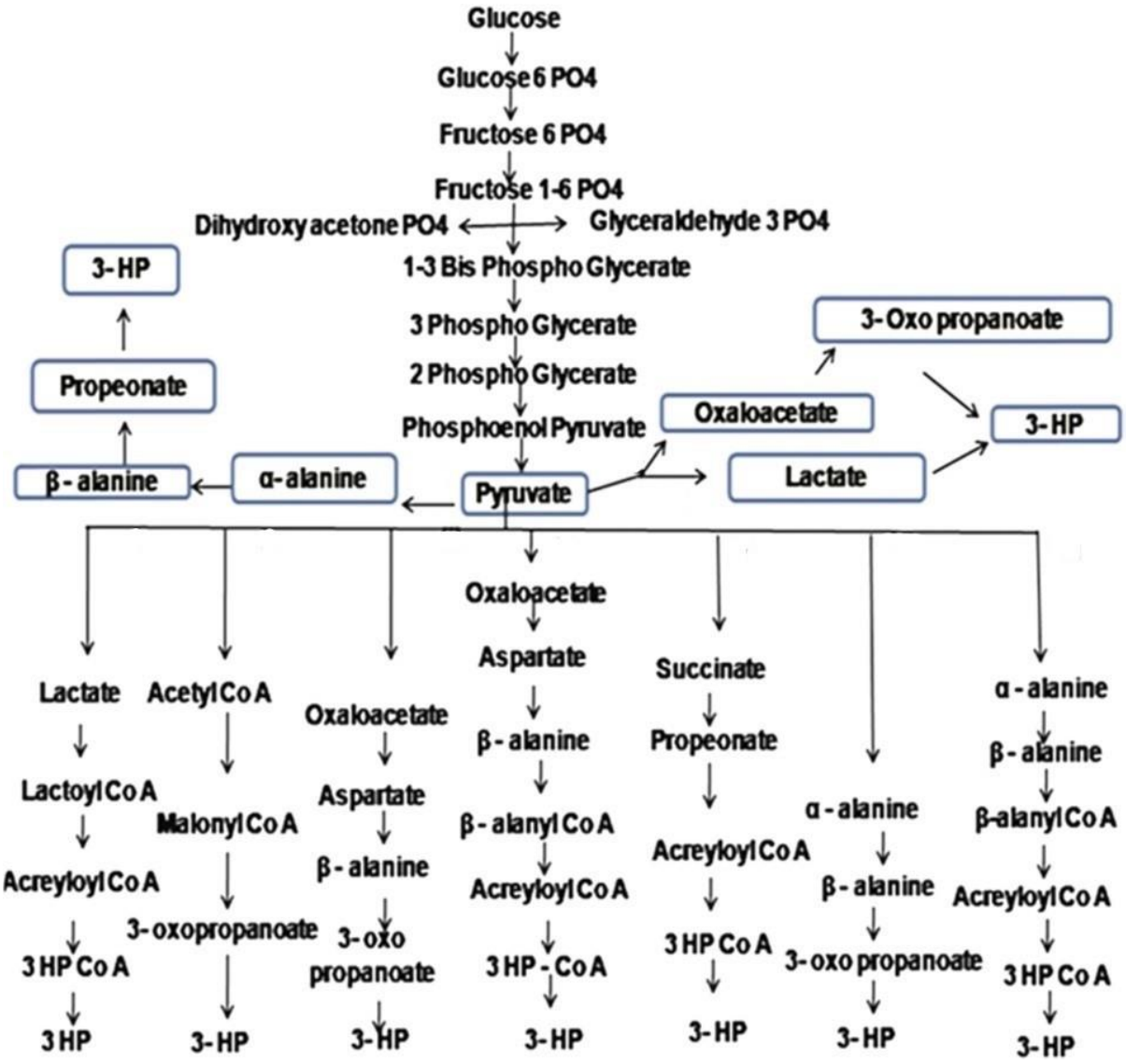


Figure 1.3. Biosynthetic pathways from glucose to 3-HP (Tingirikari *et al.*, 2016)

These pathways lead to ATP production for cell growth and to the generation of the desired product, with phosphoenolpyruvate (PEP) and pyruvate being important intermediates. From a stoichiometric point of view, one mole of glucose gives two moles of pyruvate, two moles

of ATP, together with the reduction of 2 moles of NAD^+ into NADH. The further reactions occur for the re-oxidation of NADH as the redox balance has to be equilibrated.

Among these pathways, the one involving malonyl-CoA (Figure 1.3) is promising and has been intensively studied in various hosts: bacteria (*Escherichia coli*) (Lynch *et al.*, 2014), yeasts (Ji *et al.*, 2018) and cyanobacteria (Wang *et al.*, 2016). Most of the studies concern carbon flux redirection, screening and engineering of enzymes, or cofactors and energy supply. In 2010, the Biochemical Network Integrated Computational Explorer (BNICE) software proposed three novel pathways for 3-HP production, from the intermediate pyruvate via (1) *α*-alanine, (2) oxaloacetate or (3) lactate (Henry *et al.*, 2010) (Figure 1.3).

However, until now, no wild microorganism has been found to produce 3-HP from sugars without genetic modifications.

1.2.3 Bioproduction of 3-HP from glycerol

Glycerol (propane-1,2,3-triol) is most widely studied in the literature as a substrate to produce 3-HP, either in the form of pure glycerol or in the form of glycerin that normally contains about 95 % (v/v) of glycerol. The pure glycerol is colorless, water-soluble, odorless, non-toxic, viscous, hygroscopic, with a high boiling point at 290°C (Nda-Umar *et al.*, 2019).

Most of glycerol on the market is obtained from biodiesel production as it is the main by-product from transesterification reaction of a triglyceride with ethanol, in the presence of NaOH as a catalyst (Matsakas *et al.*, 2018). By considering that the biodiesel market represents 26 000 tons per year in the world (OECD-FAO, 2020) and that 100 kg glycerol are generated per one ton of biodiesel produced (Yang *et al.*, 2012), the glycerol availability is around 2600 tons per year worldwide. In order to decrease the cost of biodiesel biorefineries, valorization of glycerol into valuable platform chemicals is compulsory. Among the proposed valorization routes, 3-HP production is an interesting opportunity. The interest of glycerol as 3-HP precursor is also due to its competitive price (even if it increased between 2010 and 2020[1]) but also to its non-toxicity against microorganisms (Matsakas *et al.*, 2014). Hence, it can be said that glycerol is an interesting renewable resource for 3-HP production.

Some studies are devoted to use crude glycerol instead of refined one (Lindlbauer *et al.*, 2017) (Szymanowska-Powalowska, 2014). However, the impurities present in crude glycerol may prevent microorganisms to utilize efficiently this substrate as demonstrated by (González *et al.*, 2019) in the context of 3-HPA bioproduction.

The metabolic pathways that convert glycerol into 3-HP are strongly dependent on the microorganisms involved in the biotransformation. Several microorganisms are able to naturally use glycerol as a carbon source to produce 3-HP, such as *Klebsiella pneumoniae* (Lee *et al.*, 2018), *Citrobacter freundii* (Seifert *et al.*, 2001), *Clostridium butyricum* (Abbad-

[1] https://www.radiantinsights.com/research/glycerol-market

Andaloussi *et al.*, 1998), *Clostridium pasteurianum* (Biebl, 2001), *Enterobacter agglomerans (Barbirato and Bories, 1997)*, *Lactobacillus reuteri* (Burgé *et al.*, 2015c; Dishisha *et al.*, 2014), *Lactobacillus collinoides* (Garai-Ibabe *et al.*, 2008) and *Alcaligenes faecalis* (Ethica and Raharjo, 2014). The pathways used in these various cases are described in the next parts.

1.2.3.1 Transport of glycerol into the cells

In all cases, the transport of glycerol is done through a passive diffusion mechanism, as it is an uncharged molecule that can pass the cytoplasmic membrane. However, due to the limitation of passive uptake at a low substrate concentration, a membrane protein named glycerol facilitator (GlpF), which acts as a selective channel for permeation of small molecules such as glycerol, has been identified (da Silva *et al.*, 2009). The influx of glycerol transported by GlpF is 100- to 1000-fold higher compared to passive diffusion. In addition, it does not saturate at a glycerol concentration of 200 mM (da Silva *et al.*, 2009; Millet and Lonvaud-Funel, 2000).

Afterward, depending on the microorganism under consideration, the biotransformation of glycerol is achieved according to various metabolic pathways that will be presented below.

1.2.3.2 Utilization of glycerol for growth

Bacteria can use glycerol as a growing substrate. Glycerol can enter the oxidative route to be metabolized into pyruvate that is further converted into lactic acid, ethanol acetic acid or butanediol together with ATP and biomass production, thus being a carbon source of growth (Figure 1.4) (Ashok *et al.*, 2011; Matsakas *et al.*, 2014).

However, *L. reuteri* cannot use glycerol as a carbon source that has been ascribed to gene regulation of enzyme dihydroxyacetone kinase to convert dihydroxyacetone into dihydroxyacetone phosphate prior to metabolism via the glycolysis pathways (Kristjansdottir *et al.*, 2019). Although all genes related to conversion of glycerol into dihydroxyacetone phosphate are present in genome of *L. reuteri* (Chen *et al.*, 2016), several genes show to be downregulated in the presence of glycerol (Santos *et al.*, 2008) that explains the lack of growth on glycerol (Kristjansdottir *et al.*, 2019).

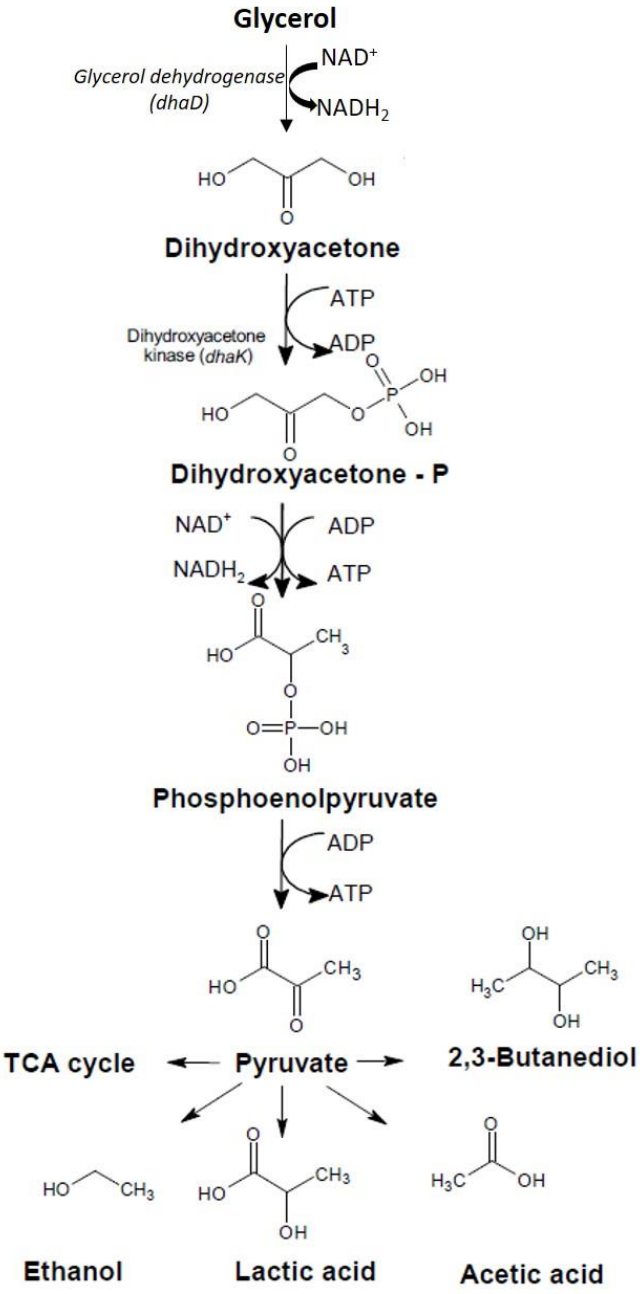


Figure 1.4. Glycerol catabolism via the oxidative pathways (adapted from (da Silva *et al.*, 2009))

1.2.3.3 Bioproduction of 3-HP from glycerol via the Dha pathway

Some bacterial species belonging to the *Enterobacteriaceae*, for instance *Klebsiella oxytoca*, *Klebsiella pneumoniae* or *Citrobacter freundii*, are able to transform glycerol into 3-HP by using the enzymes coded by the dihydroxyacetone (*dha*) operon (Homann *et al.*, 1990). This operon includes a glycerol dehydratase, an aldehyde dehydrogenase and a 1,3-propanediol oxidoreductase (Matsakas *et al.*, 2018).

The metabolic pathway illustrated in Figure 1.5 shows that the bioconversion of glycerol into 3-HP is performed in two stages. Initially, glycerol is dehydrated to form 3-HPA through the action of the enzyme glycerol dehydratase, which is a coenzyme vitamin B12 (cobalamin)-dependent enzyme (Vollenweider *et al.*, 2003). The generated 3-HPA is further reduced into 1,3-PDO thanks to the action of a 1,3-propanediol oxidoreductase (Nakamura and Whited, 2003), together with the oxidation into 3-HP through the coenzyme A-independent enzyme. These two reactions are coupled to balance the $NADH_2/NAD^+$ equilibrium. The molar balance of the Dha pathway shows that 1 mole of glycerol generates the equimolar proportion of 0.5 mole of 3-HP and 0.5 mole of 1,3-PDO.

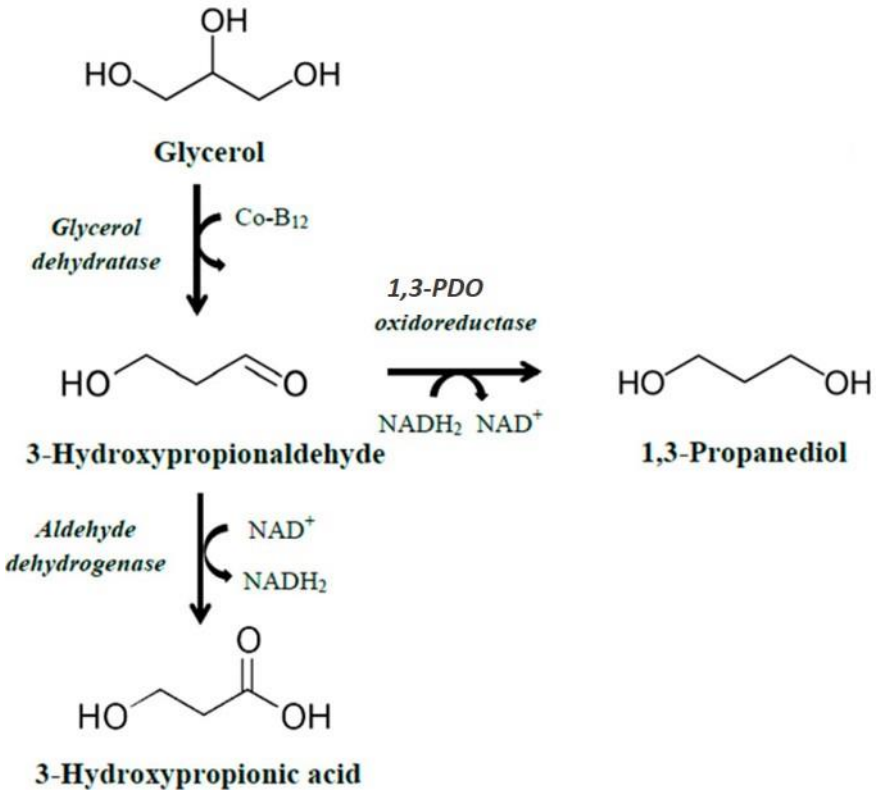


Figure 1.5. Bioconversion of glycerol to 3-HP via the Dha pathway (adapted from (Matsakas *et al.*, 2018))

1.2.3.4 Bioproduction of 3-HP from glycerol via the Pdu pathway

In the nature, the propanediol utilization pathway (*pdu*) operon can be found in *Salmonella typhimurium* (Bobik *et al.*, 1997) and in some *Lactobacillus* (Chen and Hatti-Kaul, 2017; Matsakas *et al.*, 2018). This operon has been identified by (Ramakrishnan *et al.*, 2015) and is shown in Figure 1.6. It includes the genes coding for a vitamin B12-dependent glycerol dehydratase (*pduCDE*), a phosphotransacylase (*pduL*), a propionaldehyde dehydrogenase (*pduP*) which is a Coenzyme-A dependent enzyme, a 1,3-propanediol oxidoreductase (*pduQ*) and a propionate kinase (*pduW*). These genes are associated to a catabolite repression site.

This organization justifies the simultaneous production of 3-HP and 1,3-PDO by the co-expression of the genes *pduCDE* and *pduQ* along with *pduP*, *pduL* and *pduW.*

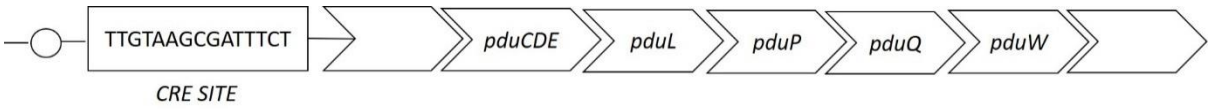


Figure 1.6. Genes involved in the Pdu pathway of glycerol bioconversion into 3-HP (adapted from (Ramakrishnan *et al.*, 2015))

Compared to the Dha pathway, the conversion of glycerol into 3-HP by the enzymes encoded in the *pdu* operon requires more intermediate steps (Figure 1.7) (Matsakas *et al.*, 2018).

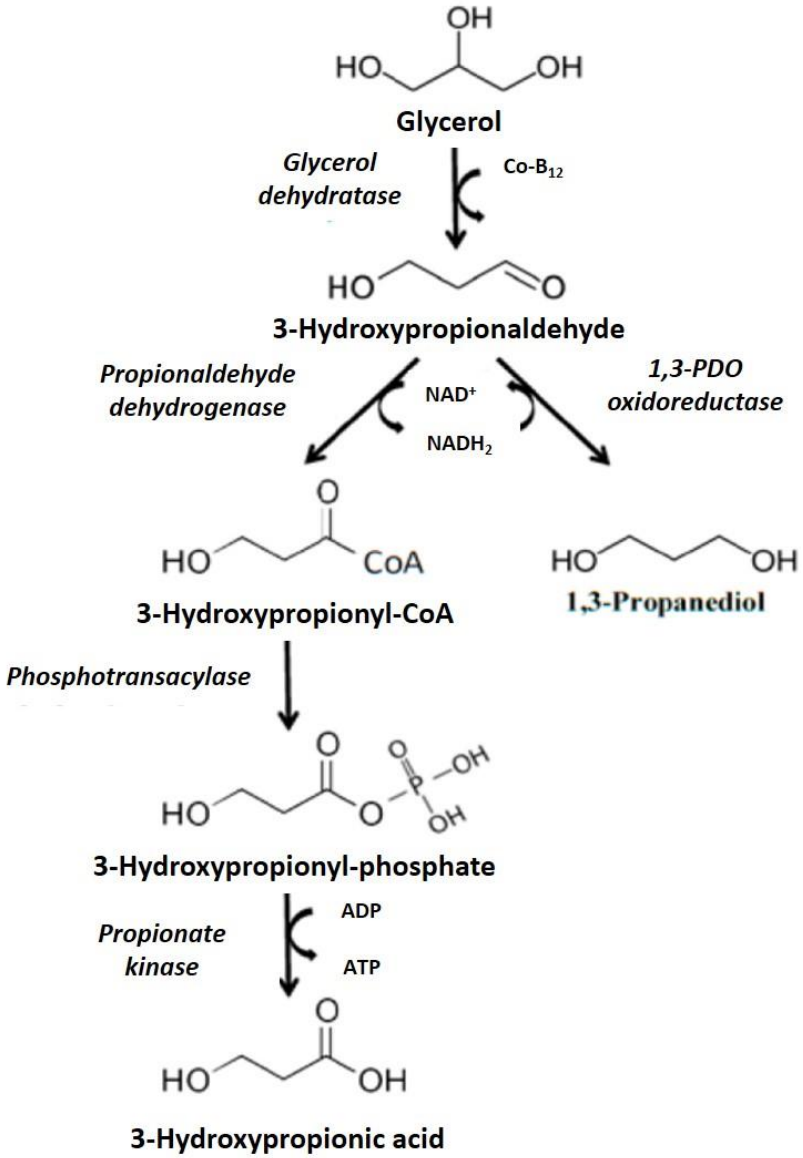


Figure 1.7. Bioconversion of glycerol to 3-HP via the Pdu pathway (Matsakas *et al.*, 2018)

Firstly, glycerol is converted into 3-HPA with the help of a diol dehydratase in the presence of vitamin B12 as a coenzyme. The 3-HPA is then transformed into 3-hydroxypropionyl-CoA (3-HP-CoA) by the action of a coenzyme A (CoA)-dependent, ATP-producing pathway. The third step corresponds to the phosphorylation of 3-HP-CoA to 3-hydroxypropionyl phosphate (3-HP-P) by a phosphotransacylase. Subsequently, the 3-HP-phosphate is dephosphorylated to form 3-HP thanks to the action of the enzyme propionate kinase, together with the formation of 1 ATP from 1 ADP. The reductive branch leading to 1,3-PD formation from 3-HPA is coupled to balance the cofactor ratio $NADH_2$ and NAD^+ (Figure 1.7).

As a result of the Pdu pathway, 1 mole of glycerol induces the equimolar proportion of 0.5 mole of 3-HP and 0.5 mole of 1,3-PDO. In other words, a theoretical molar ratio of 1:1 is observed between the substrate and the two metabolites and, if the redox balance between $NADH_2$ and NAD^+ is maintained during the process, the ratio between the products 1,3-PDO and 3-HP is also 1:1. The energy efficiency of this reaction is low, with only 1 mole of ATP produced per mole of glycerol (Matsakas *et al.*, 2018).

1.2.4 Bioproduction of 3-HP from other carbon sources

3-HP bioproduction can be achieved from other carbon sources than glycerol or glucose. The main alternative substrates are CO_2 (Lan *et al.*, 2015; Liu *et al.*, 2017; Wang *et al.*, 2016), propionic acid (Luo *et al.*, 2016), 3-HPA (Dishisha *et al.*, 2019), acrylic acid (Lee *et al.*, 2009), uracil (Kim *et al.*, 2010), or β-alanine (Borodina *et al.*, 2015). However, applying these substrates for 3-HP bioproduction requires metabolic engineering of the cells that will be discussed in the part 1.2.6.

Meanwhile, various non-GMO (genetically modified organisms) are natural 3-HP producers from precursors of 1,3-PDO like *Acetobacter* sp. (Li *et al.*, 2016a), *Gluconobacter oxydans* (Zhao *et al.*, 2015) and 3-hydroxypropionitrile (3-HPN) like *Comamonas testosteroni* (Hann *et al.*, 2003).

Being a co-product of the glycerol metabolism, 1,3-PDO can enter an oxidation step to form 3-HP. The bacterium *Acetobacter sp.* CGMCC 8142 has also been recognized to perform the oxidation of 1,3-PDO into 3-HP, by using immobilized cells that show better substrate tolerance, pH stability, thermal stability and storability.(Li *et al.*, 2016a).

The 3-hydroxypropionitrile is not a carbon source for cell growth but can be a substrate for 3-HP bioproduction in *C. testosteroni.* This microorganism owns a combination of nitrile hydratase and amidase activities that are key enzymes to convert 3-hydroxypropionitrile (3-HPN) into 3-HP (Hann *et al.*, 2003). 3-HPN is first dehydrated into 3-hydroxypropionamide (3-HPAm) by a nitrile hydratase, then the enzyme amidase converts 3-HPAm into 3-HP.

The recent achievements about 3-HP bioproduction from glycerol by the natural producer *Lactobacillus reuteri* will be summed up in the part 1.2.5.3.

However, despite these good results obtained at lab scale, these resources are less readily available as compared to glucose or glycerol.

1.2.5 The bacterium *Lactobacillus reuteri*: a promising strain for glycerol bioconversion

1.2.5.1 General characteristics of *L. reuteri*

The bacteria *L. reuteri* is a Gram-positive lactic acid bacterium that is Bacillus-shaped. It was first described by Kandler and Stetter in 1980, with *L. reuteri* DSM 20016 being the type strain (Kandler *et al.*, 1980). Under standard conditions, it is anaerobic aero-tolerant, acid tolerant and heterofermentative, producing lactic acid as a major end-product (Alazzeh *et al.*, 2009). The strain *L. reuteri* DSM 17938, that is highly referenced, is the daughter strain that results from the removal of antibiotic resistance gene-carrying plasmids of *L. reuteri* ATCC 55730 (Rosander *et al.*, 2008).

L. reuteri possesses safety characteristics, as demonstrated by its G.R.A.S (Generally Recognized as Safe) status (FDA, 2008) and Q.P.S. (Qualified Presumption of Safety) status (EFSA, 2009). Thanks to these safety properties, it is widely used as a probiotic species.

1.2.5.2 General metabolic pathways for growth of *L. reuteri*

As a heterofermentative bacterium, the use of sugars by *L. reuteri* for growth is achieved through glycolysis by using either the phosphoketolase (PK) pathway or the Embden Meyerhof Parnas (EMP) pathway (Årsköld *et al.*, 2008; Burgé *et al.*, 2015b). Figure 1.8 presents the schemes of these pathways from glucose.

They differ by considering the final products (only lactic acid in the case of EMP pathway, a mix of lactic acid, CO_2 and acetic acid or ethanol in the case of PK pathway). Another difference is found by considering the production of ATP, that is lower in the case of PK pathway (1 ATP, sometimes 2 when acetic acid is produced) as compared to EMP pathway (2 ATP). The dominant pathway depends on the growth conditions, but generally the PK pathway prevails (Burgé *et al.*, 2015b).

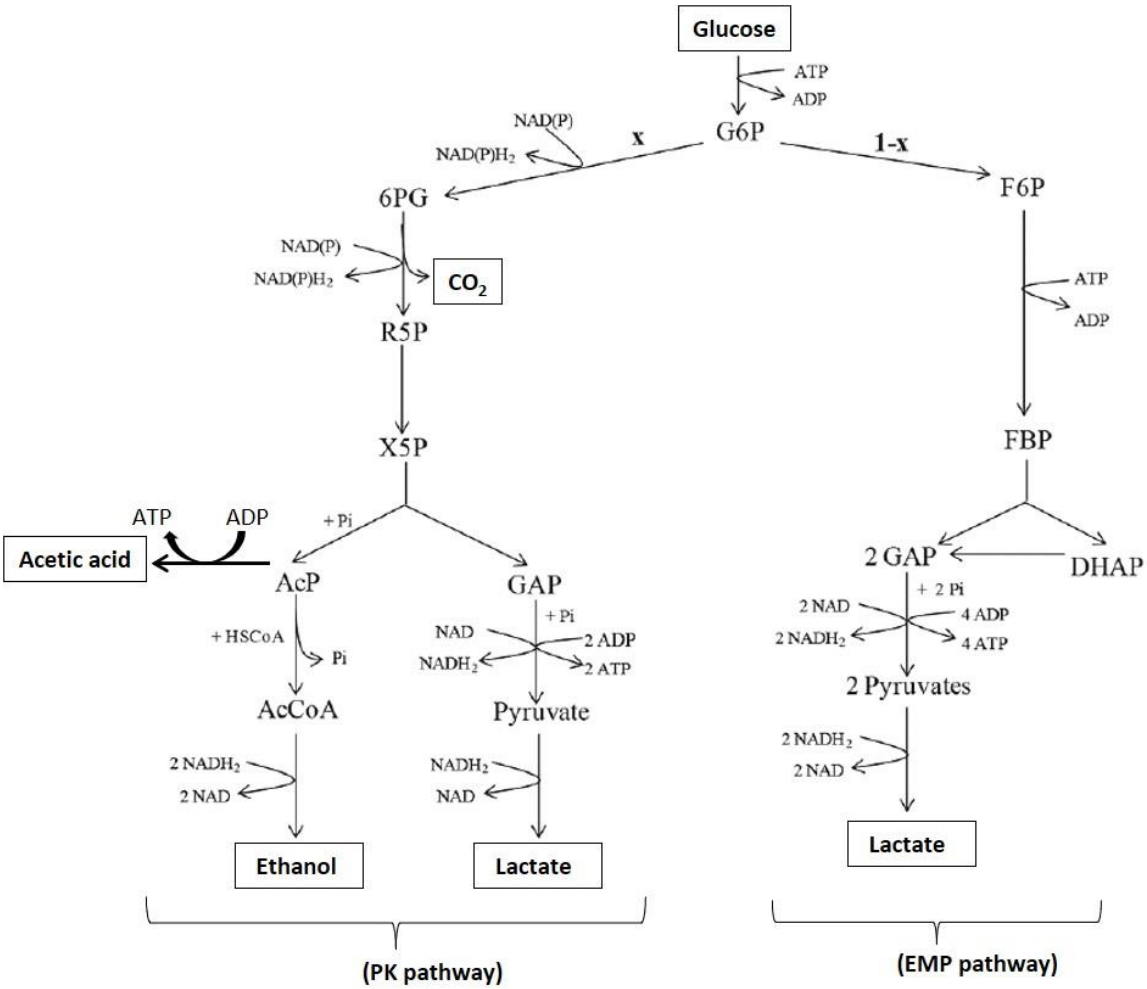


Figure 1.8. Scheme of the PK and EMP pathways in *L. reuteri* (adapted from (Burgé *et al.*, 2015b)

L. reuteri does not catabolize glycerol through its central metabolism as the cells lack the enzyme dihydroxyacetone kinase that is required for channeling glycerol into the central carbon metabolism (Dishisha *et al.*, 2014). As a reminder, this enzyme acts prior to the glycolysis and phosphoketolase pathways, to convert dihydroxyacetone into dihydroxyacetone phosphate (Figure 1.4). Consequently, glycerol cannot be used by *L. reuteri* as a carbon source for growth but only as an electron acceptor. This particularity is of interest in the context of 3-HP production as is allows avoiding the synthesis of undesirable growth by-products during the bioconversion, particularly lactic acid that can hinder conversion yield and further *in situ* extraction.

1.2.5.3 Bioproduction of bacteriocins by *L. reuteri*

The probiotic properties of *L. reuteri* are related to its ability to produce antimicrobial molecules, such as reuterin, organic acids, or ethanol, thus being able to inhibit undesirable microorganisms in the gastro-intestinal tract (Mu *et al.*, 2018). Some healthcare products containing *L. reuteri* strains have been introduced in the market by the Swedish biotechnology

company BioGaia (Stockholm, Sweden). Besides, *L. reuteri* is used as food preservative and has been formulated in probiotic ice creams (Lüthi-Peng *et al.*, 2002).

A lot of works has been published on *L. reuteri*, as this species is able to produce reuterin, a bacteriocin that acts as an antimicrobial compound. Reuterin, which is the dimeric form of 3-HPA, is an intermediate product in the Pdu metabolic pathway of glycerol to 3-HP (Lüthi-Peng *et al.*, 2002; Vollenweider *et al.*, 2010). The mechanism of action by which reuterin inhibits cell growth is linked to an oxidative stress, that modifies the thiol groups in proteins and peptides (Schaefer *et al.*, 2010). The inhibition spectrum of reuterin produced by *L. reuteri* SD2112 varies, depending on the targeted species. The minimal inhibitory of reuterin is 7.5–15 mM for *Escherichia coli*, 15–50 mM for Lactobacilli and *Clostridium clostridioforme* (Cleusix *et al.*, 2007). Beside, as an aldehyde, 3-HPA in the form of monomer is also toxic, as detailed in Section 1.4.1.4

1.2.5.4 Bioproduction of 3-HP by *L. reuteri*

The bioproduction of 3-HP by *L. reuteri* strains has been studied by many authors. As indicated in Section 1.2.3.4, this bacterial species transforms glycerol into 3-HP via the Pdu pathway, without using glycerol for growth. However, according to the culture conditions and the strain used (including natural and GMO strains), the performances strongly vary. Detailed information will be given in Table 1.4 in Section 1.4.2.

The glycerol bioconversion into 3-HP by *L. reuteri* strains has been conducted in different modes: batch, fed-batch and repeated batch processes, mostly at 37 °C and at controlled pH between 5.5 and 7.0 or at free pH. The highest 3-HP titer of 14 $g \cdot L^{-1}$ was obtained after 58 h of bioconversion at 37 °C, pH 7.0 in fed-batch mode at a glycerol feeding rate of 0.75 $g \cdot h^{-1}$ with the wild-type *L. reuteri* DSM 20016 (Dishisha *et al.*, 2015). The higher conversion yield of 3-HP from glycerol was close to 1.3 $mol_{3\text{-}HP} \cdot mol_{glycerol}^{-1}$ with the GMO strain *L. reuteri* HR2 (Suppuram *et al.*, 2019) and 0.49 $mol_{3\text{-}HP} \cdot mol_{glycerol}^{-1}$ with the wild type *L. reuteri* DSM 20016 (Dishisha *et al.*, 2015). The highest value of 3-HP volumetric productivity was equal to 2.91 $g \cdot L^{-1} \cdot h^{-1}$ and was obtained from a short bioconversion duration of 3 h in batch mode at 30 °C and free pH (Zabed *et al.*, 2019).

1.2.6 Metabolic engineering approaches for 3-HP bioproduction

Metabolic engineering of microorganisms implies either genetic improvements of natural producers (such as *K. pneumoniae*) or construction of new strains (for example with *E. coli*) by introduction of genes coding for new enzymes. Many studies on these engineered bacteria have been published. They are summarized in Table 1.1.

Table 1.1. Performances of 3-HP bioproduction by genetically modified microorganisms (adapted from (de Fouchécour *et al.*, 2018))

Microorganisms	**Carbon substrate**	**Bioproduction mode**	**3-HP titer ($g \cdot L^{-1}$)**	**3-HP conversion yield ($mol_{3\text{-}HP}.mol_S^{-1}$)**	**3-HP productivity ($g \cdot L^{-1} \cdot h^{-1}$)**	**References**
E. coli SH501_E209Q/E269Q	Glycerol + Glucose	Fed-batch in 5 L bioreactor	71.9	na	1.8	(Chu *et al.*, 2015)
*K. pneumoniae ldh1*Δ*ldh2*Δ*pta*(pTAC-*puuC*)	Glycerol	Fed-batch in 5 L bioreactor	83.8	0.54	1.16*	(Li *et al.*, 2016b)
K. pneumoniae (p3tac-PuuC)	Glycerol	Fed-batch in 5 L bioreactor	102.6	0.43*	1.07*	(Zhao *et al.*, 2019)
E. coli TA214	Glycerol	Batch in 60 mL baffled flask	5.15*	0.54	0.11*	(Honjo *et al.*, 2015)
E. coli BTnoxplw	3-HPA	Batch in 100 mL shaken flask	1.98*	na	0.16	(Dishisha *et al.*, 2019)
E. coli BW2511	Glucose	Fed-batch in 1.8 L bioreactor	48.4	na	-	(Lynch *et al.*, 2014)
E. coli Q2186	Glucose	Fed-batch in 5 L bioreactor	40.6	0.38*	0.56*	(Liu *et al.*, 2016)
E. coli Ec-ΔY-ΔP-PPH	Propionate	Batch mode in 100 mL flask	2.17*	0.34	0.09*	(Luo *et al.*, 2016)
E. coli W3110	Glucose	Fed-batch in 6.6 L bioreactor	31.1	0.84*	0.63*	(Song *et al.*, 2016)
S. cerevisiae SCE-R2-200	Glucose	Fed-batch in 1 L bioreactor	13.7	na	0.17	(Borodina *et al.*, 2015)

na: not available

(*) data obtained by calculation

From Table 1.1, the most interesting information concerns the 3-HP titer that was much higher by using recombinant *E. coli* or *K. pneumoniae* strains (up to 100 $g_{3\text{-HP}}\cdot L^{-1}$). However, the 3-HP conversion yields and volumetric productivities were close to those obtained with natural strains. In addition, many of these constructions exhibit some negative issues. *K. pneumoniae* species do not display good safety characters that may complicate its use in industrial plants. *E. coli* species is aerobic and requires the addition of vitamin B12 to produce 3-HP, thus leading to higher costs for the process at large scale.

1.2.7 Summary of information

3-HP bioproduction gains more and more interest in a context of bioeconomy that aims at reducing our carbon footprint. Different carbon sources can be used to produce 3-HP by a biotechnological route: glucose, glycerol, CO_2, propionic acid, 3-HPA, acrylic acid, uracil or β-alanine being the main ones. By considering the titers, yields and productivities on the one hand, and economic issues about the abundance and the cost of the substrates on the other hand, glycerol is recognized as having a high potential for further industrial application. Depending on the microorganism involved in the biotransformation, different metabolic pathways are described. The Dha pathway, which concerns *K. pneumoniae* allows obtaining a very high 3-HP titer of 102.6 $g\cdot L^{-1}$. However, as the species is pathogenic, industrial application is more complicated. The Pdu pathway that concerns *L. reuteri* led to lower titers (14 $g\cdot L^{-1}$ or less), but this species has the advantage of being not pathogenic and it is already used to produce bacteriocins. In addition to these naturally 3-HP producers, many works have been done to develop genetically modified microorganisms in order to improve the performance of the bioprocess. However, their industrial application is not yet implemented. Consequently, the use of *L. reuteri* to convert glycerol into 3-HP is of great interest and will be deeply examined in this study.

1.3 Effect of nutritional and environmental conditions on the growth of *L. reuteri*

1.3.1 Effects of nutritional conditions on *L. reuteri* growth

During a bioprocess, nutritional conditions have to be defined in order to maximize the bacterial concentration and the specific growth rate, and to optimize the physiological state of the microorganism in order to produce efficiently the desired biomolecule. *L. reuteri* species has complex nutritional requirements. In addition to the common carbon and nitrogen sources, the growth of the bacteria needs to be supplied with specific amino acids, peptides, salts, fatty acids or fatty acid esters, nucleic acid derivatives and vitamins.

1.3.1.1 Carbon sources

As one of the first requirements for bacterial growth, the carbon source is necessary for biomass growth, product formation and maintenance of cell physiological state. When the cells enter the stationary phase as a consequence of substrate depletion, they are progressively exposed to carbohydrate starvation. The exhaustion of essential nutrients induces a decrease in the specific growth rate until cessation of growth. This is explained by a downregulation of nucleic acid and protein synthesis and a degradation of proteins as an adaptive response (De Angelis and Gobbetti, 2011; Wang *et al.*, 2011). Besides, an increase in membrane fluidity, ascribed to a higher content of branched, unsaturated, and cyclic fatty acids was found in starved cells (Wang *et al.*, 2011).

Different carbon sources have been used to trigger *L. reuteri* growth, such as glucose alone (Årsköld *et al.*, 2008; Burgé *et al.*, 2015b), glucose plus fructose, saccharose (Årsköld *et al.*, 2008), galactose, lactose, melibiose, raffinose, saccharose (Alazzeh *et al.*, 2009) and industrial wheat or sugar beet syrup by-products that contained glucose and fructose (Couvreur *et al.*, 2017). Among them, glucose is most widely used in research studies. As *L. reuteri* displays a heterofermentative metabolism, glucose is catabolized through the PK and EMP pathways that operate simultaneously and lead to the production of lactic acid, acetic acid, ethanol, CO_2 and energy (Årsköld *et al.*, 2008).

From (Bengtsson, 2020), the presence of glucose into the culture medium led to the highest biomass production of *L. reuteri* DSM 17938 ($OD_{620\ nm}$ of 6.4) in comparison to the other carbon sources tested (maltose, maltose plus fructose, glucose plus fructose, sucrose alone and sucrose plus fructose. This was confirmed later by (Polak-Berecka *et al.*, 2010) with *Lactobacillus rhamnosus* and studied as a component of an industrial byproduct by (Couvreur *et al.*, 2017) with *L. reuteri* DSM 17938. One of advantage of using this sugar is its wide availability on the market. The suggested concentrations of glucose were comprised between 20 $g{\cdot}L^{-1}$ (Bengtsson, 2020) and 30 $g{\cdot}L^{-1}$ (Couvreur *et al.*, 2017) in total.

1.3.1.2 Nitrogen sources

Nitrogen sources are required for bacterial growth as they are involved in the building of proteins and nucleic acids. The nitrogen sources that are commonly referenced for *L. reuteri* are yeast extract, meat peptone and more recently, phytone peptone. Yeast extract is widely used in culture media (Alazzeh *et al.*, 2009; Atilola *et al.*, 2015; Couvreur *et al.*, 2017; Ichinose *et al.*, 2020). It contains a mix of peptides, free amino acids, purine and pyrimidine bases and water soluble vitamins of B group (Proust *et al.*, 2019), which are favorable to biomass production. Meat peptone provides a source of nitrogen, amino acids, vitamins and carbon (Merck, 2020).

Recently, phytone peptone has been identified as a component leading to higher cell counts for *L. reuteri* among other nitrogen sources: meat peptone, tryptone, proteose peptone, tryptic soy broth, yeast extract and beef extract (Atilola *et al.*, 2015). Phytone peptone

provides a broad range of nutrients (Ayad *et al.*, 2020), including carbohydrates and many amino acids, excepted asparagine and glutamine (Merck Microbiology Manual 12th Edition, 2018). The amino acid composition of yeast extract and phytone peptone is listed in Table 1.2.

Table 1.2. Amino acid composition in yeast extract and phytone peptone (adapted from (Oganotechnie, 2008) and (Merck, 2020))

Amino acids	**Concentrations (g·100 g^{-1} product)**	
	Yeast extract	**Phytone peptone**
Aspartic acid	6.0	7.82
Threonine	2.6	2.39
Serine	2.7	3.18
Glutamic acid	11.0	12.4
Proline	2.2	3.34
Glycine	2.5	4.77
Alanine	4.1	2.69
Cysteine	0.6	0.82
Valine	3.1	2.82
Methionine	0.8	0.82
Isoleucine	2.6	2.82
Leucine	3.7	4.71
Tyrosine	0.8	2.55
Phenylalanine	2.5	3.12
Histidine	1.1	3.64
Lysine	4.2	3.91
Arginine	2.8	8.65
Tryptophan	0.8	0.60
Glutamine	0	0
Asparagine	0	0

By considering this table, it can be observed that the amino acids glycine, tyrosine, histidine and arginine are more present in phytone peptone than in yeast extract, which may affect positively the bacterial growth.

1.3.1.3 C/N ratio

If the role of carbon (C) and nitrogen (N) sources are quite well described to support the growth of *L. reuteri*, the C/N ratio is another important factor that has to be taken into account. In fact, the C/N ratio was recognized as a sensitive factor for the growth of *Lactobacillus paracasei* HCT, which was maximized for a C/N ratio of 8.6 (Xu *et al.*, 2010). By considering *L. reuteri*, the Table 1.3 summarizes the growth performances of different strains obtained from different C/N values.

Table 1.3. C/N ratio in culture media of *L. reuteri*

***L. reuteri* strain**	**Culture conditions**	**Carbon source and concentration**	**C/N ratio***	**Final $OD_{600\ nm}$**	**Reference**
ATCC 55730	Batch culture 37 °C, pH 5.5	Glucose, 50.4 $g{\cdot}L^{-1}$	8.70	4.3*	(Årsköld *et al.*, 2008)
CG001	Batch culture 38.6 °C, initial pH 6.2	Glucose, 20 $g{\cdot}L^{-1}$	1.00	3.7	(Chen *et al.*, 2010)
DSM 17938	Batch culture 37°C, initial pH 6.8	Wheat extract, 30 $g{\cdot}L^{-1}$	0.86	12.8	(Couvreur *et al.*, 2017)
DSM 20016	Batch culture 37 °C, pH 5.5	Glucose, 40 $g{\cdot}L^{-1}$	1.60	11.7*	(Dishisha *et al.*, 2014)
DSM 20016	Batch culture 37 °C, initial pH 6.5	Dextrose, 10 $g{\cdot}L^{-1}$	0.80	1.35	(Atilola *et al.*, 2015)
DSM 20016	Batch culture 37 °C, free pH	Glucose, 20 $g{\cdot}L^{-1}$	0.8	9.2*	(Mota *et al.*, 2018)

* Data obtained by calculation

From this table, the C/N ratio varies from 0.80 to 8.70 according to the authors. The best results were obtained with low ratios comprised between 0.86 (Couvreur *et al.*, 2017) and 1.6 (Dishisha *et al.*, 2014). But, for a given C/N ratio, other conditions may also affect the growth performances (strain used, detailed composition of the substrate, pH, temperature…). To date, no study already determined the optimum C/N ratio to maximize *L. reuteri* growth.

1.3.1.4 Salts

Salts are necessary elements for living organisms. For *L. reuteri* cultivation, the better salt composition has not been properly determined. However, from previous studies (Alazzeh *et al.*, 2009; Atilola *et al.*, 2015; Dishisha *et al.*, 2014), the common media used for growth of *L. reuteri* strains typically include: monopotassium phosphate (KH_2PO_4, 0-2 $g{\cdot}L^{-1}$), dipotassium phosphate (K_2HPO_4, 0-2 $g{\cdot}L^{-1}$), sodium acetate (CH_3COONa, 5 $g{\cdot}L^{-1}$), diammonium citrate ($(NH_4)_2C_6H_5O_7$, 2 $g{\cdot}L^{-1}$), magnesium sulfate ($MgSO_4$, 0.2 $g{\cdot}L^{-1}$) and manganese sulfate ($MnSO_4$, 0.05 $g{\cdot}L^{-1}$).

1.3.1.5 Vitamins

Vitamins are essential micronutrients for the metabolism of all living organisms. Among them, B-group vitamins play an important role in nutrition and general metabolism in lactic acid

bacteria (LeBlanc *et al.*, 2011). This group includes biotin (B8), cyanocobalamin (B12), folic acid (B9), niacin (B3), pantothenic acid (B5), pyridoxal (B6) and thiamine (B1).

Results from previous studies show that vitamins B group did no impact the growth of *L. rhamnosus* PEN (Polak-Berecka *et al.*, 2010). The supplementation of the culture medium with the vitamins pyridoxine hydrochloride, pantothenic acid, niacin, riboflavin and folic acid only little affected the growth of *L. rhamnosus* ATTC7469 (Liew *et al.*, 2005). Consequently, they were not included into the optimized medium for the growth of this strain (Chang and Liew, 2013). By considering *L. reuteri*, the study of (Couvreur *et al.*, 2017) recently showed that supplementation of cyanocobalamin did not improve the cell density during the growth phase.

Consequently, as vitamins are expensive and complex to prepare, the growth media for *L. reuteri* are generally not complemented with vitamins. In the culture media used for lactic acid bacteria, the vitamins are generally brought with the complex components, such as peptones or yeast extracts.

1.3.1.6 Tween 80

Tween 80, also known as polyoxyethylene sorbitan monooleate, is frequently used to supplement culture media, such as HHD (homofermentative-heterofermentative differential) medium (Reque *et al.*, 2020) or MRS medium (Burgé *et al.*, 2015b), where it serves as an emulsifier and a dispersing agent. It is also considered as a growth factor, especially for *Lactobacillus delbrueckii* species (Partanen *et al.*, 2001). This was confirmed by the recent study of (Reitermayer *et al.*, 2018) who showed, using a transcriptomic analysis, that the growth-enhancing effect of Tween 80 in *Lactobacillus plantarum* is based on energy and resource savings, thanks to the downregulation of *de novo* fatty acid synthesis. Compared to Tween 40, Tween 60 and other free fatty acids, Tween 80 and free oleic acid also conferred resistance against high hydrostatic pressure to *L. plantarum* (Reitermayer *et al.*, 2018). The authors showed that Tween 80 diminishes the pressure-induced loss of metabolic activity, the protein release as well as the membrane permeabilization. In addition, the presence of Tween 80 in the growth medium of *Streptococcus thermophilus* modified its intracellular fatty acid content, thus leading to a better survival after freezing and during frozen storage (Béal *et al.*, 2001). Finally, the work of (Al-Naseri *et al.*, 2013) showed that *Lactobacillus casei* was able to use Tween 80 and citrate as alternative carbon sources under carbohydrate starvation, which sustained the cell survival for weeks.

From these studies, Tween 80 can be considered as a key component of culture media for lactobacilli, where it can be added in a range of concentrations comprised between 5 $g \cdot L^{-1}$ (Al-Naseri *et al.*, 2013) and 7.5 $g \cdot L^{-1}$ (Reitermayer *et al.*, 2018). For *L. reuteri*, Tween 80 is generally added into the culture media in concentration comprised between 1 $g \cdot L^{-1}$ (Atilola *et al.*, 2015) and 5 $g \cdot L^{-1}$ (Couvreur *et al.*, 2017).

1.3.1.7 Cysteine

Cysteine is a sulfur-containing amino acid whose thiol group (-SH) works as a functional group and is responsible for the formation of disulfide bonds in proteins (Piste, 2013). It also displays an antioxidant effect (Mokhtari *et al.*, 2017) and acts as a redox potential reducing agent (Santiesteban-López *et al.*, 2013). Cysteine can be utilized as a medium component for lactic acid bacteria cultures in order to build the corresponding proteins and to reduce the oxygen content in the media (Feng and Wang, 2020; Rodrigues *et al.*, 2011). It is also known to help the bacteria to survive under cold stress in fermented milks (Dave and Shah, 1997).

From these considerations, cysteine can be considered to supplement the culture medium of *L. reuteri*. It has already been included into *L. reuteri* culture media at concentrations of 0.1 $g \cdot L^{-1}$ (Santos *et al.*, 2009) to 0.5 $g \cdot L^{-1}$ (Alazzeh *et al.*, 2009).

1.3.1.8 Betaine

Betaine is a natural amino acid compound, also known as trimethylglycine or glycine betaine that is naturally found in sugar beet (Drobny *et al.*, 2020). Functionally, betaine can work as a methyl donor since it is composed by three methyl groups. Additionally, it is considered as an osmolyte that helps the cells to counteract osmotic shocks. This factor is an important compatible solute for lactic acid bacteria and it shows a protective effect against drying. The study of (Kets and De Bont, 1994) on *L. plantarum* demonstrated that the addition of 2 mM betaine into the culture medium helped the cells to survive an osmotic stress of 0.6 M sodium chloride. By considering *Lactobacillus buchneri* R1102*,* a moderate osmotic stress was applied by combining the addition of betaine 2 $mmol \cdot L^{-1}$ to that of KCl 0.1 $mol \cdot L^{-1}$ at the beginning and KCl 0.6 $mol \cdot L^{-1}$ at the end of fermentation. This method led to the highest accumulation of betaine and improved the cell survival to freeze-drying (Louesdon *et al.*, 2014).

The presence of betaine in culture medium at a low level supplementation (1 - 4 $g \cdot L^{-1}$) was reported as a factor leading to an increase of cell growth and L-lactate production by *L. casei* ZW-63A (Zou *et al.*, 2013).

From this information, as betaine helps the cells to counteract various stresses, its addition should be interesting in the context of *L. reuteri* growth for further 3-HP bioproduction.

1.3.2 Effects of environmental conditions on the growth of *L. reuteri* in bioreactors

Environmental conditions have to be controlled during any bioprocess, in order to potentiate the metabolic activity of the microorganisms. Agitation rate, temperature, pH, osmolarity and atmosphere are the most important factors that have to be taken into account (Hammes and Hertel, 2006).

1.3.2.1 Agitation speed

Agitation is compulsory in a bioreactor, to permit homogenization and avoid cell agglomeration and decantation, to facilitate temperature control and to reduce gradients (substrates and oxygen concentrations, pH). However, it can generate some shear stress, thus affecting membrane integrity and modifying cell functions. In addition, increasing agitation speed leads to the increase of dissolved oxygen concentration, which can engender an oxidative stress during anaerobic bioprocesses.

In the case of anaerobic bacteria like *L. reuteri*, the agitation speed only tends to ensure medium homogenization and microaerobic conditions. In addition, as *L. reuteri* does not form filaments unlike filamentous bacteria or fungi, the cells are not sensitive to shear stress. According to (Ju *et al.*, 2020), low stirring rates (50 or 100 rpm) are more adapted to the production of biomass of *L. reuteri* CH53 than higher agitation rates (300 rpm). Consequently, a moderate value of stirring rate is commonly used during *L. reuteri* cultures, such as 100 rpm (Burgé *et al.*, 2015c; Couvreur *et al.*, 2017; Ju *et al.*, 2020; Ortiz *et al.*, 2015) or 200 rpm (Chen and Hatti-Kaul, 2017; Dishisha *et al.*, 2014).

1.3.2.2 Culture temperature

Temperature is one of the most important environmental conditions that affect bacterial growth. Basically, increasing the temperature until the optimum value enhances the specific growth rate by improving the enzyme activities, modifying the membrane fluidity and altering the saturation level of the fatty acids of the phospholipids, both at intracellular and membrane levels. It also modifies the affinity between the microorganism and the substrates (Nedwell, 1999). However, at too high temperatures, denaturation of macromolecules occurs, thus leading to cell death.

Most lactobacilli species are mesophilic, with an optimum temperature ranging between 30 and 45 °C. They can also grow at lower temperatures (Van De Guchte *et al.*, 2002) and until an upper limit of 53 °C, depending on the species (Ahmed *et al.*, 2006).

Previous studies have investigated the optimal temperature of *L. reuteri*. By considering the DSM 17938 strain, it was demonstrated that 37 °C is the optimal temperature for cell growth. The specific growth rate was two-fold increased at 37 °C as compared to 32 °C, irrespective of culture pH (Hernández *et al.*, 2019). A slight different value was found for *L. reuteri* CG001 that exhibited an optimal temperature of 38.6 °C (Chen *et al.*, 2010).

It should be noticed that the optimum temperature for growth may differ from that maximizing a given metabolic activity. For example, the optimal temperature for lactic acid production by *Lactobacillus acidophilus* RD758 is 3 °C lower than the optimal growth temperature (Wang, 2005). In addition, *L. reuteri* I5007 cultured at 47 °C showed a better survival after freeze-drying, as compared to the optimum growth temperature of 37 °C (Liu *et*

al., 2014). Consequently, this environmental factor has to be well defined for each strain and each purpose of bioprocess.

1.3.2.3 pH and base used for pH control

To ensure a high metabolic activity, the cells have to maintain their intracellular pH at a suitable value, even though the external pH is more acid or alkaline, since both disturb bacterial growth and survival (Wall *et al.*, 2007). This necessity is explained by the mechanisms linked to the proton motive force (Chen *et al.*, 2019). As a lactic acid bacterium, *L. reuteri* produces lactic acid as the main product of the glycolysis pathway. Lactic acid is excreted into the extracellular medium to help the cells to maintain their intracellular pH, thus leading to an acidification of the extracellular medium. Although *L. reuteri* is relatively acid tolerant (Teixeira *et al.*, 2014), the accumulation of lactic acid and other organic acids associated, or not, with a pH decrease considerably affects the physiological state of the cells. Cytoplasmic acidification causes the inhibition of enzyme activities, the decrease in intracellular energy production, together with the increase in energy expenditure to cope with cytoplasmic acidification (Even *et al.*, 2002). In these conditions, energetic limitations may occur, thus diminishing the specific growth rate and in some cases, the final biomass concentration (Schepers *et al.*, 2002). The inhibition is more important at low pH, i.e. when the proportion of undissociated lactic acid is high as compared to the lactate form (Schepers *et al.*, 2002). To enhance the bacterial growth, it is recommended to maintain the pH at a constant value corresponding to the optimum pH of bacterial cells. In that condition, the growth is improved (Bai *et al.*, 2004) as a consequence of better enzymatic activities (Ampatzoglou *et al.*, 2010).

By considering *L. reuteri*, the growth can occur in a large range of pH, between pH 5.0 and pH 7.5, depending on the strain. From (Kandler *et al.*, 1980), the optimum pH is generally comprised between pH 6.0 and pH 6.8 for *L. reuteri* sp. It was identified at pH 5.5 for *L. reuteri* DSM 12246 (El-Ziney, 2018) and *L. reuteri* DSM 17938 (Hernández *et al.*, 2019) and at pH 5.7 for *L. reuteri* I5007 (Liu *et al.*, 2014). In addition, the maximum pH at which growth stopped has been reported at pH 8.1 for the *L. reuteri* JCM1112^{T} strain (Sawatari and Yokota, 2007).

As for the temperature, the optimal pH for a given functionality may differ from the one for growth. For example, *L. reuteri* ATCC 55730 was more resistant to freeze-drying when the cells were cultured at pH 5.0 as compared to pH 6.0 (Palmfeldt and Hahn-Hägerdal, 2000). Three kinds of base have been reported to control the pH during *L. reuteri* growth: KOH (Burgé *et al.*, 2015c), NaOH (Doleyres *et al.*, 2005; Hernández *et al.*, 2019; Kristjansdottir *et al.*, 2019) and NH_4OH (Dishisha *et al.*, 2014; Ricci *et al.*, 2015; Sardari *et al.*, 2013a). However, the issue of whether the kind of supplied base affects or not the bacterial growth has not yet been studied.

1.3.2.4 Osmolarity

Osmolarity that defines the water-electrolyte balance, corresponds to the number of particles in 1 L of solution and is given in $mOsm \cdot L^{-1}$. Lactic acid fermentation is generally associated to an osmotic stress that is linked to high concentrations of salts that accumulate during controlled-pH cultures. These salts can be either sodium lactate, ammonium lactate or potassium lactate (De Angelis and Gobbetti, 2011; Louesdon *et al.*, 2014). The use of high initial carbon substrate concentrations during batch cultures is another reason of osmolarity issues.

Regarding lactobacilli, high osmolarity caused by high concentrations of sugar or metabolites may inhibit cell growth and lactate production (Zou *et al.*, 2013). In fact, the cell membrane is readily permeable to water molecules, but an effective barrier to most other solutes (Glaasker *et al.*, 1996). Under high external osmolarity, water is removed out of the cell to maintain the osmotic balance, which results in a reduction of cell volume, an increase in intracellular solute concentration and a modification of the turgor pressure (Glaasker *et al.*, 1996). In order to relieve osmolarity problems, different approaches may be used. By considering lactate production in *L. casei*, the osmotic stress caused by high substrate concentration was reduced by applying a fed-batch fermentation strategy (Ding and Tan, 2006). As another approach, the selection of the osmotolerant strain *L. casei* G-03 resulted in an increase in L-lactate concentration as compared to the parent strain (Ge *et al.*, 2011). But most of the works that deal with osmotic stress consider the ability of lactic acid bacteria, mainly lactococci and lactobacilli, to uptake or synthesize some compatible solutes including amino acids (glutamine, glutamate, proline) and amino acid derivatives (betaine, peptide, N-acetylated amino acids) (Csonka, 1989) in order to retain water within the cell and thus, to maintain turgor pressure. In most of the cases, the addition of betaine in the culture medium is then carried out in order to reduce the osmotic stress caused by high salt concentration or dehydration (Guillot *et al.*, 2000; Kets and De Bont, 1994; Louesdon *et al.*, 2014).

1.3.2.5 Gaseous atmosphere

As an anaerobic aerotolerant microorganism, *L. reuteri* generally prefers a reduced oxygen tension or anaerobiosis during growth (Kandler *et al.*, 1980). From a positive point of view, oxygen can act as an electron acceptor during sugar metabolism thus favoring energy synthesis (Condon, 1987). The presence of small concentrations of oxygen or other oxidants during the growth of heterofermentative lactic acid bacteria causes a metabolic shift to acetate at the expense of lactate or ethanol, together with the production of one additional mole of ATP from the acetate kinase reaction (Årsköld *et al.*, 2008). Oxygen also permits co-factor regeneration via NADH oxidase and peroxidase (Hammes and Vogel, 1995).

Practically, most of the cultures of *L. reuteri* are performed without air sparging and without addition of any gas (Burgé *et al.*, 2015b; Couvreur *et al.*, 2017), which is the most simple and less expensive situation. In few studies, anaerobic conditions have been maintained during

the growth by continuous bubbling nitrogen gas (Dishisha *et al.*, 2015; Doleyres *et al.*, 2005). The study of (Doleyres *et al.*, 2005) indicated that adding N_2 during the growth phase slightly improved the 3-HPA production by glycerol bioconversion. This was explained by a reduction of the inhibition of glycerol dehydratase by oxygen (Doleyres *et al.*, 2005). However, as some CO_2 is produced during cell growth by the heterofermentative metabolism, the conditions are naturally anaerobic (Årsköld *et al.*, 2008), which could explain the small difference in 3-HPA production that resulted from the two studied conditions (Doleyres *et al.*, 2005).

1.3.3 Summary of information

The growth of *L. reuteri* in bioreactor is affected by many operating conditions. The availability of nutrient sources including carbohydrates, nitrogen sources, salts and vitamins, as well as the addition of some growth factors like Tween 80, cysteine and betaine modify the growth performances of the bacterium and its physiological state. Among these components, glucose is considered as a good carbon source, yeast extract and/or phytone peptone can be chosen as complex nitrogen sources, together with salts such as KH_2PO_4, K_2HPO_4, CH_3COONa, $(NH_4)_2C_6H_5O_7$, $MgSO_4$, and $MnSO_4$. Tween 80, cysteine and betaine not only benefit for cell growth but may also help the cells to cope with stresses encountered during the fermentation or during the subsequent processes they are involved in. The main environmental conditions that act during growth include temperature, pH, osmolarity, aeration and agitation. A moderate agitation speed (100 rpm), a temperature of 37 °C, a pH controlled between 5.5 and 6.8 and an anaerobic or microaerobic atmosphere are promising conditions for biomass production of *L. reuteri*.

1.4 Glycerol bioconversion into 3-HP by *L. reuteri*

1.4.1 Major challenges of 3-HP bioproduction from glycerol by *L. reuteri*

The bioconversion of glycerol into 3-HP by *L. reuteri*, according to the Pdu pathway shown in Section 1.2.3.4, shall take into account five main barriers that have to be controlled in order to achieve high performances during the bioprocess. These barriers are presented below, as well as the answers to avoid them.

1.4.1.1 Separation of growth and bioconversion steps

Due to the characteristics of the metabolic pathway that transforms glycerol into 3-HP (Figure 1.7), the growth stage of *L. reuteri* must be separated from the subsequent bioconversion phase, during which the cells will be used as whole-cell biocatalysts. Indeed, when glucose and glycerol are simultaneously brought in the medium, the cofactor NADH generated from the glycolytic pathway leads to a redox imbalance between NAD^+ and NADH in the Pdu

pathway (Dishisha *et al.*, 2014), thus resulting in 1,3-PDO production only (Lüthi-Peng *et al.*, 2002; Sriramulu *et al.*, 2008). Besides, the biosynthesis of coproducts during the growth, such as lactic acid, ethanol and acetic acid will be a hindrance to the further 3-HP extraction processes (Burgé, 2015). Consequently, to separate the growth from the 3-HP bioproduction, a specific step of biomass harvesting coupled with a washing of cell pellets is necessary to eliminate the growth medium that contains metabolites and residual glucose. The solution used for washing and re-suspending cells is required to be not or less deleterious to cell physiological state. This solution can be a saline buffer, physiological water, or osmosis water that was recently used to re-suspend the resting cells *L. reuteri* DSM 17938 (Burgé, 2015). A recent study in our lab reported that cell pellets of *L. reuteri* DSM 17938 re-suspended directly in osmosis water without washing showed a better physiological state than cells suspended and washed by potassium phosphate buffer or by physiological water (Görge, 2016).

As a direct consequence of the separation of growth and bioconversion steps and of the dilution of the cells in osmosis water, a lack of intracellular energy may occur in the resting cells during the bioconversion, as there is no more carbon source for maintenance. Depletion of intracellular energy in resting cells may reduce the bioreaction kinetics. However, during the last step of the Pdu pathway that refers to the transformation of 3-hydroxypropionyl-phosphate into 3-HP, some ATP regeneration occurs (Figure 1.7) that may limit this energy depletion (Matsakas *et al.*, 2018).

1.4.1.2 Limited conversion yield

The second challenge that 3-HP bioproduction shall take up concerns the limitation of the conversion yield of glycerol into 3-HP, that cannot be higher than a maximum value of 50 % in mole (Dishisha *et al.*, 2014). This limitation is the result of the redox balance requirement that directs the catabolism of glycerol into equimolar proportions of 3-HP and 1,3-PDO (Figure 1.7). One solution to overcome this barrier is to couple the bioconversion process with another bioprocess to convert 1,3-PDO into 3-HP. This was proposed by (Dishisha *et al.*, 2015) and (Spinnler *et al.*, 2020) who demonstrated that 1,3-PDO produced by *L. reuteri* can subsequently be converted into 3-HP using an acetic acid bacterium such as *G. oxydans* (Dishisha *et al.*, 2015) or *Acetobacter* sp. CIP 58.66 (Spinnler *et al.*, 2020).

1.4.1.3 Vitamin B12 supplementation

The mandatory presence of vitamin B12 constitutes the third challenge of the glycerol bioconversion process into 3-HP. Vitamin B12 is as a required cofactor of glycerol dehydratase, the enzyme involved in the dehydration of glycerol into 3-HPA (Toraya, 2002). Although *L. reuteri* owns the advantage of natural ability to synthesize this component (Matsakas *et al.*, 2018), a supplementation of the bioconversion medium with an exogenous source of vitamin B12 may boost the glycerol metabolism (Couvreur *et al.*, 2017). However, this supplementation is costly, which could be a serious impediment in the context of industrial production of 3-HP.

1.4.1.4 Inhibition of bioconversion by 3-HPA

The fourth challenge of the 3-HP production from glycerol is linked to the toxicity of the metabolic intermediate 3-HPA, which is an antibacterial agent (Schaefer *et al.*, 2010) that conferred the probiotic properties to *L. reuteri*. The aldehyde group of 3-HPA is highly reactive and thus can be converted into additional compounds in aqueous solution (Vollenweider *et al.*, 2003). 3-HPA can be dimerized, thus forming HPA dimer, or hydrated to form HPA hydrate. These different forms of 3-HPA constitute the "HPA system" (Figure 1.9). The 3-HPA dimer is known as reuterin (Schaefer *et al.*, 2010; Vollenweider *et al.*, 2003). In the context of 3-HPA bioproduction, 3-HPA is an inhibitor of the enzyme propionaldehyde dehydrogenase, which transforms 3-HPA into 3-hydroxypropionyl CoA. The minimum inhibitory concentration (MIC) of the bioconversion step was shown to be equal to 0.6 $g \cdot L^{-1}$ (equivalent to 8.11 mM) for *L. reuteri* 20016 (Sabet-Azad *et al.*, 2013). The MIC of 3-HPA for *L. reuteri* growth has been reported at 30 to 50 mM (or 2.2 $g \cdot L^{-1}$ to 3.7 $g \cdot L^{-1}$) for *L. reuteri* DSM 20016 (Cleusix *et al.*, 2007).

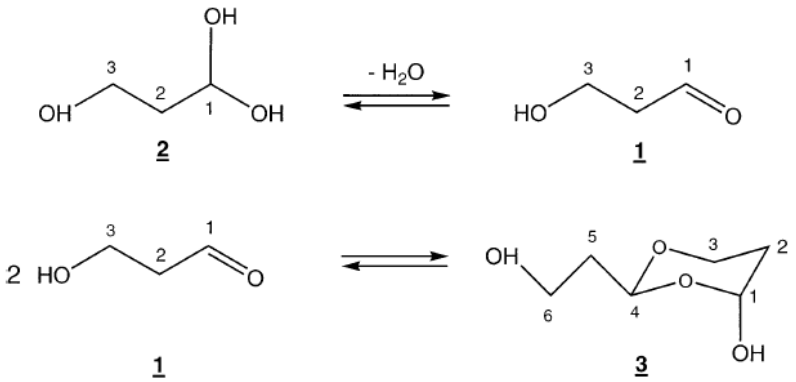


Figure 1.9. HPA system in aqueous solution: 3-HPA (1); HPA hydrate (2), and HPA dimer (3) (adapted from (Vollenweider *et al.*, 2003))

As an intermediate metabolite in the Pdu pathway, the accumulation of intracellular 3-HPA can be deleterious to the bacterial cells. The toxicity of 3-HPA is caused by unspecific reactions of the aldehyde with sulfhydryl groups of cellular proteins, thus resulting in their inactivation (Krauter *et al.*, 2012; Schaefer *et al.*, 2010). The problem is that the dehydration of glycerol into 3-HPA by the enzyme glycerol dehydratase is more rapid than the subsequent reduction and oxidation of 3-HPA into 1,3-PDO and 3-HP, as demonstrated by (Dishisha *et al.*, 2014). By using metabolic flux analysis, these authors demonstrated that the dehydration rate was 10 times higher than the rates of the subsequent reactions. In order to counteract this problem, the glycerol has to be progressively supplied and its concentration shall be maintained at a low value during the bioconversion to limit the activity of the glycerol dehydratase and to maintain a low level of 3-HPA in the cytoplasm. Consequently, fed-batch bioprocesses must be employed instead of batch processes, to limit the glycerol concentration value all along the bioconversion (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015). In that case, the glycerol feeding rate shall be optimized in order to prevent the toxicity caused by 3-HPA accumulation.

1.4.1.5 Inhibition of bioconversion by 3-HP

In the whole-cell bio-catalysts, inhibition by metabolic products is often a regulatory process during bioconversion (Ramakrishnan *et al.*, 2015). As other organic acids, 3-HP exhibits a toxic effect to the cells (Burgé *et al.*, 2015c; van Maris *et al.*, 2004). This toxic effect is due to the consecutive acidification of the medium, that lowers the 3-HP production rate (Burgé *et al.*, 2016). Moreover, cell needs more ATP to excrete the 3-HP produced in order to maintain the pH homeostasis. Thus, these phenomena imply that the intracellular ATP concentration may decrease when 3-HP accumulates.

In addition, when the pH is not controlled during the bioconversion, the proportion between the dissociated and non-dissociated forms of 3-HP may vary. At low pH, the non-dissociated form predominates, which is more deleterious as indicated by (Schepers *et al.*, 2002).

The end product-related inhibition occurred when using *L. reuteri* RPRB3007 as a biocatalyst for 3-HP bioproduction (Ramakrishnan *et al.*, 2015). These authors reported that addition of 35 mM 3-HP in the bioconversion medium decreased the production of 1,3-PDO by 73 % and hindered the synthesis of 3-HP (Ramakrishnan *et al.*, 2015).

1.4.2 Production modes used for glycerol bioconversion into 3-HP by *L. reuteri*

The bioproduction of 3-HP by *L. reuteri* has been carried out through three kinds of bioprocesses: batch mode, fed-batch mode and chemostat by using free cells or immobilized cells. Table 1.44 summarizes the main results that will be discussed below.

1.4.2.1 Bioconversion in batch process

The simplest mode for metabolite production is the batch process. In the context of 3-HP bioproduction by *L. reuteri*, it has been applied with both natural and engineered strains, either in flasks, Schott bottles or bioreactors.

From Table 1.44, the bioconversion performed by the wild type *L. reuteri* DSM 17938 in batch mode always led to a short bioconversion phase, between 1 h (Burgé *et al.*, 2015c) and 3 h (Couvreur *et al.*, 2017). This is explained by the fast accumulation of the toxic intermediate 3-HPA that reached 5.63 $g \cdot L^{-1}$ and 1.0 $g \cdot L^{-1}$ in the culture, as a consequence of high initial glycerol concentrations (i.e., 18.2 $g \cdot L^{-1}$, (Burgé *et al.*, 2015c) and 10 $g \cdot L^{-1}$, (Couvreur *et al.*, 2017). The longest bioconversion in batch mode achieved so far, lasted up to 6 hours, thanks to a supplementation with carbohydrazide (Dishisha *et al.*, 2014) that will be discussed in Section 1.4.2.4. This work led to a 3-HP titer of 6.9 $g \cdot L^{-1}$, along with a 3-HPA accumulation of 25.8 $g \cdot L^{-1}$. The highest 3-HP titer (8.74 $g \cdot L^{-1}$) reached in batch mode was obtained with *L. reuteri* FXZ014 after 3 h of bioconversion, together with a 3-HPA titer of 4.16 $g \cdot L^{-1}$ (Zabed *et al.*, 2019).

Table 1.4. Performances of 3-HP bioproduction from glycerol by *L. reuteri*

***L. reuteri* strains**	**Bioproduction modes**	**Operation conditions**	**3-HP titer ($g \cdot L^{-1}$)**	**3-HP productivity ($g \cdot L^{-1} \cdot h^{-1}$)**	**3-HP yield ($mol \cdot mol_{glycerol}^{-1}$)**	**3-HPA titer ($g \cdot L^{-1}$)**	**Duration of bioconversion (h)**	**References**
DSM 20016	Batch mode in bioreactor 1 L	37 °C, pH 7.0, NH_4OH 5N	6.9	0.38*	0.14*	25.8*	6	(Dishisha *et al.*, 2014)
DSM 20016	Batch then fed-batch mode in bioreactor 3 L	37 °C, pH 7.0, NH_4OH 5N	10.6	0.50*	nd	nd	21	(Dishisha *et al.*, 2014)
DSM 20016	Batch then fed-batch mode in bioreactor 3 L	37 °C, pH 5.5, NH_4OH 5N	14	0.24*	0.49*	0	58	(Dishisha *et al.*, 2015)
DSM 17938	Batch mode in Schott bottle 250 mL	37 °C, pH 6.0, KOH 10N	2.34	1.56*	0.17	5.0	1.5	(Burgé *et al.*, 2015c)
DSM 17938	Batch mode in bioreactor 5 L	37 °C, pH 6.0, KOH 10N	0.90	0.90*	0.11	5.6	1.0	(Burgé *et al.*, 2015c)
RPRB3007**	Repeated batch biotransformation in static flask 250 mL	37 °C, initial pH 7.4	nd	nd	0.28*	nd	16	(Ramakrishnan *et al.*, 2015)
DSM 17938	Batch mode in bioreactor 2 L	37 °C, initial pH 6.8	3.0	0.99*	nd	1.0	3	(Couvreur *et al.*, 2017)
RPRB3007**	Fed-batch mode in glass reactors 12 mL	37 °C, pH 7.0	3.30	0.09	0.48	nd	35	(Zaushitsyna *et al.*, 2017)
HR2**	Batch mode in vial 50 mL	37 °C, initial pH 7.4	5.20	1.04*	1.30	3*	4	(Suppuram *et al.*, 2019)
FXZ014	Batch mode in flask 500 mL	30 °C, initial pH 6.5	8.74	2.91	0,28*	4.16	3	(Zabed *et al.*, 2019)

nd: not determined; (*) data obtained by calculation from published data; (**) GMO strain

Repeated batch biotransformation has been used to partially overcome the inhibition by 3-HPA. This mode allowed reaching a 3-HP production yield that was 3.3 times higher than that obtained at the end of a non-repeated batch (Ramakrishnan *et al.*, 2015). However, a decline of the volumetric consumption rate of glycerol after two cycles had been observed. This was explained by a the lack of vitamin B12 *de novo* synthesis and a by loss of cellular viability (Ramakrishnan *et al.*, 2015).

Consequently, from this information, the batch mode was not a good choice to reach good performances for the bioconversion of glycerol into 3-HP by *L. reuteri*.

1.4.2.2 Bioconversion in fed-batch process

To avoid the limitation of 3-HP production due to the 3-HPA accumulation arising with high glycerol concentration in the bioconversion medium, the fed-batch mode is a more suitable option. This kind of bioprocess is characterized by a continuous feeding of sterile substrate, without harvesting the fermented medium containing the bioproducts. Consequently, an increase in the volume occurs during the process, which induces a dilution of the medium and consequently of the product concentration.

In fed-batch mode, the glycerol feeding rate is controlled to maintain a low residual glycerol concentration in the bioconversion medium. Some studies applied this mode of operation, by associating a first step in batch mode dedicated to *L. reuteri* growth to a second step in fed-batch mode to subsequently perform the glycerol bioconversion with a whole-cell biocatalyst approach (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015).

During a fed-batch bioconversion, the glycerol feeding rate strongly affects the performances of the bioprocess, and then the ratio between the intermediate product (3-HPA) and the final products (3-HP and 1,3-PDO). From Table 1.4, depending on the *L. reuteri* strain and glycerol feeding rate, an accumulation of 10.6 and 14 $g \cdot L^{-1}$ of 3-HP was achieved, depending on the pH of the medium (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015). The 3-HP productivity was comprised between 0.24 and 0.5 $g \cdot L^{-1} \cdot h^{-1}$. These results were linked to low values of accumulated 3-HPA that did not exceed 1.36 $g \cdot L^{-1}$, thus leading to a low molar ratio between 3-HPA and 3-HP of 0.11 $mol \cdot mol^{-1}$ compared to 4.5 $mol \cdot mol^{-1}$ in batch mode (Dishisha *et al.*, 2014).

1.4.2.3 Bioconversion using immobilized cells

Immobilization in biotechnology is defined as "the confinement or localization of viable microbial cells to a certain defined region of space in such a way as to exhibit hydrodynamic characteristics which differ from those of the surrounding environment" (Gungormusler-Yilmaz *et al.*, 2016). Various strategies can be applied for cell immobilization, including surface attachment (natured or enforced adsorption), entrapment (in hydrogel or natural polymers), self-aggregation (natural or induced) or containment behind a barrier (microencapsulation,

two-phases entrapment, use of synthetic barrier such as hollow-fibers, or use of membrane bioreactors) (Gungormusler-Yilmaz *et al.*, 2016).

Some advantages of cells immobilization are recognized: higher reaction rates, higher productivities, increased tolerance to high substrate concentration and product inhibition, thus allowing a prolonged activity of the cells (Gungormusler-Yilmaz *et al.*, 2016; Westman *et al.*, 2012). Another advantage consists in the possibility of re-using the biocatalysts (i.e., the immobilized cells) for many successive bioproduction steps (Yu *et al.*, 2016). In the case of 3-HP bioproduction, a longer bioconversion is observed by immobilizing the cells (Yu *et al.*, 2016). These authors demonstrated that bioconversion duration increased from 24 h with free cells to 150 h with immobilized cells of recombinant *E. coli* BL21(DE3) harboring a nitrilase gene used for hydrolysis of 3-HPN into 3-HP. Recently, whole-cells of *L. reuteri* RPRB3007 were immobilized in crosslinked and cryostructured monoliths by (Zaushitsyna *et al.*, 2017). When being operated in a continuous plug flow reactor, these immobilized cells co-produced 3-HP and 1,3-PDO equimolarly, with a molar ratio of 91 %, corresponding to 3.3 $g \cdot L^{-1}$ of 3-HP and 2.5 $g \cdot L^{-1}$ of 1,3-PDO (Zaushitsyna *et al.*, 2017). In addition, cell immobilization also allows easier product recovery (Outram *et al.*, 2017), which may help separating the 3-HP from the bioconversion medium.

However, besides the above-mentioned advantages, cell immobilization also shows some disadvantages that concern low mass transfer through bacterial cell membrane, diffusion limitation, possible deactivation of some functionalities during immobilization and additional cost for operation (Westman *et al.*, 2012), which may limit the use of this technique at industrial scale.

1.4.2.4 Bioconversion associating a complexation of 3-HPA

In order to alleviate the inhibitory effect of 3-HPA, a complexation of the molecule with scavengers can be considered. This approach was achieved by complexing 3-HPA with sodium bisulfite (Sardari *et al.*, 2013b), carbohydrazide (Dishisha *et al.*, 2014; Krauter *et al.*, 2012) or semicarbazide (Talarico *et al.*, 1988), with the aim to increase 3-HPA bioproduction or to protect cells from 3-HPA detrimental effects. When the complexation was performed with sodium bisulfite (43.4 $g \cdot L^{-1}$) during a fed-batch bioconversion with *L. reuteri* DSM 20016, the specific 3-HPA production and the overall molar ratio of 3-HPA to 1,3-PDO and 3-HP were increased by 2.2 times as compared to the fed-batch process without *in situ* complex formation (Sardari *et al.*, 2013b). The protective effect of carbohydrazide has been studied during 3-HPA production by *L. reuteri* SD2112 (Krauter *et al.*, 2012). The maximal titer of 150 $g \cdot L^{-1}$ 3-HPA was achieved in batch biotransformation with an initial glycerol concentration of 184 $g \cdot L^{-1}$ and an initial carbohydrazide concentration of 180 $g \cdot L^{-1}$. These conditions led to the concomitant achievement of 3-HP 5.9 $g \cdot L^{-1}$ and 1,3-PDO 6.4 $g \cdot L^{-1}$. These authors showed that when 3-HPA was entrapped, its production was improved, without benefiting to 3-HP bioproduction. The component semicarbazide was utilized during reuterin production by *L. reuteri* 1063 (Talarico *et al.*, 1988). Nevertheless, this complexation displayed an inhibition of

the production of reuterin (i.e., the dimer form of 3-HPA) due to the covalent combination of semicarbazide with 3-hydroxypropionaldehyde. From this information, the addition of 3-HPA scavengers did not lead to an increase in 3-HP bioproduction.

1.4.3 Effects of environmental conditions during bioconversion on the performances of 3-HP production from glycerol

During the bioconversion step, *L. reuteri* cells are sensitive to environmental conditions such as temperature, pH and atmosphere composition. In addition, the substrate feeding conditions of the fed-batch process and the complementation with some specific components are also influent.

1.4.3.1 Composition of bioconversion medium

Before entering the bioconversion phase, cells resulting from the growth phase have to be harvested and washed to eliminate growth products and remaining glucose. It is also possible to concentrate the cells to maximize the biomass concentration before starting the bioconversion. Cells are then re-suspended in a specific bioconversion medium that is supplied with glycerol in fed-batch. Different bioconversion media have been proposed to perform 3-HP bioproduction.

As previously mentioned, a non-growing medium has to be used for 3-HP bioproduction, in order to prevent the synthesis of undesirable fermentation products (i.e. lactic acid, ethanol, acetic acid) (Burgé, 2015). In fact, the absence of glucose was shown to reduce the production of 1,3-PDO and to permit that of 3-HP, because of the balance between redox cofactors (Chen and Hatti-Kaul, 2017; Dishisha *et al.*, 2014). Consequently, only glycerol shall be present as a carbon source in the bioconversion broth.

Moreover, from the study of (Görge, 2016), the re-suspension of washed cells of *L. reuteri* DSM 17938 in osmosis water gave better results compared to re-suspension in potassium phosphate buffer or in physiological water. Cells were able to better maintain their enzymatic activity and to ensure the maintenance of cell energy.

The addition of Na^+ has also been documented. It leads to a decrease in the activity of the enzyme 1,3-PDO oxidoreductase (Malaoui *et al.*, 2000) that explained the decrease of 3-HPA production by *L. reuteri* ATCC 53608 (Lüthi-Peng *et al.*, 2002). However, as less 3-HPA was synthesized, it was less available for oxidation into 3-HP.

The presence of some free amino groups of peptides, that are included in complex media such as sodium caseinates, casein hydrolysates, peptones and Amicase (i.e., casein acid hydrolysate) was considered by (Lüthi-Peng *et al.*, 2002). These components may react with 3-HPA as soon as it is produced, thus leading to a decrease in its concentration in the bioconversion medium.

Finally, one has to be considered the fact that the medium composition changes during bioconversion, mainly because of the increased concentrations of metabolites such as 3-HP and 1,3-PDO. The increase in 3-HPA concentration may also occur when glycerol concentration is not maintained at a low value. As 3-HPA and 3-HP accumulation can be deleterious to the bioconversion at a certain threshold, 0.6 $g.L^{-1}$ for 3-HPA (Sabet-Azad *et al.*, 2013) and 3.2 $g.L^{-1}$ for 3-HP (Ramakrishnan *et al.*, 2015), this can lead to a decrease in the bioprocess performances.

1.4.3.2 Temperature

Glycerol metabolism using resting cells of *L. reuteri* is affected by the temperature that acts directly on the cell physiological state and on enzymatic activities (Nedwell, 1999). The optimum temperature for the dehydration of glycerol to 3-HPA was reported as 37 °C for resting cell *L. reuteri* ATCC 53608 (Lüthi-Peng *et al.*, 2002) and *L. reuteri* CG001 (Huiliang *et al.*, 2013). Meanwhile, the optimum temperature for the reduction of 3-HPA to 1,3-PDO thanks to the enzyme 1,3-PD oxidoreductase was equal to 45 °C that is probably due to a higher enzyme stability in the resting cells (Lüthi-Peng *et al.*, 2002). The impact of temperature on the enzymatic activity of the enzyme propionaldehyde dehydrogenase in Pdu pathway of *L. reuteri* KCTC 3594 was observed at 37 °C in a context of 3-HP bioproduction (Luo *et al.*, 2011). Thus, overall, the temperature 37 °C is the most convenient temperature that has been reported for 3-HP bioproduction by *L. reuteri* so far.

1.4.3.3 pH

Environmental pH affects *L. reuteri* cells during bioconversion, as it acts directly on intracellular pH, as well as on enzymatic activities. Optimum pH during glycerol bioconversion by *L. reuteri* may differ from that observed during growth. The optimum pH for growth of *L. reuteri* sp. nov. in MRS medium is comprised between 6.0 and 6.8 (Kandler *et al.*, 1980), but the optimum pH for 3-HPA bioproduction differs according to the bioproduction medium. It was shown to be pH 5.0 in osmosis water, pH 6.0 in skim milk and pH 7.0 in MRS (Lüthi-Peng *et al.*, 2002).

The optimal pH of the bioconversion stage is also related to the optimum pH of the enzymes involved in the Pdu pathway in *L. reuteri*. The enzymatic activity of the Pdu proteins was shown to be sensitive to pH changes (Lüthi-Peng *et al.*, 2002). Glycerol dehydratase displayed the highest activity at pH 7.2 for the conversion of 1,2-propanediol (1,2-PDO) to propionaldehyde (Talarico and Dobrogosz, 1990). The most rapid reactions of oxidation and reduction of 3-HPA to 3-HP and 1,3-PDO, respectively, thanks to the enzymes propionaldehyde dehydrogenase and propanediol oxidoreductase, were observed at pH 7.0 (Sabet-Azad *et al.*, 2013) and pH 6.2, respectively (Talarico *et al.*, 1990). This was confirmed by (Dishisha *et al.*, 2014) who showed that increasing the pH from pH 5.0 to pH 7.0 enhanced the specific production rates of both 3-HP and 1,3-PDO during the glycerol transformation by *L. reuteri* DSM 20016 (wild type) and by recombinant *L. reuteri* RPRB3007. Moreover, a higher

molar ratio of 3-HP to 1,3-PDO was achieved at pH 7.0 than at pH 5.0 (0.49 mol.mol^{-1} compared to 0.43 mol·mol^{-1}).

Finally, in the case of 3-HP accumulation, the pH effect is stronger as it modifies the dissociation level of the acid, whose pK_a is equal to 4.51. At low pH, the acid form becomes dominant in the medium thus generating a stronger inhibition by pH, as explained by (Schepers *et al.*, 2002) with *L. helveticus*.

1.4.3.4 Gaseous atmosphere

In the context of 3-HP bioproduction, the gaseous conditions are important not only for bacterial growth but also for the bioconversion stage. Particularly, they significantly affect the expression of enzymes involved in the glycerol pathway as well as the regeneration of the necessary cofactors (Zabed *et al.*, 2019).

It is noticeable that the enzyme glycerol dehydratase is sensitive to oxygen (Zhao *et al.*, 2015). It is expressed under anaerobic conditions but inactivated in the presence of oxygen (Huang *et al.*, 2012). Besides, the presence of oxygen reduces or even stops the synthesis of the coenzyme vitamin B12 that is necessary for glycerol metabolism (Ye *et al.*, 1996). More recently, it has been reported that oxygen inactivates the coenzyme B12 of the enzyme glycerol dehydratase, by a reaction between the activated Co–C bond of the coenzyme with oxygen (Xu *et al.*, 2009). Finally, a low dissolved oxygen level can also reduce the NAD^+ regeneration during glycerol bioconversion (Ashok *et al.*, 2013).

Maintaining anaerobic conditions by continuous bubbling of nitrogen gas was applied through biotransformation of glycerol to achieve the highest 3-HP titer (14 g·L^{-1}) obtained by resting cells of *L. reuteri* DSM 20016 so far (Dishisha *et al.*, 2015; Ramakrishnan *et al.*, 2015). No comparison with other gaseous conditions was however presented. Finally, a complete study analyzed the effect of aerobic, micro-aerobic and anaerobic environments on the bioproduction of 3-HP, 1,3-PDO and 3-HPA by *L. reuteri* FXZ014 (Zabed *et al.*, 2019). The authors recommended to use micro-aerobiosis in order to improve 3-HP bioproduction, whereas anaerobiosis intensified 1,3-PDO bioproduction.

From these results, the use of micro-aerobic conditions was shown to be the better choice to stimulate the glycerol bioconversion into 3-HP.

1.4.3.5 Glycerol feeding rate and specific glycerol feeding rate

As a precursor of 3-HP, the glycerol concentration in the bioconversion medium clearly impacts the bioconversion performances in fed-batch cultures. However, only few studies are presently published on this subject.

A range of glycerol feeding rates has been studied with *L. reuteri* DSM 20016 (Dishisha *et al.*, 2014) who applied a multistep fed-batch conditions. The authors showed that increasing the glycerol feeding rate from 0.6 to 1.6 g·h^{-1} enhanced the 3-HP productivity, from 0.29 g·L^{-1}·h^{-1}

to 0.64 $g \cdot L^{-1} \cdot h^{-1}$. By that, 3-HP titer reached 2.92 and 6.37 $g \cdot L^{-1}$, respectively, after 10 hours for each step whereas 3-HPA did not accumulate. A recent study of glycerol bioconversion operated by *L. reuteri* CICC 6118 in batch mode highlighted the relationship between glycerol concentration and 3-HP bioproduction (Zabed *et al.*, 2019). In this work, the best 3-HP titer of 8.55 $g \cdot L^{-1}$ was achieved (but with concomitant 3-HPA accumulation) when glycerol was supplemented at 2.97 $g \cdot L^{-1}$ for three hours that corresponded to a glycerol feeding rate of 0.99 $g_{glycerol} \cdot h^{-1}$.

Because of the differences observed in the enzymatic activities involved in the glycerol catabolism according to the Pdu pathway (Dishisha *et al.*, 2015), the rate of glycerol supply directly affects the production of intermediate metabolites, mainly 3-HPA. More precisely, the activity of the glycerol dehydratase is higher than that of the aldehyde dehydrogenase (Dishisha *et al.*, 2015). Due to this difference, it is required to control the glycerol supply rate to avoid or reduce detrimental 3-HPA accumulation. The concept of specific glycerol feeding rate is then highly relevant to characterize the bioconversion in fed-batch mode as it takes into account the bacterial concentration in the bioreactor, in addition to the substrate feeding rate (Dishisha *et al.*, 2015).

Table 1.5 summarizes the results of the two studies that have been published on 3-HP production from glycerol in fed-batch mode.

Table 1.5. Specific rates characterizing the glycerol bioconversion by resting cells of *L. reuteri* DSM 20016

Glycerol feeding rate ($g_{glycerol} \cdot h^{-1}$)	0.6	1.0	0.6	1.6	0.75
pH	5.0	5.0	7.0	7.0	5.0
Specific glycerol feeding rate ($mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	100.0*	167.7*	100.0*	267.7*	62.5
Specific 3-HP production rate ($mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	43.0	62.4	49.3	110.8	31.0*
Specific 1,3-PDO production rate ($mg_{1,3\text{-}PDO} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	36.3	52.7	41.7	93.7	28.0*
Specific 3-HPA production rate ($mg_{3\text{-}HPA} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	0	25.9	0	29.0	0
References	(Dishisha *et al.*, 2014)				(Dishisha *et al.*, 2015)

* Data obtained by calculations from the results given in the article

From Table 1.5, it can be seen that the higher specific feeding rate resulted in the higher specific production rate of final products (3-HP and 1,3-PDO) along with higher accumulation of the toxic intermediate 3-HPA, irrespective of pH. Consequently, the specific feeding rate of glycerol dedicated to 3-HP bioproduction without undesirable accumulation of 3-HPA was recommended at 62.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ when the culture was conducted at pH 5.0 or at 100

$mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ when the culture was conducted at pH 7.0 (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015).

1.4.4 Effects of growth conditions on glycerol bioconversion into 3-HP

1.4.4.1 Harvesting time during growth

With a view of their subsequent use, the time at which the cells are harvested during growth in batch cultures influences the physiological state of bacteria. This has been clearly demonstrated by (Rault, 2009), who showed that the viability and the acidification activity of different lactobacilli species differed when the cells were recovered at the end of exponential phase, the beginning of stationary phase or in late stationary phase. (Rault *et al.*, 2008) explained that enzymatic activities and membrane integrity, which were measured using fluorescent labeling of the cells coupled to flow cytometry detection, can be used to define the physiological state of the cells, and that these properties were affected by harvesting time. Their results show that harvesting cells of *L. delbrueckii* subsp. *bulgaricus* CFL1 in stationary phase helped bacteria to better survive and maintain their acidification activity (Rault *et al.*, 2009). This was confirmed with *Lactococcus lactis* subsp. *lactis* TOMSC161, that exhibited better survival and resistance to freeze-drying and storage when the cells were harvested 6 h after entering the stationary phase instead of at the beginning of this phase or after 3 h (Velly *et al.*, 2014). However, when the cells of *L. bulgaricus* CFL1 stayed for a longer time in stationary phase, a significant loss of cultivability was observed, thus indicating that a degradation of the physiological state occurred (Rault *et al.*, 2008).

By considering *L. reuteri* ATCC 55730, cells collected 2.5 h after entering the stationary phase showed higher viability (80 %) after freeze-drying than when they were harvested after 5 h (50 %) (Palmfeldt and Hahn-Hägerdal, 2000). In addition, from the study of Ramakrishnan et al. (2015), cells recovered in late stationary (12 h) showed an enhancement of glycerol bioconversion, which increased the specific 3-HP production rate (0.17 $g_{3\text{-HP}} \cdot g_{CDW}^{-1} \cdot h^{-1}$) as compared to cells recovered in exponential phase (0.07 $g_{3\text{-HP}} \cdot g_{CDW}^{-1} \cdot h^{-1}$) (Ramakrishnan *et al.*, 2015). These results were recently confirmed with *L. reuteri* FXZ014 by (Zabed *et al.*, 2019). These authors achieved the highest concentrations of 3-HP and 1,3-PDO with cells collected in stationary phase (sampling after 12 h of culture). In addition, the accumulation of 3-HPA was significantly lower than with cells recovered in exponential phase (sampling at 6 h and 9 h) or in late stationary phase (sampling at 18 h) (Zabed *et al.*, 2019). The increase in 3-HP and 1,3-PDO production was explained by an increase in some enzyme activities when the cells reached the stationary phase. For 3-HP, the expression of *pduP*, *pduL* and *pduQ* genes was 3.7-fold up-regulated in these cells (Ramakrishnan *et al.*, 2015). The simultaneous over-production of 1,3-PDO was explained by the higher expression of the genes *pduCDE* and *pduQ* along with *pduP*, *pduL*, *pduQ* (Ramakrishnan *et al.*, 2015).

From this information, a diversity of results was demonstrated by considering the effect of the harvesting time on 3-HP bioproduction, depending on the species, the strains and the growth conditions. However, a consensus emerged with a better production when the bacterial cells are recovered in early stationary phase.

1.4.4.2 Addition of vitamin B12 in growth medium

The addition of exogenous vitamin B12 (or cyanocobalamin) has been considered for the glycerol bioconversion into 3-HP by *L. reuteri*. Regarding the Pdu pathway (Figure 1.7), cyanocobalamin is an essential cofactor of the enzyme glycerol dehydratase. However, as it is occasionally inactivated during the reaction catalyzed by this enzyme (Kumar *et al.*, 2013; Toraya, 2002), the addition of exogenous vitamin may be required (Kumar *et al.*, 2013). Moreover, the moment at which the supplementation with vitamin B12 is done may affect the ability of the cells to uptake the molecule at the intracellular level. It has been reported that the introduction of vitamin B12 (0.1 $mg \cdot L^{-1}$) in the culture medium of *L. reuteri* DSM 17938 although not influencing the bacterial growth, positively impacted the 3-HP bioproduction in batch mode (Couvreur *et al.*, 2017).

Consequently, the medium supplementation with vitamin B12 shall be considered, either in growth or in bioconversion media, with the aim of ensuring a real improvement of 3-HP production, while taking care to limit the production cost as cobalamin is quite expensive.

1.4.4.3 Addition of 1,2-propanediol in the growth medium

1,2-PDO, also called propylene glycol, owns a similar chemical structure to glycerol. As previously seen, the metabolic pathway for glycerol bioconversion into 3-HP by *L. reuteri* follows the Pdu pathway (Figure 1.7). The addition of 1,2-PDO in the growth medium has been studied by (Chen *et al.*, 1994) as it is involved in the activation of genes of the Pdu pathway. For this reason, (Sriramulu *et al.*, 2008) added 1,2-PDO at a concentration of 5 $g \cdot L^{-1}$ into *L. reuteri* DSM 20016 growth medium prior to glycerol conversion. Cells incubated in this 1,2-PDO-containing medium showed the maximal glycerol dehydratase activity in comparison with those incubated in media containing only glucose or glucose plus glycerol. This was confirmed by the high 3-HP titer of 14 $g \cdot L^{-1}$ that was achieved by *L. reuteri* DSM 20016 resting cells previously grown in MRS medium containing 1.5 $g \cdot L^{-1}$ 1,2-PDO (Dishisha *et al.*, 2015).

From these studies, the early addition of 1,2-PDO in the growth medium is expected to prepare the enzymatic pool of the bacterial cells and to further stimulate the glycerol bioconversion into 3-HP. However, the definition of an appropriate concentration of 1,2-PDO can be useful for the optimum expression of the enzymes. Besides, its mechanism on glycerol metabolism has not yet been elucidated.

1.4.5 Summary of information

The 3-HP bioproduction from glycerol by *L. reuteri* faces five major challenges that have to be alleviated: (1) the separation of the growth step from the bioconversion step that is accompanied by a limitation of intracellular energy and a loss of cellular viability; (2) the limitation of the conversion yield at 50 % as a result of cofactor balance; (3) the necessity of vitamin B12 as an expensive cofactor for the first enzyme of the Pdu pathway; (4) the deleterious inhibition by the intermediate metabolite 3-HPA and (5) the deleterious inhibition by the final product 3-HP.

In order to overcome these issues, the fed-batch and continuous culture modes are advantageous as they allow maintaining low concentrations of 3-HPA during the biotransformation. At that time, mainly fed-batch mode was studied to produce 3-HP. The nutritional and environmental conditions have to be selected in order to prepare the enzymatic machinery of the cells for further bioconversion. The growth medium composition can be improved by adding 1,2-PDO and exogenous vitamin B12, and the harvesting time of the cells has to be defined probably in stationary phase. Finally, the effects of temperature, pH, gaseous atmosphere and substrate feeding rate have been studied in order to increase 3-HP bioproduction.

1.5 Knowledge synthesis

The increasing interest for 3-HP as a building block recently led to some achievements in the bioproduction of this renewable chemical. Overall, glucose and glycerol are the two most common carbon sources that have been used depending on the microbial species. Even if biochemical pathways to produce 3-HP from glucose exist, the most interesting microorganisms own naturally the enzymes involved in the glycerol metabolism. Among them, *L. reuteri* is a promising candidate since this species harbors all enzymes of the Pdu pathway, together with the ability to synthesize vitamin B12 as an essential cofactor. The bioproduction of 3-HP by *L. reuteri* has to be performed through a two-steps process that includes (1) a growth phase in batch mode to increase the cell concentration and (2) a bioconversion phase in fed-batch mode, during which the precursor glycerol is converted into 3-HP and 1,3-PDO by resting cells of *L. reuteri*. If many studies investigated the ability of this species to produce 3-HP from glycerol, the optimum conditions for this bioproduction have not been fully determined. Consequently, conducting studies on the effect of culture and environmental conditions is important since this will help to optimize the bioproduction process. These conditions may act either during the first step of the bioprocess, i.e. during bacterial growth, or during the second step, namely the bioconversion.

In that context, the present work aims at defining the conditions that affect the glycerol bioconversion into 3-HP. These conditions have to be considered first during the growth

phase, at the end of which the physiological state of the bacterial cells as well as the biomass concentration shall be maximized. Secondly, the conditions employed during the bioconversion step also have to be regarded, in order to increase the bioprocess performances. To address these issues, the bibliographic synthesis has brought to light some important medium components and environmental conditions during the growth that will be studied in the first chapter of the experimental study. In the second chapter of the experimental work, some conditions used during the bioconversion step will be explored, with the objective to improve 3-HP bioproduction by *L. reuteri*.

Chapter 2. Materials and methods

2.1 Overall methodology

This work aims at studying the effects of several nutritional and environmental factors during the growth step and the bioconversion step, in order to achieve the best conditions to obtain 3-HP by *L. reuteri* DSM 17938. The overall scheme of the study is illustrated in Figure 2.1.

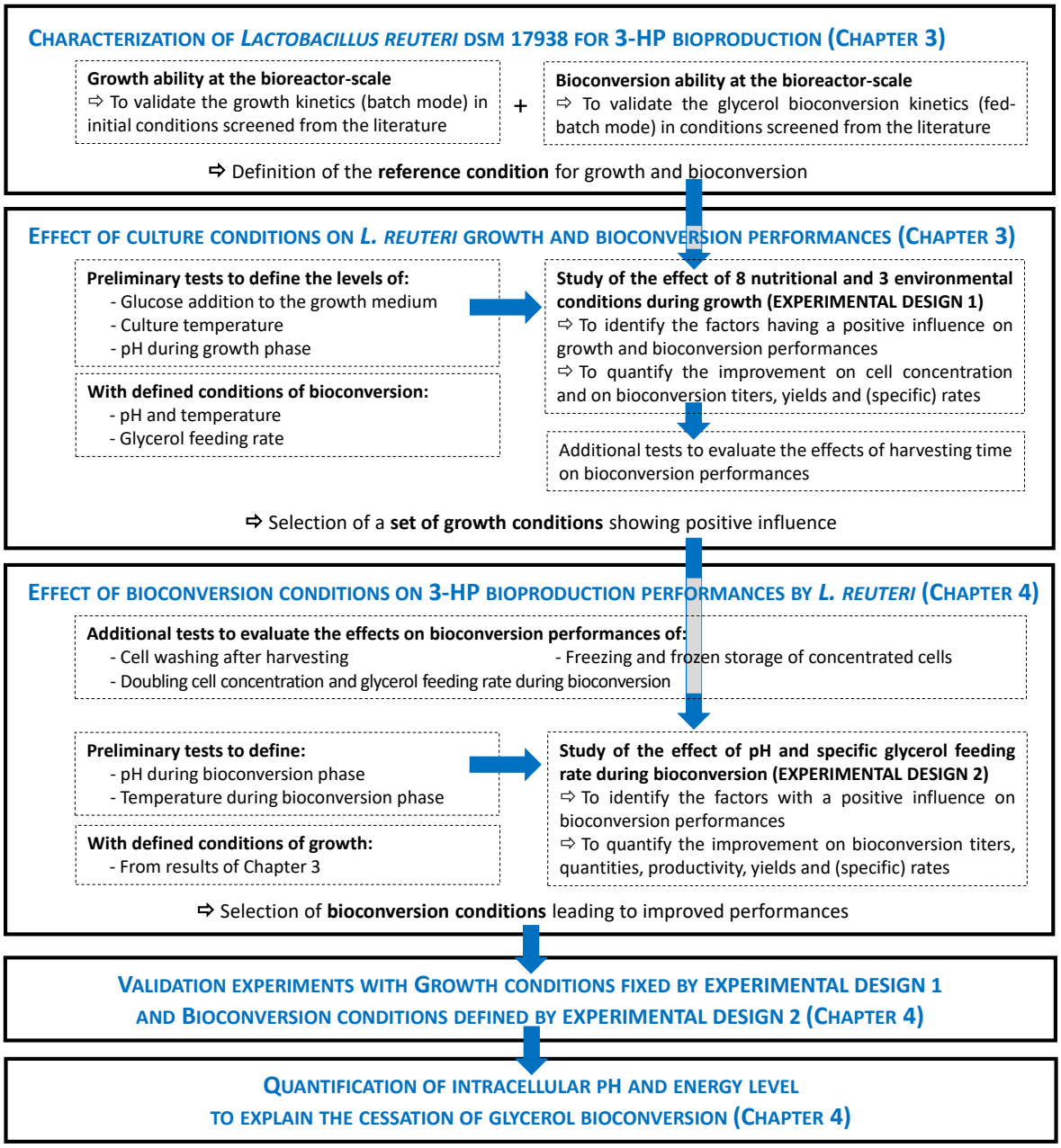


Figure 2.1. Scheme of the experimental strategy performed in the present study

Firstly, the conditions for growth of *L. reuteri* DSM 17938 and glycerol bioconversion into 3-HP by resting cells were selected from the bibliographic study. These conditions were carried out to characterize the bacterial growth and the glycerol metabolism in bioreactors, as well as the repeatability of the experiments. Then, some preliminary tests were processed before each experimental design to define the factors to investigate and the lower and upper limits of the tested factors.

Then, a first experimental design allowed studying the effects of nutritional and environmental conditions during growth on the biomass production and on the cell ability to subsequently perform glycerol bioconversion. These experiments were carried out using fixed conditions during bioconversion.

Thirdly, with this new set of growth conditions, a second experimental design was conducted with the aim to study the effects of some environmental conditions during the bioconversion step on the 3-HP bioproduction. These experiments were performed using the set of growth conditions fixed as a result of the first experimental design.

Finally, the selected growth and bioconversion conditions issued from the two experimental designs were used simultaneously to improve the 3-HP bioproduction process.

2.2 Materials

2.2.1 Bacterial strain

The strain *Lactobacillus reuteri* DSM 17938 was obtained from BioGaia AB, Stockholm, Sweden (Figure 2.2). It was stored at - 80 °C in cryotubes with glycerol 20 % (v/v) as cryoprotectant. The same work bank was used during the whole study.

(A) (B)

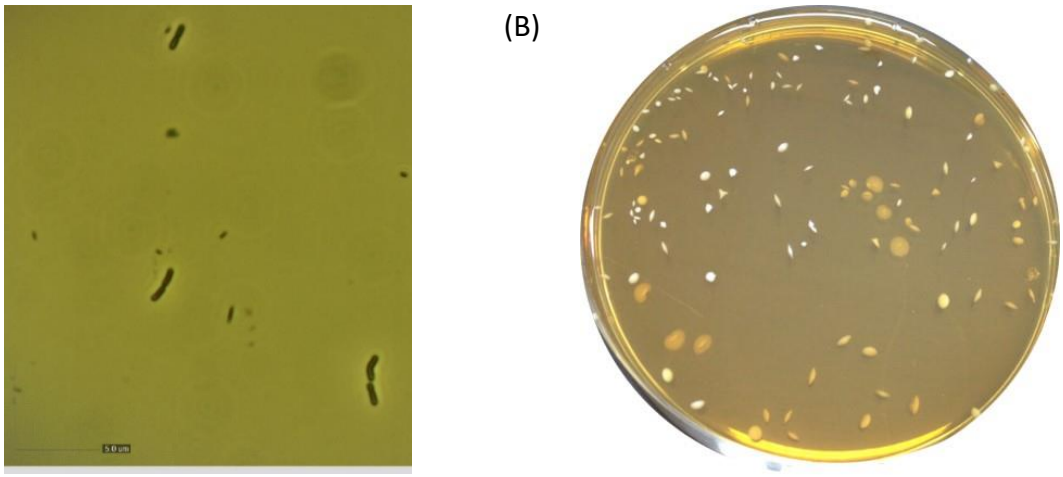


Figure 2.2. Cell morphology of *L. reuteri* DSM 17938 under optical microscope x 100 (A) and their colonies on and inside a MRS agar plate (B)

The purity of the strain was verified by 16S rRNA gene sequencing using Sanger sequencing (Eurofins Genomics, Les Ulis, France). The detail of the method is described in Appendix 1.

The BLAST algorithm (Figure 2.3) was used to compare the 16S rDNA sequences with known sequences found in NCBI GenBank (https://www.ncbi.nlm.nih.gov/). Results obtained from two samples (i.e., stored cells of the work bank and fresh cells after culture in MRS medium) were the same. The 16S rRNA nucleotide sequences matched at 99.9 % with that of *L. reuteri* ATCC 55730 and at 99.8 % with the type strain *L. reuteri* DSM 20016. It was reported in the literature that *L. reuteri* DSM 17938 is the daughter strain of *L. reuteri* DSM 55730 (i.e. a strain of plasmid-cured ATCC 55730 with two deleted antibiotic genes) (Rosander *et al.*, 2008) and that the strain *L. reuteri* DSM 20016 is close to *L. reuteri* DSM 55730.

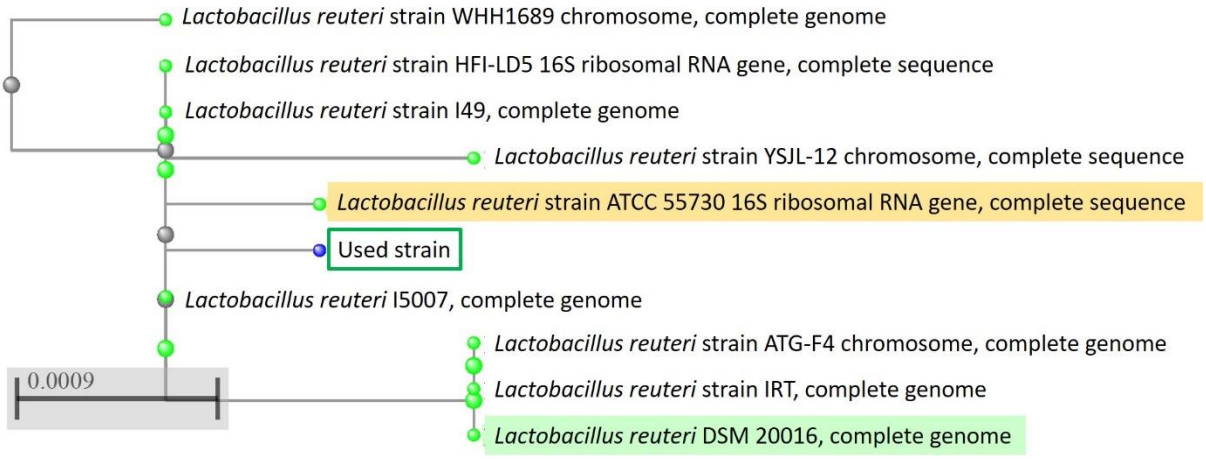


Figure 2.3. Phylogenetic tree allowing the comparison of the used strain *L. reuteri* DSM 17938 with sequences available in NCBI GenBank

2.2.2 Chemicals used

The chemicals used in this study were purchased or synthesized as listed in Table 2.1.

Table 2.1. Chemicals used in the study

Chemical	Chemical formula	CAS-No.	Purity	Supplier
1,2 PDO	$C_3H_8O_2$	57-55-6	99.5 %	Sigma-Aldrich, Saint-Quentin-Fallavier, France
1,3-PDO	$C_3H_8O_2$	504-63-2	98 %	Sigma-Aldrich
3-HP	$C_3H_6O_3$	503-66-2	~ 30 %	TCI Europe, Zwijndrecht, Belgium
3-HPA	$C_3H_6O_2$	na	100 % [a]	URD ABI, Pomacle, France [b]
Acetic acid	$C_2H_4O_2$	64-19-7	≥ 99.8 %	Sigma-Aldrich

Acetone	C_3H_6O	67-64-1	> 99.5 %	Fisher Scientific, Illkirch, France
Ammonium hydroxide	NH_4OH	1336-21-6	28 – 30 %	Sigma-Aldrich
Betaine	$C_5H_{11}NO_2$	107-43-7	≥ 98 %	Sigma-Aldrich
Carboxyfluorescein diacetate (Chemchrom V8)	na	306-R1002-01[d]	na	Biomérieux, Marcy-l'Etoile, France
Carboxyfluorescein diacetate succinimidyl ester	$C_{29}H_{19}NO_{11}$	150347-59-4	≥90%	Thermo Fisher Scientific, Villebon sur Yvette, France
Disodium dihydrogenphosphate	$Na_2HPO_4.H_2O$	10028-24-7	99 %	VWR, Fontenay-sous-Bois, France
Ethanol	C_2H_5O	64-17-5	≥ 99.9 %	Carlo Erba, Chaussée du Vexin, France
Glucose	$C_6H_{12}O_6$	50-99-77	99 %	VWR
Glycerol	$C_3H_8O_3$	56-81-5	≥ 99 %	Sigma-Aldrich
L-cysteine hydrochloride hydrate	$C_3H_7NO_2S.HCl.H_2O$	345909-32-2	≥ 99 %	Sigma-Aldrich
Lithium L-lactate	$C_3H_5LiO_3$	27848-80-2	≥ 98 %	Sigma-Aldrich, Saint Louis, MO, USA
Monohydrate citric acid	$C_6H_8O_7$, H_2O	77-92-9	99.5 %	Acros Organics, Geel, Belgium
MRS Broth	$CH_{1.98}O_{0.82}N_{0.14}S_{0.01}$[c]	na	na	Biokar Diagnostic, Beauvais, France
Phytone peptone (from soymeal)	$CH_{1.85}O_{0.71}N_{0.22}S_{0.02}$[c]	391079-46-8	na	Merck KGaA, Darmstadt, Germany
Potassium chloride	KCl	7447-40-7	≥ 97 %	VWR
Propidium iodide	$C_{27}H_{34}I_2N_4$	25535-16-4	≥ 94 %	Sigma-Aldrich
Sodium hydroxide	NaOH	1310-73-2	35 %	VWR
Trichloroacetic acid	$C_2HCl_3O_2$	76-03-9	> 99 %	Sigma-Aldrich
Tween 80	na	9005-65-6	≥ 99 %	VWR
Vitamin B12 (cyanocobalamin)	$C_{63}H_{88}CoN_{14}O_{14}P$	68-19-9	≥ 98 %	Sigma-Aldrich
Yeast extract	$CH_{2.02}O_{0.67}N_{0.24}S_{0.01}$[c]	na	na	Organotechnie, La Courneuve, France

([a]) The purity is considered as 100 %

([b]) The chemical 3-HPA is not commercially available. It was synthesized according to the protocol of (Burgé, 2015) in URD ABI-AgroParisTech, Pomacle, France as described in Appendix 2

([c]) The chemical formula has been determined in this study as explained below

([d]) Stock Keeping Unit (SKU) number

na: information not available

2.2.3 Elemental analysis of *L. reuteri* DSM 17938 and complex media

An elemental analysis was performed to determine the cell formula of *L. reuteri* DSM 17938 and the elemental composition of yeast extract and phytone peptone used as components of the growth medium. A 2400 Series II CHNS/O Elemental Analyzer (PerkinElmer, Waltham, MA, USA) was used by the Institute of Chemistry of Natural Substances to determine the percentages of C, H, O and N elements (Service de microanalyses, C.N.R.S, Gif-sur-Yvette, France). The detailed analysis is given in Appendix 3.

2.2.3.1 Elemental analysis of complex media

The chemical formula of MRS was found to be $CH_{1.98}O_{0.82}N_{0.14}S_{0.01}$. Those of phytone peptone and yeast extract were respectively determined as $CH_{2.02}O_{0.67}N_{0.24}S_{0.01}$ and $CH_{1.85}O_{0.71}N_{0.22}S_{0.02}$. This information is summarized in Table 2.1.

2.2.3.2 Elemental analysis of *L. reuteri* DSM 17938

Duplicate analyses were carried out from three samples of cell pellets prepared independently. The cells were previously obtained from a batch culture in MRS medium added with glucose 20 $g \cdot L^{-1}$ (37 °C, free pH, 8 h), then concentrated by centrifugation at 5000 *g*, 10 min, 4 °C. The cell pellets obtained after discarding supernatant were transferred to cryotubes and freeze-dried in a SMH 15 freeze-drier (Usifroid, Maurepas, France). They were stored at 4 °C until chemical analysis.

The cell formula of *L. reuteri* DSM 17938 was defined as $CH_{1.89}O_{0.62}N_{0.24}$. This result is consistent with the general formula of bacteria, this one being $CH_{1.75}O_{0.5}N_{0.25}$ (Metcalf, 2003).

From this cell formula, the cell molecular weight was calculated as 27.17 $g \cdot mol^{-1}$. It allowed determining the number of moles of carbon incorporated in bacterial cells during growth, from the difference between the final and initial cell dry weights (CDW), using the following equation:

$$n_{C\ in\ biomass}\ (mol) = \frac{FinalCDW - InitialCDW}{27.17} \qquad \text{(eq. 2.1)}$$

This value was used to determine the carbon mass balances during the cultures, as explained in Section 2.5.5.

2.3 Two-step bioprocess for 3-HP bioproduction

As explained in Chapter 1, the glycerol bioconversion shall be separated from the bacterial growth. This was necessary to allow the cells to use different carbon sources to favor either the growth or the bioconversion, and to keep a favorable redox balance during glycerol metabolism. Thus, a two-step process was performed, as illustrated in Figure 2.4 and

described below. It includes mainly a growth phase, performed in batch mode with glucose as the carbon source, and a bioconversion phase by resting cells obtained from the first step, performed in fed-batch mode with glycerol as the carbon source.

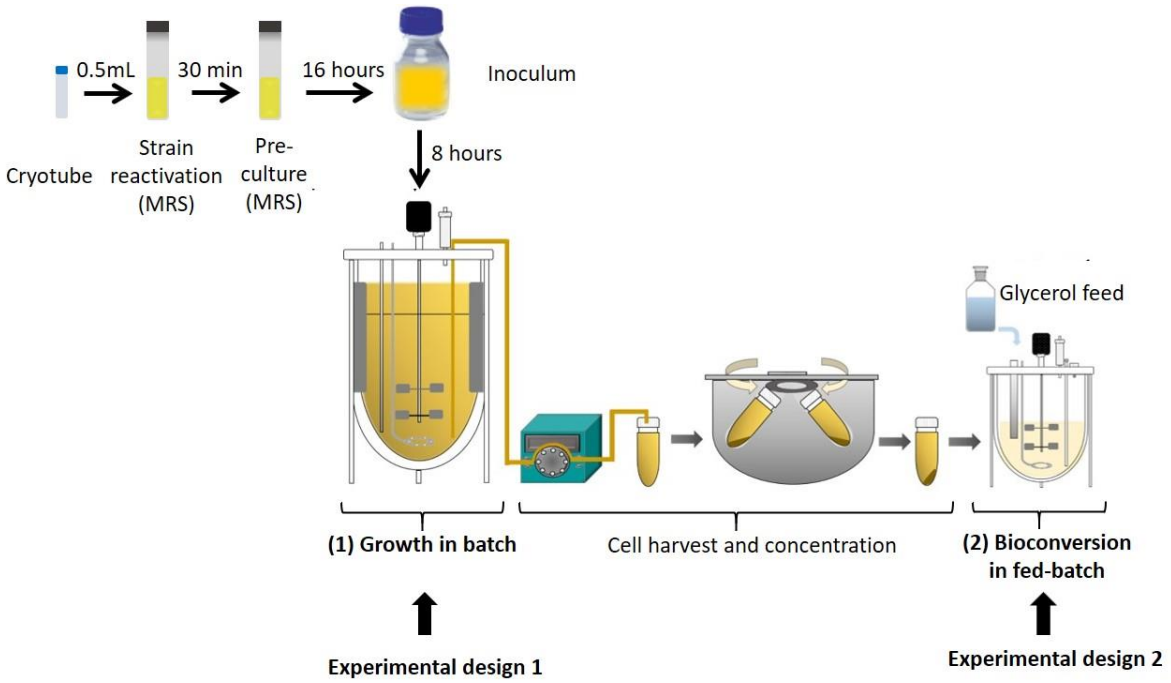


Figure 2.4. Two-step process for 3-HP bioproduction by *L. reuteri* DSM 17938

2.3.1 Bacterial growth in batch mode

Bacterial cells were first reactivated by introducing 0.5 mL of a work bank cryotube (gently thawed from - 80 °C to ambient temperature) into 9 mL of MRS broth in a test tube that was incubated for 30 minutes at 37 °C and 100 rpm. Then, a volume of 0.5 mL was transferred into another test tube containing 9 mL of MRS broth. After a 16-h incubation at 37 °C and 100 rpm, cells from this pre-culture were used to prepare the inoculum in MRS broth supplemented with 20 $g \cdot L^{-1}$ of glucose, incubated for 8 hours in the same conditions. Inoculation of the culture medium was performed at a concentration of 5.1 x 10^{-6} g of CDW per liter ($g_{CDW} \cdot L^{-1}$), corresponding to 5.8 x 10^{3} $cells \cdot mL^{-1}$.

Bacterial growth was conducted in a 5-L bioreactor Sartorius B^{+} (Sartorius, Dourdan, France), controlled by the software BioPAT® MFCS (Sartorius). The agitation rate was supported by two Rushton propellers working at 100 rpm. Temperature was controlled using a Pt100 probe, associated to a cooling finger and a heating coat. The temperature was defined in each experimental design (33 °C or 37 °C; Table 2.2). The pH was controlled using a pH probe (Hamilton, Bonaduz, Switzerland) at pH 5.5 or 6.0 (Table 2.2), by adding either NH_4OH 14.8 $mol \cdot L^{-1}$ (Sigma-Aldrich) or NaOH 8.75 $mol \cdot L^{-1}$ (VWR), according to the experimental design (Table 2.2). Neither gas nor air was transferred into the bioreactor during the cultures to

generate micro-aerobiosis during the cultures. However, a condenser was employed to avoid evaporation.

The growth was stopped when the cells reached stationary phase. This was defined by the time when the base consumption stopped.

Sampling was done periodically to monitor cell concentration by optical density (OD) measurement at 600 nm and by flow cytometry (FCM), and to determine the cell physiological state by flow cytometry combined with double fluorescent staining . It allowed quantifying also the substrate and product concentrations by high-performance liquid chromatography (HPLC).

For some experiments, the cell concentration was quantified *on-line* by a biomass probe EXcell 230 (CellD, Roquemaure, France).

2.3.2 Cell harvesting and concentration

At the end of growth, the fermented broth was harvested aseptically by a peristaltic pump (Watson-Marlow Fluid Technology Group, Falmouth, UK). Cell pellets were separated from the supernatant by centrifugation (Avanti® J-E centrifuge, Beckman Coulter, Fullerton, CA) for the experimental design 1 or (Avanti® J-26-XP centrifuge; Beckman Coulter, Fullerton, CA) for the experimental design 2, at 6,200 *g* for 10 min at 4 °C. Cell pellets were suspended in the same volume of sterile osmosis water to reach between 17 and 36 $g_{CDW}{\cdot}L^{-1}$, corresponding to 2 to $5{\cdot}10^{10}$ cells·mL^{-1} in the experimental design 1, and between 20 and 24 $g_{CDW}{\cdot}L^{-1}$, corresponding to 2 to $3{\cdot}10^{10}$ cells·mL^{-1} in the experimental design 2.

2.3.3 Glycerol bioconversion in fed-batch mode

Two kinds of bioreactors were used for the bioconversion stage, due to the need of various working volumes. They were used in fed-batch mode, by controlling the glycerol feeding rate.

For the preliminary assays and the first experimental design, the bioconversion was achieved in a 2-L bioreactor (Sétric Génie Industriel, Toulouse, France), associated with the software WCidus V.3 (INRAE, Grignon, France). The initial working volume was set at 0.8 L. A pH probe (Mettler Toledo), a temperature probe (Sétric Génie Industriel) and a pO_2 probe (Mettler Toledo) were used to monitor the bioprocess. The pH was controlled at 6.0 by addition of NH_4OH 1.48 mol·L^{-1}, the temperature was set at 37 °C and the agitation rate was maintained at 100 rpm with two Rushton propellers. Glycerol was added during the fed-batch process using a peristaltic pump (Watson-Marlow Fluid Technology Group, Falmouth, UK) that was calibrated in advance for a glycerol concentration fixed at 100 g·L^{-1}. The glycerol feeding rate was equal to 0.5 g·h^{-1} to avoid 3-HPA accumulation.

For the second experimental design and for the validation experiments, 500-mL mini-bioreactors (Global Process Concept, La Rochelle, France) were used with an initial working volume of 200 mL in order to launch the bioproduction with smaller volumes (Figure 2.5). They were associated to the software C-Bio 2 (Global Process Concept). Temperature and agitation were controlled at 37 °C and 100 rpm, respectively. The pH was controlled using a pH probe (Mettler Toledo) at a value defined by the matrix of the second experimental design (Table 2.4) by adding NH_4OH 1.48 M. The pO_2 was measured by an optical dissolved oxygen probe (Hamilton, Bonaduz, Switzerland). Glycerol was supplied using a peristaltic pump (Watson Marlow 120U-DV) that was calibrated in advance for a glycerol concentration of 50 $g \cdot L^{-1}$. As the pump can deliver the glycerol solution with a precision of 0.1 rpm corresponding to 0.8 $mL \cdot h^{-1}$, the concentration of the glycerol feeding solution was defined for each experiment, to obtain the glycerol feeding rate targeted.

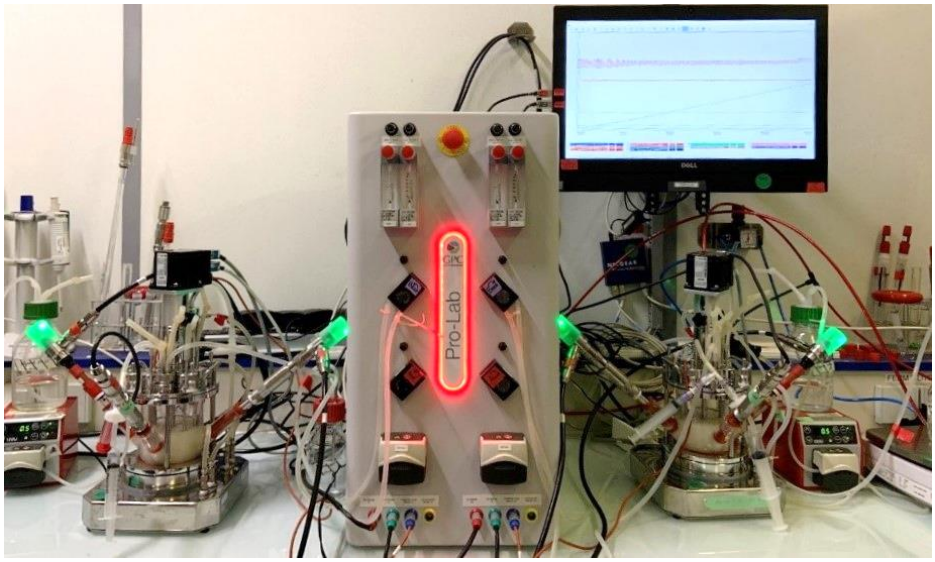


Figure 2.5. Photo of the mini-bioreactors (GPC-ProLab) used in parallel to perform the bioconversion step

In all cases, bioconversion was stopped when no more 3-HP was produced, which was evaluated by the cessation of base consumption. Concentrations of glycerol, 3-HP, 1,3-PDO and 3-HPA were measured by sampling every 1.5 h until the end of the bioprocess, followed by HPLC analysis. These regular samples were also used to monitor cell concentration and cell physiological state by flow cytometry.

2.4 Experimental designs

The principle of defining an experimental design is to achieve the targeted goals by minimizing the number of required experiments. In the context of biological transformations, experimental designs can help identifying relevant parameters that significantly act on the bioprocess or allow defining the optimal conditions to be carried out to maximize the

performances of the bioprocess. In this work, two experimental designs have been defined. The first one attempts to identify nutritional and environmental conditions during the growth of *L. reuteri* DSM 17928 that act on the ability of the cells to subsequently convert glycerol into 3-HP. Due to the high number of factors to be tested, a Plackett and Burman experimental design (Plackett and Burman, 1946) was selected in order to identify the key factors that influence cellular biomass production and glycerol bioconversion. The second one aims at determining some optimal environmental conditions that act during the bioconversion stage. A central composite rotatable design was retained to select optimal conditions for pH and glycerol feeding rate on 3-HP bioproduction

2.4.1 Plackett and Burman experimental design and statistical analysis

From the bibliographic analysis, 11 factors have been identified as providing potential effect on the growth of *L. reuteri* and its capacity to produce 3-HP from glycerol. These factors are listed in Table 2.2: glucose and yeast extract supplementation, addition of phytone peptone, 1,2-propanediol, cysteine, betaine (plus KCl), Tween 80, vitamin B12 and temperature, pH and base used for pH control.

2.4.1.1 Experimental factors and their levels

The Plackett and Burman design (Plackett and Burman, 1946) was selected to test a high number of factors, allowed creating a matrix with 11 medium components and culture conditions, with the aim to test their effects on growth and subsequent 3-HP bioproduction by *L. reuteri* DSM 17938. All factors have been tested within two levels: a Minus level and a Plus level, as described in Table 2.2. A reference condition (T1) was defined by the Minus levels of all variables, except for the pH and the temperature that were fixed at their Plus levels (corresponding to the preliminary experiments). This reference condition was carried out in triplicate.

The Minus level for glucose corresponded to a concentration of 20 $g \cdot L^{-1}$ added to MRS medium and the Plus level to an addition of 50 $g \cdot L^{-1}$, according to the results of the preliminary tests for glucose addition in Section 3.3.1.1. Adding glucose in MRS medium was done to avoid substrate limitation.

The Minus level of the other components that were added in the medium was defined at 0 $g \cdot L^{-1}$ and the Plus levels were fixed according to information from the bibliographic review (see part 1.3). The Plus levels for additional yeast extract and phytone peptone were fixed at 25 $g \cdot L^{-1}$. 1,2-PDO was added at a concentration of 3 $g \cdot L^{-1}$, cysteine at 1 $g.L^{-1}$, betaine (+ KCl) at 0.234 $g \cdot L^{-1}$ (plus 0.745 $g \cdot L^{-1}$), Tween 80 at 4 $g \cdot L^{-1}$ (MRS medium already contains Tween 80 at 1 $g \cdot L^{-1}$) and vitamin B12 at 0.1 $mg \cdot L^{-1}$.

Table 2.2. Factors included in the Plackett and Burman experimental design and their levels to study the effects of growth conditions on growth and bioconversion performances of *L. reuteri* DSM 17938

Run code	Experimental factors										
	Additional glucose ($g \cdot L^{-1}$)	Additional yeast extract ($g \cdot L^{-1}$)	Phytone peptone ($g \cdot L^{-1}$)	1,2-propanediol ($g \cdot L^{-1}$)	Cysteine ($g \cdot L^{-1}$)	Betaine ($g \cdot L^{-1}$) and KCl ($g \cdot L^{-1}$)	Additional Tween 80 ($g \cdot L^{-1}$)	Vitamin B12 ($mg \cdot L^{-1}$)	Temperature (°C)	pH	Base type (concentration, in $mol \cdot L^{-1}$)
T1	20	0	0	0	0	0 and 0	0	0	37	6	NH_4OH (14.8)
T2	50	0	25	0	0	0 and 0	4	0.1	33	6	NaOH (8.75)
T3	50	25	0	3	0	0 and 0	0	0.1	33	5.5	NH_4OH (14.8)
T4	20	25	25	0	1	0 and 0	0	0	33	5.5	NaOH (8.75)
T5	50	0	25	3	0	0.234 and 0.745	0	0	37	5.5	NaOH (8.75)
T6	50	25	0	3	1	0 and 0	4	0	37	6	NaOH (8.75)
T7	50	25	25	0	1	0.234 and 0.745	0	0.1	37	6	NH_4OH (14.8)
T8	20	25	25	3	0	0.234 and 0.745	4	0	33	6	NH_4OH (14.8)
T9	20	0	25	3	1	0 and 0	4	0.1	37	5.5	NH_4OH (14.8)
T10	20	0	0	3	1	0.234 and 0.745	0	0.1	33	6	NaOH (8.75)
T11	50	0	0	0	1	0.234 and 0.745	4	0	33	5.5	NH_4OH (14.8)
T12	20	25	0	0	0	0.234 and 0.745	4	0.1	37	5.5	NaOH (8.75)

The Minus level of temperature was defined thanks to a preliminary experiment (Section 3.3.1.2) at 33 °C, and the Plus level at 37 °C which is the optimum temperature for *L. reuteri* growth. The Minus level of pH value was assigned at 5.5 as a result of a preliminary experiment (Section 3.3.1.3) and the Plus level at pH 6.0 as optimal value for growth (Section 1.3.2.2). The base solutions for pH control were defined as NH_4OH (14.8 $mol \cdot L^{-1}$) and NaOH (8.75 $mol \cdot L^{-1}$) for the Minus and Plus levels, respectively.

In order to help the reader to better understand the results obtained from this experimental design, the pH and temperature have been introduced at their Plus level in the reference condition. This means that in the Plackett and Burman matrix, all factors of the reference condition were set at their low level except for these two factors. This did not change the calculations but facilitated the interpretation of the results.

All experiments were performed according to the two-step bioprocess that corresponded to experimental design 1 mentioned in section 2.1. They were randomized, and the reference condition was carried out in triplicate.

2.4.1.2 Responses variables and statistical analyses

The effects of the 11 factors were tested on various response variables, which were expressed according to the general model as follows:

$$Y_i = k_i + \Sigma\, a_i \cdot Y_{(+)i} \qquad \text{(eq. 2.2)}$$

Where Y_i is the response variable; k_i, the constant value of Y_i when all 11 variables are at their Minus levels (except for the factors pH and temperature); a_i, the linear coefficient of each variable to express the effect of the Plus level; and $Y_{(+)i}$, the value of the Plus level of each variable i.

Data treatment was performed by multiple regression analyses using the XLSTAT software (Addinsoft, Paris, France).

Six response variables have been selected to describe the effects of the factors on the growth and bioconversion steps. Three of them concerned the growth step:

- Cell concentration at the end of the growth step ($g_{CDW} \cdot L^{-1}$)
- Lactic acid concentration ($g_{LA} \cdot L^{-1}$)
- Molar ratio of the sum of [acetic acid + ethanol] to lactic acid which informed about the relative part of PK pathway in glycolysis ($mol_{(acetic\ acid\ +\ ethanol)} \cdot mol_{lactic\ acid}^{-1}$), as described by (Burgé *et al.*, 2015b).

Three response variables concerned the bioconversion step:

- Total quantity of 3-HP produced at the end of the fed-batch ($g_{3\text{-}HP}$)
- Specific 3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)
- Duration of the bioconversion (h)

2.4.2 Central composite design

A central composite rotatable experimental design (CCRD) with two factors at five levels was employed to search for optimized conditions during the bioconversion step. This kind of experimental design allowed generating surfaces responses by quantifying the single and quadratic effects of the factors as well as their interactions (Box and Wilson, 1951).

2.4.2.1 Experimental factors and their levels

According to the bibliographic study and results of the preliminary tests, two factors influencing the bioconversion step were included in the CCRD: the pH and the specific glycerol feeding rate (q_S, $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$). They were tested at five different levels, whose coded values and corresponding real values are given in Table 2.3. The lower limit, the central point and the upper limit of the factors were defined from the literature and according to the results of a preliminary test (Section 4.4.1).

Table 2.3. Factors of the central composite rotatable design and their five levels

Factor	Unit	Levels				
		-1.4142	**-1**	**0**	**1**	**1.4142**
pH	---	4.8	5.15	6.0	6.85	7.2
q_S	$mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$	20	29	50	71	80

q_S: Specific glycerol feeding rate

The design allowed creating a matrix of 11 combinations, including a triplicate repetition at the central point. The matrix of the experimental design is shown in Table 2.4.

All experiments were performed using the same conditions during the growth step. These conditions were selected from the results obtained in the first Plackett and Burman experimental design. They corresponded to: MRS broth supplemented with glucose 20 $g \cdot L^{-1}$, phytone peptone 25 $g \cdot L^{-1}$, 1,2-PDO 3 $g \cdot L^{-1}$, betaine 0.234 $g \cdot L^{-1}$ plus KCl 7.455 $g \cdot L^{-1}$, Tween 80 4 $g \cdot L^{-1}$. The growth temperature was set at 37 °C and the pH was controlled at 6.0 by addition of NH_4OH at 14.8 M.

The bioconversion step was conducted at 37 °C, at an agitation rate of 100 rpm and at pH and specific glycerol feeding rates defined by the experimental design.

Table 2.4. Matrix showing the coded levels of the factors tested during the bioconversion step

Run code	pH	Specific glycerol feeding rate ($mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$)
R1	5.15	29
R2	5.15	71
R3	6.85	29
R4	6.85	71
R5	4.8	50
R6	7.2	50
R7	6.0	20
R8	6.0	80
R9	6.0	50
R10	6.0	50
R11	6.0	50

2.4.2.2 Response variables and statistical analyses

The effects of the two factors have been tested on various response variables, which were expressed according to the general second order polynomial equation as follows:

$$Y = a_0 + a_1 \cdot pH_c + a_2 \cdot q_{Sc} + a_{12} \cdot pH_c \cdot q_{Sc} + a_{11} \cdot pH_c^2 + a_{22} \cdot q_{Sc}^2 \qquad \text{(eq. 2.3)}$$

Where Y is the predicted value of the response variable; pH_c and q_{Sc} are the coded values of the factors; a_0 is the regression coefficient corresponding to the central point (where all coded variables were set at 0); a_1 and a_2 are the linear coefficients; a_{11} and a_{22} are the quadratic coefficients; a_{12} is the cross-product coefficient.

Analyses of variance (ANOVA) were performed to highlight the significant effects of the factors and their interaction at $P < 0.05$. The software Statgraphics 3.0 (Statistical Graphics Corp., Warrenton, USA) was used for designing the experiments and analyzing the data.

The model has been tested on seven variables that concerned the 3-HP bioproduction:

- Maximal 3-HP titer ($g_{3\text{-}HP} \cdot L^{-1}$)
- Maximal 3-HP quantity ($g_{3\text{-}HP}$)
- 3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)
- Glycerol specific consumption rate ($mg_{Glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$)
- 3-HP production rate ($g_{3\text{-}HP} \cdot h^{-1}$)
- 3-HP specific production rate ($g_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$)
- Volumetric productivity of 3-HP ($g_{3\text{-}HP} \cdot L^{-1} \cdot h^{-1}$)

The volumetric productivity of 3-HP was calculated after 24 h of bioconversion, in order to better compare the different conditions.

2.5 Analytical methods

2.5.1 Characterization of cell concentration and cell physiological state

2.5.1.1 Quantification of cell dry weight

Cell dry weight was determined by gravimetry. A defined volume of sample was filtered on a pre-dried and pre-weighted polyethersulfone filter of pore size 0.22 µm (Sartorius, Göttingen, Germany) using a vacuum pump.

The filter was then rinsed with deionized water and dried in an oven (Memmert GmbH, Büchenbach, Germany) at 90 °C for at least 24 h until constant weight. Filters were then weighted on a precision balance (Sartorius ED224S, Göttingen, Germany). The CDW was obtained by subtracting the weight of the empty filter to that of the filled filter and by dividing by the sample volume. It was expressed in $g_{CDW}.L^{-1}$.

As CDW measurements were not completed during all the cultures because of the time frame to obtain the results, they have been correlated to turbidity measurements.

2.5.1.2 Measurement of optical density

OD value was assessed *off-line* by spectrophotometry, using a visible spectrophotometer (UV-Vis Evolution™ 201, Fisher Scientific SAS, Illkirch, France) at 600 nm (OD_{600}). Culture samples were diluted so that the measured value was comprised in the linear working range of the spectrophotometer. The results were correlated to CDW, as presented in section 2.5.1.4.

Some experiments benefitted from the *on-line* measurement of medium turbidity by a biomass probe EXcell 230 (CellD).

The system is based on the measurement of optical density with an infrared-LED beam light at 880 nm that was registered every minute.

2.5.1.3 Quantification of cell concentration and cell physiological state by flow cytometry

Cell concentration and physiological state were measured by combining FCM and a double fluorescent staining of the samples to differentiate active cells from permeabilized cells, according to (Burgé *et al.*, 2015c; Rault *et al.*, 2007).

The carboxyfluorescein diacetate (cFDA) allowed assessing the intracellular esterase activity of the cells. It is a small molecule that can easily enter into the cells, where it can be cleaved by cytoplasmic esterases to form carboxyfluorescein (cF) that fluoresces in green after excitation. As esterases are enzymes of the basal metabolism of micro-organisms, this measurement gives an image of the metabolic activity of cells (Rault *et al.*, 2008). The nucleic acid dye propidium iodide (PI) was used to characterize cells with permeabilized membranes. As a large molecule, PI only enters inside the cells whose membrane is permeabilized, where

it forms a DNA-complex that fluoresces in red after excitation. By combing these two fluorescent dyes, it is possible to differentiate, in a bacterial population, four sub-populations that correspond to active and not permeabilized cells, not active and permeabilized cells, active and permeabilized cells and unstained cells. Unstained cells correspond either to particles of same size and granularity than cells or to cells that were not active and that definitively lost their nucleic acid (Rault *et al.*, 2007). Figure 2.6 presents a scheme that summarizes the principle of the double fluorescent staining.

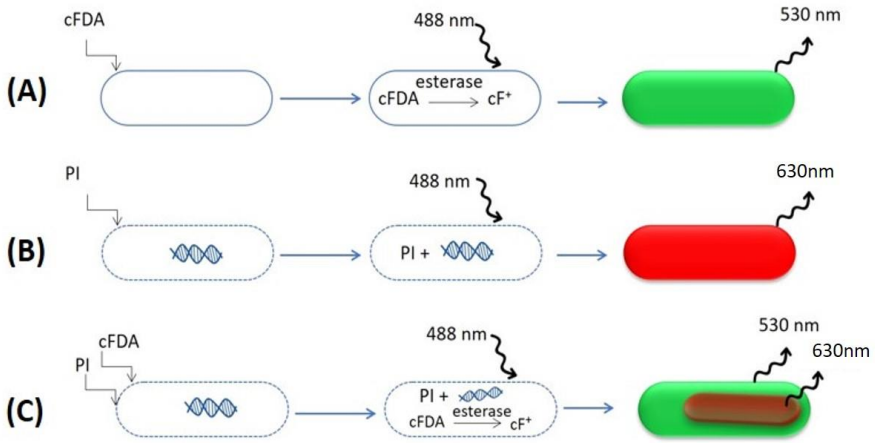


Figure 2.6. Principle of the fluorescent probes (cFDA and PI) simultaneously used to quantify the cellular physiological state.

(A) Enzymatically-active cells, stained with only cFDA; (B) Permeabilized cells, stained only with PI; (C) Damaged cells, stained with both cFDA and PI

Samples were carefully prepared before FCM measurements and were kept at 4 °C except for the incubation step. They were first diluted in McIlvaine (MI) buffer pH 7.3 to reach a concentration of about 10^6 cells·mL^{-1}. MI buffer was composed of 0.2 mol·L^{-1} disodium dihydrogenphosphate (J. T. Baker, Deventer, NL) and 0.1 mol·L^{-1} citric acid (Acros Organics, Geel, Belgium). Dual staining was carried out by simultaneously adding to one milliliter of diluted sample 10 µL cFDA from the commercial solution Chemchrom V8 (Biomérieux, Marcy-l'Etoile, France) diluted in acetone (Fisher Scientific, Leicestershire, UK) at 10 % (v/v) and 10 µL PI (1 g·L^{-1} in distilled water, Sigma-Aldrich). After mixing, samples were incubated for 10 min at 40 °C and cooled in an ice-bath. Subsequently, FCM analyses were performed with a BactiFlow cytometer (Sysmex Partec, Roissy, France) working with a sheath fluid (Sysmex Partec). Cell fluorescence was measured at 530 nm for cF and at 630 nm for PI, after excitation by a 488 nm emitting laser (Figure 2.6).

The BactiFlow cytometer (Sysmex Partec) was equipped with an air-cooled argon ion laser that emitted at 488 nm. The signal obtained from a forward-angle light scatter (FSC, 0° from the incident light) gave indications about the cell size. The signal obtained from a side-angle light scatter (SSC, 90° from the incident light) informed about the cell granularity.

Fluorescence intensity was measured by two photomultipliers, at 530 nm to collect the green fluorescence of cF (FL1 channel) and at 630 nm to collect the red fluorescence of PI (FL2 channel).

Data were acquired and analyzed thanks to the Flowmax software (Sysmex Partec) by using logarithmic gains and specific detectors settings and thresholds. The bacterial population and subpopulations were identified using dot plots. A dot plot combining the FSC and SSC signals was used to discriminate the bacterial population (corresponding to the region of interest 1, R1) from the background signal (Figure 2.7A). Gates were defined on the dot plot FL1/FL2 to differentiate the events that corresponded to the distinct sub-populations: enzymatically-active cells with a non-permeabilized membrane (quadrant Q4), permeabilized and inactive cells (quadrant Q1), active but permeabilized cells (quadrant Q2) and unstained particles (quadrant Q3) (Figure 2.7B).

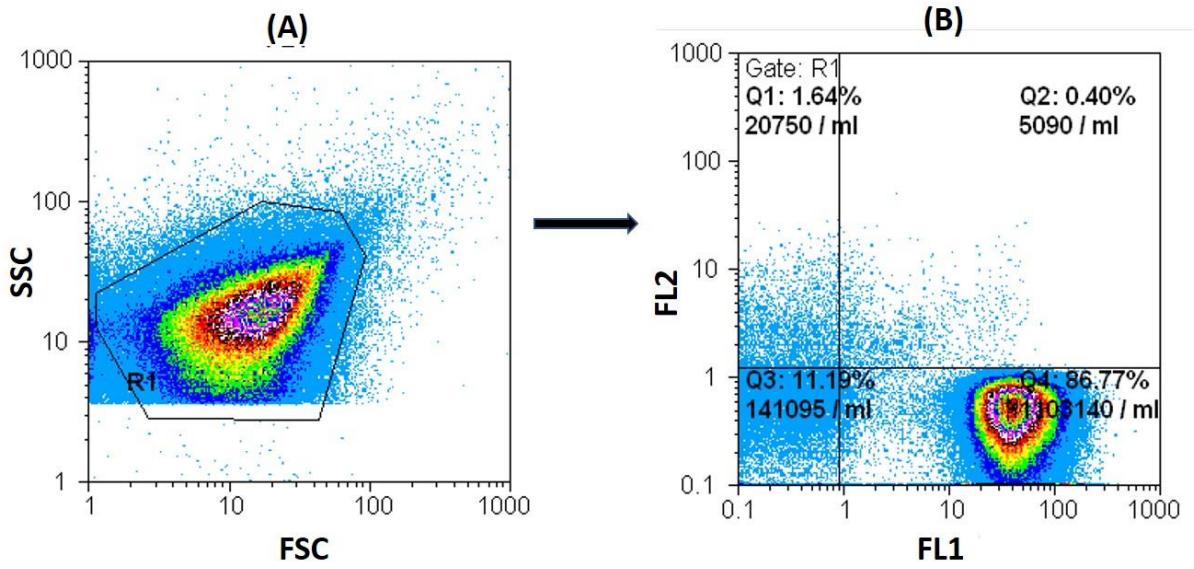


Figure 2.7. Cytograms obtained from FCM detection of *L. reuteri* DSM 17938 bacterial cells thanks to (A) the FSC and SSC signals, and (B) double fluorescent staining to differentiate sub-populations

Q1: PI-stained cells corresponding to permeabilized and inactive cells; Q2: PI- and cFDA-stained cells corresponding to damaged cells; Q4: cFDA-stained cells corresponding to enzymatically-active cells with a non-permeabilized membrane; Q3: unstained particles

The cell concentrations (in cells·mL^{-1}) of the region of interest R1 and the percentages of each subpopulation were calculated and displayed by the software, as illustrated in Figure 2.7B.

2.5.1.4 Correlations between optical density, cell dry weight and cell concentration obtained by flow cytometry

Three correlations were established between (1) optical density at 600 nm and cell dry weight, (2) optical density at 600 nm and cell concentration obtained by flow cytometry (CFCM) and (3) cell dry weight and cell concentration obtained by FCM. These correlations allowed an easy switch from one to the other method of cell concentration determination and expression.

Specific cultures were performed to establish these correlations. Bacterial cells were grown in a 5-L bioreactor with MRS broth supplemented with glucose 20 $g \cdot L^{-1}$ at pH 6.0, 37 °C and 100 rpm until the beginning of stationary phase. Cells were then harvested and concentrated by centrifugation to obtain 10^{11} cells$\cdot mL^{-1}$ before processing triplicate dilutions from 10^{11} cells$\cdot mL^{-1}$ to 10^{5} cells$\cdot mL^{-1}$.

All measurements of OD_{600}, CFCM and CDW were performed in triplicate.

The corresponding equations are given below:

$$CDW = 0.26 \times OD_{600} \quad (R^2 = 1.0) \quad \text{(eq. 2.4)}$$

$$CFCM = 2.99.10^{8} \times OD_{600} \quad (R^2 = 1.0) \quad \text{(eq. 2.5)}$$

$$CDW = 8.82.10^{-10} \times CFCM \quad (R^2 = 1.0) \quad \text{(eq. 2.6)}$$

With CDW ($g_{CDW} \cdot L^{-1}$) and CFCM (cells$\cdot mL^{-1}$).

2.5.2 Assessment of intracellular energy

According to (Bunthof *et al.*, 1999; Rault *et al.*, 2009), the measurement of intracellular energy level is associated to the capacity of the cells to excrete the intracellular cF after staining with cFDA as cells use ATP for cF excretion. These authors proposed to measure the intracellular energy level by combining flow cytometry to a fluorescent staining with cFDA and by measuring the fluorescence intensity of the cells before and after incubation for 20 min to allow cF excretion.

Samples of 2 mL were first prepared at the desirable concentration of 10^{6} cells$\cdot mL^{-1}$ by dilution in MI buffer pH 7.3, then added with 10 µL of cFDA (Chemchrom V8, Biomérieux). The mixture was incubated in an immersion circulator (Fisher Scientific) at 40 °C for 15 min before centrifugation (Eppendorf, Fisher Scientific) at 14,500 rpm for 90 seconds to eliminate the supernatant. The cell pellets were suspended in 2 mL of MI buffer pH 7.3. Half of the volume (1 mL) was directly analyzed with the flow cytometer (Sysmex Partec) to measure the initial fluorescence intensity (IF0) of cF at 530 nm, as described in Section 2.5.1.3. The remaining sample of 1 mL was incubated at 40 °C for 20 min to allow cF excretion. It was centrifuged again at 14,500 rpm for 90 seconds, re-suspended in 1 mL of MI buffer pH 7.3 and the

fluorescence intensity of cF after 20 min incubation was measured (IF20). The difference between the initial intracellular fluorescence intensity and the residual intracellular fluorescence intensity after a 20-min incubation divided by the initial intracellular fluorescence intensity allowed calculating the intracellular energy level as DeltaIF20 (in %) according to equation 2.7:

$$\text{DeltaIF20 (\%)} = \frac{\text{IF0} - \text{IF20}}{\text{IF0}} \text{ x } 100 \qquad \text{(eq. 2.7)}$$

2.5.3 Measurement of intracellular pH

The intracellular pH (pHi) was determined by FCM after staining the bacterial cells with carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The principle of this measurement is based on the preliminary establishment of a linear correlation between the fluorescence intensity of cells and their intracellular pH fixed by previous permeabilization with ionophores and incubation in buffers of various known pH. The intracellular pH of samples can then be calculated from this linear equation. The correlation has to be carried out for each sample, due to the fact that the fluorescence intensity is linked to the cell volume, which itself depends on the growing phase and cell physiological state.

To build the linear correlation, a series of 6 MI buffer solutions with pH values ranging from 5.5 to 8.0 was first prepared.

1 mL of each cell sample to be characterized was added with 10 µL of cFDA-SE (Thermo Fisher Scientific) and incubated in an immersion circulator (Fisher Scientific) for 15 min at 40 °C. It was centrifuged at 14,500 rpm for 90 seconds (Eppendorf, Fisher Scientific) at 4 °C, then re-suspended in MI buffer pH 7.3 to reach a concentration of 10^7 cells·mL^{-1}. It was then aliquoted in seven parts, six of them to establish the linear correlation and one for measuring its intracellular pH.

The first aliquot of cell sample was diluted 10 times more (to reach 10^6 cells·mL^{-1}) in MI buffer pH 7.3 to measure the fluorescence intensity without membrane permeabilization.

The six other aliquots were diluted 10 times in the six MI buffers of known pH to reach a concentration of 10^6 cells·mL^{-1} and permeabilized by adding nigericin (1 µM, Sigma Aldrich) and valinomycin (1 µM, Sigma Aldrich) to equilibrate intracellular and extracellular pH. Permeabilization was instantaneous and the samples were kept at 4 °C until fluorescence measurement.

Samples were then analyzed by flow cytometry to detect the fluorescence intensity at 530 nm on FL1. A correlation between the fluorescence intensity and the pHi of the cells was then established, as illustrated in Figure 2.8.

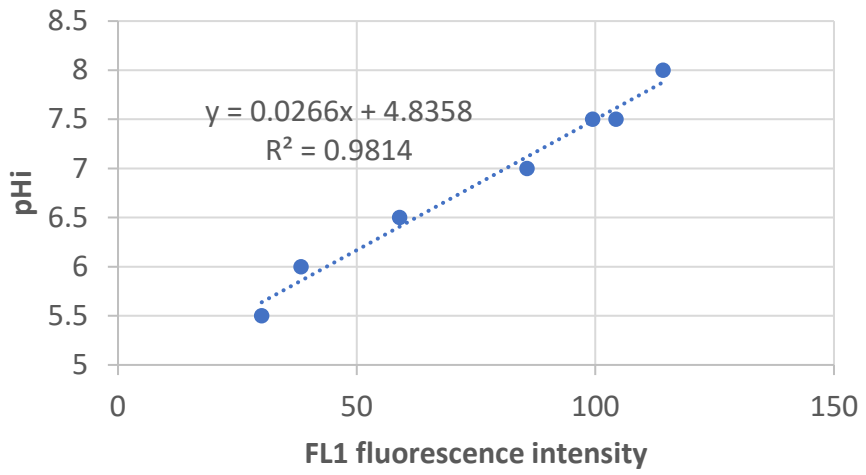


Figure 2.8. Example of a linear relationship between intracellular pH (in the range 5.5 to 8.0) and corresponding fluorescence intensities of *L. reuteri* DSM 17938 cell samples

The pHi of bacterial cells was then determined thanks to the measurement of the sample fluorescence intensity by using the linear equation given in Figure 2.8.

2.5.4 Quantification of substrates and metabolites by HPLC

The substrate of bacterial growth (i.e., glucose), the precursor of the bioconversion (glycerol) as well as the growth metabolites (lactic acid, ethanol and acetic acid) and bioconversion products (3-HP, 1,3-PDO and 3-HPA) were detected and quantified by high-performance liquid chromatography (HPLC, Waters Associates, Molsheim, France).

Before HPLC analyses, proteins have to be eliminated from the samples. Samples harvested during the growth step were diluted twice with trichloroacetic acid (Sigma-Aldrich) at 60 $g \cdot L^{-1}$, while those harvested during the bioconversion step were diluted twice with citric acid (Acros Organics, Geel, Belgium) at 5 $g \cdot L^{-1}$. A centrifugation at 13,000 rpm for 10 min at 4 °C (Eppendorf, Fisher Scientific) was performed to separate the cell pellets from the supernatant before filtration through a 0.22-µm pore-size filter (Sartorius Stedim Biotech, Göttingen, Germany). A volume of 20 µL of each sample was injected by an autosampler (Waters 717 plus) on a cation-exchange column Aminex HPX-87H (300 mm × 7.8 mm, Biorad, Richmond, USA). The analyses were done in isocratic mode, with a mobile phase composed of H_2SO_4 (Sigma-Aldrich) and at controlled temperature. Two sets of conditions were applied to separate properly the components of the samples. They differed by considering the column temperature, the H_2SO_4 concentration, the flow rate of the mobile phase and the running time, as described in Table 2.5.

Table 2.5. Conditions of HPLC analyses for quantification of substrates and products of both growth and bioconversion steps

	Condition A	**Condition B**
Temperature (°C)	35	60
H_2SO_4 concentration in mobile phase ($mmol \cdot L^{-1}$)	5	0.5
Flow rate of mobile phase ($mL \cdot min^{-1}$)	0.6	0.4
Running time (min)	35	30
Compounds detected in growth samples (and corresponding retention times, in min)	Glucose (9.3), lactic acid (13.2), acetic acid (15.6), ethanol (21.2)	
Compounds detected in bioconversion samples (and corresponding retention times, in min)	3-HPA (14.9), 1,3-PDO (17.5), acetic acid (15.6), ethanol (21.2) (the two later remaining from growth)	Lactic acid (18.7, remaining from growth), 3-HP (19.7), glycerol (20.7)

Detection was achieved by a refractive index (RI) detector (Waters 2414). Quantification of the concentrations was achieved using the Empower software (Waters Associates) with the help of external standards that were prepared in Milli-Q grade water at concentrations comprised between 0.1 and 10 $g \cdot L^{-1}$. Concentrations were given in $g \cdot L^{-1}$.

2.5.5 Assessment of molecular balance and carbon mass balance

The molecular balance corresponds to the ratio of the sum of molar quantities of metabolites to the molar quantity of consumed substrate. The carbon mass balance corresponds to the ratio of the sum of molar quantities of metabolites and molar carbon quantity in biomass to the molar quantity of substrate. They are both given in percentage. During the growth phase, they differed as the carbon contained in the biomass is not taken into account in the molecular balance whereas it is included in the carbon mass balance. During the bioconversion phase, these balances were equivalent as the biomass concentration remains constant in the form of resting cells. For this step, only the carbon mass balance will thus be considered.

The molecular weight of *L. reuteri* DSM 17938 cells was calculated from the cell formula established in section 2.2.3.2: $CH_{1.89}O_{0.62}N_{0.24}$. It is equal to 27.17 $g_{cell}.mol_{cell}^{-1}$.

During growth, the molecular balance (MB_G, in % $mol \cdot mol^{-1}$) and the carbon mass balance (CMB_G, in % $mol \cdot mol^{-1}$) have been calculated by considering that the central metabolism of *L. reuteri* used PK and EMP pathways, from the equations below:

$$MB_G\ (\%) = [1/2(n_{lactic\ acid} + n_{Ethanol} + n_{acetic\ acid})] / n_{Glucose} \quad \text{(eq. 2.8)}$$

$$CMB_G\ (\%) = (3.n_{Lactic\ acid} + 2.n_{Ethanol} + 2.n_{Acetic\ acid} + n_{CO2} + n_{C\ in\ Biomass}) / 6.n_{Glucose} \quad \text{(eq. 2.9)}$$

During the bioconversion step, the carbon mass balance (CMB_B, in % $mol \cdot mol^{-1}$) was established according to the Pdu pathway, by the next equation:

$$CMB_B = (n_{3\text{-}HP} + n_{1,3\text{-}PDO} + n_{3\text{-}HPA}) / n_{Glycerol} \quad \text{(eq. 2.10)}$$

With n being the number of moles of consumed glucose ($n_{Glucose}$) or glycerol ($n_{Glycerol}$), produced lactic acid ($n_{Lactic\ acid}$), ethanol ($n_{Ethanol}$), acetic acid ($n_{Acetic\ acid}$), CO_2 (n_{CO2}), carbon in cells produced during growth ($n_{C\ in\ Biomass}$), 3-HP ($n_{3\text{-}HP}$), 1,3-PDO ($n_{1,3\text{-}PDO}$) and 3-HPA ($n_{3\text{-}HPA}$).

The produced CO_2 molar quantity was estimated as being equal to the number of moles of produced [ethanol + acetate], in agreement with the stoichiometry of PK pathway.

Chapter 3. Effect of culture conditions on *L. reuteri* DSM 17938 growth and ability to perform glycerol bioconversion

This chapter presents the results and discussion of the study that focused on the effects of eleven culture conditions during the first step of growth, on the bacterial growth and on the 3-HP bioproduction by *L. reuteri* DSM 17938. The second step of the bioprocess was performed at fixed conditions for the glycerol bioconversion into 3-HP.

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3.1 Introduction

The bioproduction of 3-HP by *L. reuteri* has to be carried out through a two-step process, in order to separate the growth phase, done in batch with glucose as the carbon substrate, from the bioconversion phase, performed in fed-batch by resting cells with glycerol as the carbon source. This two-step bioprocess allows the cells to implement different metabolic pathways at each step, together with balancing the redox cofactors. In fact, in the presence of glucose, *L. reuteri* grows and reaches high cellular concentrations (Bengtsson, 2020). But this species cannot use glycerol as a carbon source for growth (Lindlbauer *et al.*, 2017), which allows glycerol to be completely valorized into 3-HP and 1,3-PDO through the bioconversion pathway using resting cells. The glycerol bioconversion into 3-HP is made possible by the ability of this species to synthesize vitamin B12 that is an essential cofactor for the first enzyme of the glycerol metabolism (Santos *et al.*, 2009). Besides, the simultaneous presence of glucose and glycerol favors 1,3-PDO production instead of 3-HP bioproduction (Chen and Hatti-Kaul, 2017) and leads to the synthesis of undesirable bioproducts such as lactic acid, ethanol, acetic acid, carbon dioxide. To limit this production of metabolites and to drive the metabolism towards the bioconversion into 3-HP, the separation between the two steps is compulsory. In addition, the process is performed on the basis of different substrates (glucose then glycerol) as it necessitates using resting cells during the bioconversion step that have to be first produced during the growth step.

As a consequence of this two-step process, the implementation of the bioprocess requires the determination of optimal conditions during the growth and bioconversion phases. In this chapter, we will consider only the growth phase conditions, by fixing the conditions during the bioconversion phase. The effects of these culture conditions will be studied on the performance of *L. reuteri* DSM 17938 to produce 3-HP from glycerol. As the culture conditions

also influence the growth performances that will indirectly affect the 3-HP bioproduction, their effects will also be determined on the growth characteristics.

The influence of some conditions implemented during the growth step, prior to bioconversion has been identified from the bibliographic analysis. In the vast majority of cases, the published studies focused on the improvement of the growth performances. From the literature review, glucose was demonstrated as the better carbon source to maximize *L. reuteri* growth (Couvreur *et al.*, 2017). The most common concentration used was comprised between 20 $g \cdot L^{-1}$ (Dishisha *et al.*, 2014; Ju *et al.*, 2020) and 30 $g \cdot L^{-1}$ (Couvreur *et al.*, 2017). As *L. reuteri* displays a heterofermentative metabolism, glucose is catabolized through two pathways (PK and EMP pathways) that operate simultaneously and lead to the production of lactic acid, ethanol, acetic acid, carbon dioxide and energy (Årsköld *et al.*, 2008).

Yeast extracts and peptones are commonly used as nitrogen sources for growth of lactic acid bacteria due to their composition in amino acids, vitamins and growth factors that meets cell needs (Alazzeh *et al.*, 2009; Atilola *et al.*, 2015). Yeast extracts are the most common sources employed in the published studies concerning *L. reuteri* (Couvreur *et al.*, 2017; Ichinose *et al.*, 2020). Peptones, which are obtained from papaic digestion of soybean meal, display an interesting composition with 18 of the 20 natural amino acids (asparagine and glutamine are missing) and are cheaper nutrient sources than yeast extracts. Among them, phytone peptone was demonstrated to lead to high cell concentrations by comparison with other nitrogen sources such as peptones of animal origin, tryptone, proteose peptone, tryptic soy broth, yeast extract and beef extract, in *L. reuteri* DSM 20016 (Atilola *et al.*, 2015), *L. reuteri* DSM 17938 (Couvreur *et al.*, 2017) and *L. reuteri* PTA-4965, 23272 and 55730 (Ayad *et al.*, 2020).

The addition of the amino acid cysteine in the growth medium of *L. reuteri* was also considered as this component is an essential growth factor of some lactobacilli (Lozo *et al.*, 2008). For *L. reuteri* DSM 20016 and SD 2112 strains, it was recommended at concentrations between 0.1 $g \cdot L^{-1}$ (Atilola *et al.*, 2015) and 0.5 $g \cdot L^{-1}$ (Alazzeh *et al.*, 2009). However, the addition of 2 $g \cdot L^{-1}$ cysteine in the culture medium was mentioned as decreasing the production of vitamin B12 by *L. reuteri* JCM1112 by a factor 3 to 5 (Santos *et al.*, 2009). This amino acid is also known as a potent reducing agent, thus displaying antioxidative properties that help bacterial cells to cope with the oxidative stress encountered during the recovery and concentration steps, as demonstrated with *Lactobacillus fermentum* (Turner *et al.*, 1999).

Tween 80 is a key growth factor in culture media for lactobacilli, as it brings unsaturated fatty acids that allow reducing intracellular energy consumption, thanks to the down-regulation of *de novo* fatty acid synthesis in membrane phospholipids (Reitermayer *et al.*, 2018). It is for instance present at a concentration of 1 $g \cdot L^{-1}$ in MRS medium (Biokar, 2016) or added in the culture medium to reach 5 $g \cdot L^{-1}$ (Couvreur *et al.*, 2017).

Besides, the influence of some other components in the culture medium of lactobacilli has been investigated. From (Couvreur *et al.*, 2017), supplementation of the growth medium with vitamin B12 (0.1 $mg \cdot L^{-1}$), which is a mandatory cofactor of the first enzyme in the Pdu pathway

(glycerol dehydratase), although not influencing *L. reuteri* growth, positively impacted the 3-HP bioproduction.

The addition of 1,2-propanediol (1,2-PDO) during the growth phase has also been tested as it is involved in the activation of genes of the Pdu pathway (Dishisha *et al.*, 2015; Sriramulu *et al.*, 2008). It was added at a concentration of 5 $g \cdot L^{-1}$ into *L. reuteri* DSM 20016 growth medium prior to glycerol conversion (Dishisha *et al.*, 2015).

Betaine is a methylated derivative of the amino acid glycine that acts as an osmolyte to help lactic acid bacteria to counteract the osmotic shock they face during the harvesting and concentration stages (Louesdon *et al.*, 2014). By considering *L. buchneri* R1102, these authors demonstrated that the combined supplementation of the growth medium with 2 $mmol \cdot L^{-1}$ betaine and 0.1 $mol \cdot L^{-1}$ KCl, together with a further addition of 0.6 $mol \cdot L^{-1}$ KCl at the end of the growth, led to intracellular accumulation of betaine and improved cell survival during freeze-drying.

Formulating the environmental parameters (i.e. growth temperature, pO_2, pH and base used for pH control) to improve cell growth and further bioconversion is also challenging. The temperature for *L. reuteri* growth is generally established at 37 °C (Atilola *et al.*, 2015; Burgé *et al.*, 2015b; Polak-Berecka *et al.*, 2010). However, the optimum temperature for growth may differ from that maximizing a given metabolic activity, but to date, no work has been dedicated to the determination of the optimum temperature for 3-HP bioproduction.

The effect of dissolved oxygen concentration during the growth of *L. reuteri* has been well studied by (Zabed *et al.*, 2019). From this study, the use of micro-aerobiosis is recommended in order to improve 3-HP bioproduction, whereas aerobic or anaerobic environments during growth were deleterious.

The optimal pH for *L. reuteri* growth is generally comprised between 6.0 and 6.8 (Kandler *et al.*, 1980). Recently, different pH values between 3.7 and 6.7 were tested during batch cultures of *L. reuteri* DSM 12246 and the final biomass concentration was enhanced at pH 5.5 (El-Ziney, 2018). The use of three kinds of bases has been reported for pH control during *L. reuteri* growth phase: NaOH (Hernández *et al.*, 2019; Kristjansdottir *et al.*, 2019), KOH (Burgé *et al.*, 2015c) and NH_4OH (Dishisha *et al.*, 2014; Ricci *et al.*, 2015). However, they have never been compared by considering further 3-HP bioproduction.

Finally, the moment at which the cells are harvested is of great importance as it directly affects their physiological state and their metabolic properties (Rault *et al.*, 2008). However, depending on the authors, the best moment to harvest *L. reuteri* varies from early stationary phase to late stationary phase, which could be explained by the different strains used (Lüthi-Peng *et al.*, 2002; Palmfeldt and Hahn-Hägerdal, 2000; Ramakrishnan *et al.*, 2015; Talarico and Dobrogosz, 1990; Zabed *et al.*, 2019).

From this synthetic bibliographic analysis, it can be seen that most previous works studied the effects of culture conditions on *L. reuteri* growth but not on 3-HP bioproduction. They show

that many culture conditions during the growth phase affect the growth performances of *L. reuteri*. However, the effects of these culture conditions were scarcely investigated on the cell ability to subsequently convert glycerol into 3-HP. In addition, the reported studies focus on a limited number of factors, thus making it difficult to define the optimal conditions by taking all of them into account.

In such context, this chapter aims at quantifying the effects of relevant culture conditions on *L. reuteri* capacity to produce 3-HP during the subsequent glycerol bioconversion stage. All experiments were performed in bioreactors in order to evaluate the effects of the factors in well-controlled conditions.

3.2 Experimental strategy

The overall methodology of this chapter is illustrated in Figure 3.1. The details of the methods used were introduced in Chapter 2.

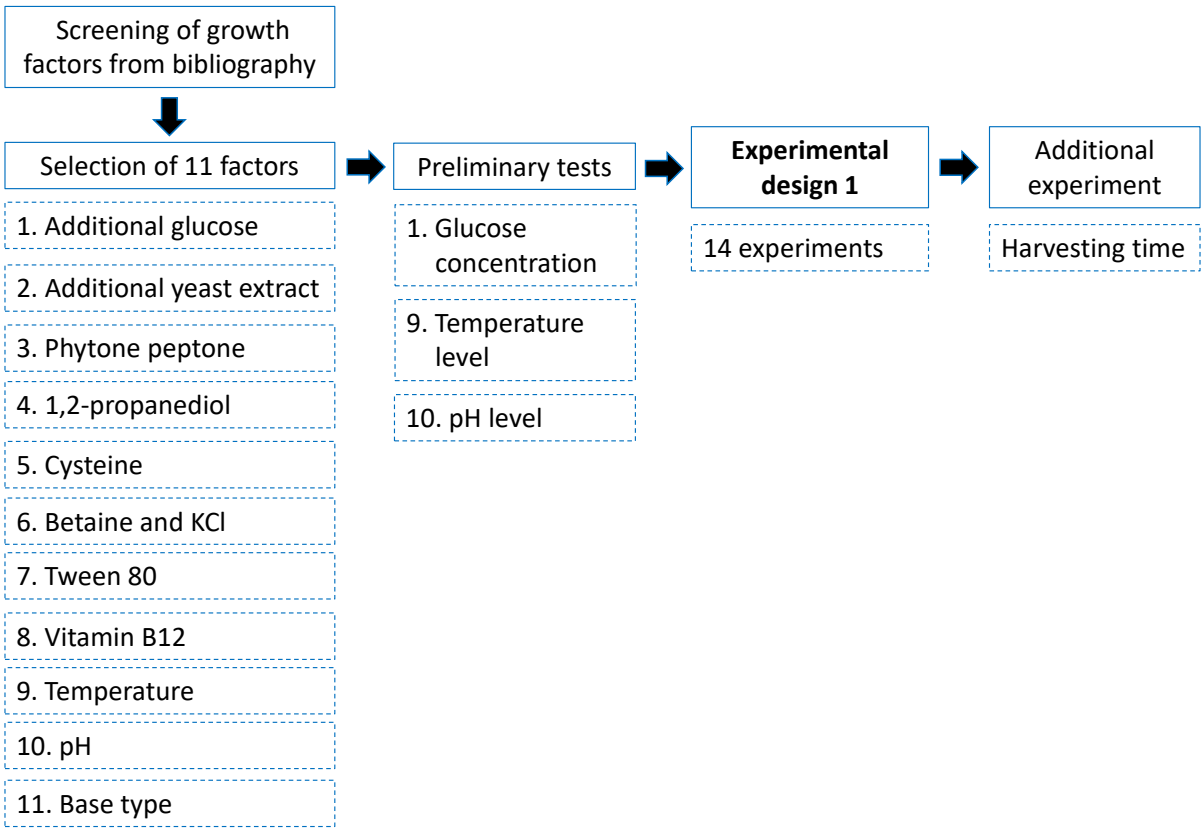

Figure 3.1. Overall methodology for the study of the effects of culture conditions during the growth step on the growth of *L. reuteri* DSM 17938 and its ability to perform glycerol bioconversion into 3-hydroxypropionic acid.

As summed-up in the scheme of Figure 3.1, eleven growth factors were screened from the thesis literature review. They were used to create a matrix of experiments according to a Plackett and Burman experimental design. The levels of most of these factors were

determined by referring to previous works, excepted for glucose addition, temperature and pH levels. For these three factors, preliminary tests were carried out during the growth phase to identify the minimum and maximum values that could be tested in the experimental design: glucose was added at concentrations of 50 $g \cdot L^{-1}$ or 80 $g \cdot L^{-1}$ in MRS medium (which already contains 20 $g \cdot L^{-1}$ of glucose); two temperatures were tested at 33 °C and 41 °C; three pH were experienced at 5.0, 5.5 and 6.7. Subsequently, the selected values were included in the experimental design that defined the conditions during growth phase as presented in (Table 2.2).

At the end of the growth, the cells were harvested and concentrated according to well standardized conditions. In order to determine the best moment at which the cells have to be recovered, additional experiments were designed to compare two various harvesting times defined at the beginning of stationary phase and in late stationary phase (i.e., 6 h later).

In order to determine the effects of the growth factors on 3-HP production from glycerol, the conditions of the bioconversion phase were fixed for all experiments, based on the literature review. The bioconversion temperature was selected at 37 °C (Burgé *et al.*, 2015c; Dishisha *et al.*, 2015), the agitation rate was fixed at 100 rpm (Burgé *et al.*, 2015c), the pH was set at 6.0 (Burgé *et al.*, 2015c) and the glycerol feeding rate was fixed at 0.5 $g \cdot h^{-1}$ (Dishisha *et al.*, 2014).

3.3 Results and Discussion

3.3.1 Preliminary tests

Seven preliminary tests were carried out to determine the minimum and maximum values to be chosen for three factors of *L. reuteri* DSM 17938 growth: glucose concentration, temperature and pH. The difference between the two levels of each factor has to be large enough to be able to quantify statistically their effects. On the other hand, it was necessary to verify that the second level of each factor really ensured the bacterial growth as well as the further glycerol bioconversion.

These preliminary tests were performed according to the reference condition, i.e. growth in MRS medium that initially contained glucose (20 $g \cdot L^{-1}$) and yeast extract (5 $g \cdot L^{-1}$), with NH_4OH (14.8 M) as the base to control the pH, but without the addition of any other component. Two different glucose additions were tested at 37 °C and pH 6.0. Two different temperatures were tested in the presence of 20 $g \cdot L^{-1}$ glucose and at pH 6.0. Three different pH were tested in the presence of 20 $g \cdot L^{-1}$ glucose and at 37 °C. All these preliminary tests have been carried out once, without repetition at this step of the study.

During these preliminary tests, the biomass concentration (in $g_{CDW} \cdot L^{-1}$) obtained at the end of growth, the corresponding concentrations of enzymatically-active and not permeabilized cells (in $cells \cdot mL^{-1}$) and the base concentration (in $mol \cdot L^{-1}$) used to control the pH were quantified. The duration of base consumption, which is indirectly linked to the organic acid production,

was also characterized to inform on the length of the growth phase. Results are displayed in the following subparts. Then, the promising levels that led to a good bacterial growth will be selected and applied in the first experimental design.

3.3.1.1 Preliminary tests about glucose concentration

The glucose availability in MRS broth (20 g·L^{-1}) is limiting for the bacterial growth and causes carbon source starvation. In order to circumvent this issue, an extra glucose of 20 g·L^{-1} was added to define the reference condition, in accordance with (Burgé, 2015). This level of complementation constitutes the low level of the experimental design. The addition of higher concentrations of glucose, at 50 g·L^{-1} or 80 g·L^{-1} was tested to define the second level of this factor. Results obtained at these three glucose levels are given in Figure 3.2 and Table 3.1.

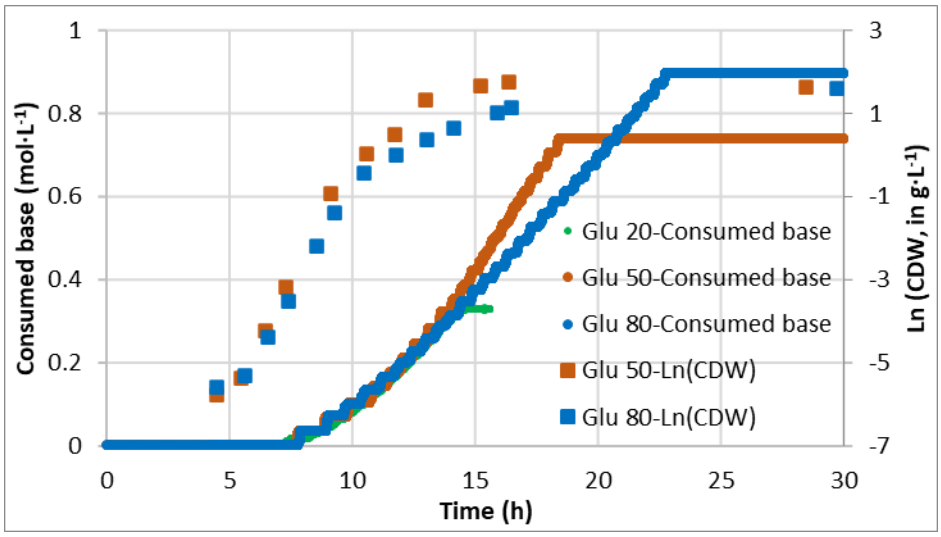

Figure 3.2. Kinetics of bacterial growth of *L. reuteri* DSM 17938 and base consumption at various glucose concentrations added to MRS broth

Figure 3.2 shows the kinetics of bacterial growth and base consumption during the growth phase of *L. reuteri* DSM 17938 at three glucose concentrations. By considering the curves of base consumption, it can be seen that the addition of 80 g·L^{-1} glucose led to a higher NH_4OH consumption and a longer fermentation duration than 20 g·L^{-1} or 50 g·L^{-1}. From Table 3.1, the concentration of base consumed increased from 0.73 mol·L^{-1} to 0.90 mol·L^{-1} and the duration of this consumption increased from 13 h to 18.5 h, when glucose addition was enhanced from 50 g·L^{-1} to 80 g·L^{-1}. This observation is easily explained by the role of glucose that acts not only as a carbon source for cell growth, but also as a substrate to produce organic acids such as lactic acid and acetic acid through the PK and EMP pathways (Burgé *et al.*, 2015b).

Table 3.1. Comparison of the growth performances of *L. reuteri* DSM 17938 in the presence of different glucose concentrations added to MRS broth

	Additional glucose ($g \cdot L^{-1}$)		
	20	**50**	**80**
Cell concentration ($g_{CDW} \cdot L^{-1}$)	3.0	5.1	5.0
Active and not permeabilized cells ($cell \cdot mL^{-1}$)	$2.9 \cdot 10^9$	$4.8 \cdot 10^9$	$5.1 \cdot 10^9$
Consumed base ($mol \cdot L^{-1}$)	0.35	0.73	0.90
Lactic acid produced ($g \cdot L^{-1}$)	15.1	27.3	40.1
Acetic acid produced ($g \cdot L^{-1}$)	0.9	0.9	1.5
Ethanol produced ($g \cdot L^{-1}$)	6.9	13.0	18.3
$\mu_{max\ 1}$ (h^{-1})	nd	1.21	1.02
$\mu_{max\ 2}$ (h^{-1})	nd	0.57	0.24

μ_{max}: maximum specific growth rate; nd: not determined

By considering the biomass production, the final concentrations were not significantly different between 50 $g \cdot L^{-1}$ and 80 $g \cdot L^{-1}$ of added glucose (Table 3.1). However, these results were higher than the cell dry weight reported at 20 $g \cdot L^{-1}$ of added glucose, and than the value of 3.1 $g_{CDW} \cdot L^{-1}$ obtained by (Dishisha *et al.*, 2014) with *L. reuteri* DSM 20016 grown in MRS medium.

The growth finished earlier when 50 $g \cdot L^{-1}$ glucose was added, as compared to the condition with 80 $g \cdot L^{-1}$ glucose, as the stationary phase was reached after 12 h instead of about 15 h (estimated value). This result is linked to the lower availability of carbon source for bacterial growth. Meanwhile, the organic acids were produced continuously some hours later, thus characterizing an intermediary metabolism. These productions completely ended after 14.1 h, 18.6 h and 22.8 h, with increasing glucose addition (Figure 3.2). The longer duration of base consumption was linked to the higher concentrations of metabolites produced during the growth when more glucose was added in the MRS medium (Table 3.1).

From Figure 3.2, two successive exponential growth phases can be observed in both conditions, with different specific growth rates. From Table 3.1, the maximum specific growth rate that characterized the 50 $g \cdot L^{-1}$ glucose addition was calculated at 1.21 h^{-1} from 5 h to 9 h, then 0.57 h^{-1} between 9 h and 15 h. When 80 $g \cdot L^{-1}$ glucose was added, the maximum specific growth rate switched from 1.03 h^{-1} between 5 h and 10.5 h, to 0.24 h^{-1} between 10.5 h and 16 h. This decrease in the maximum specific growth rate over time indicates that a limitation occurred. It may concern one or several medium components necessary for growth, excepted the carbon source, as glucose still remained present in the medium after 9 h of cell culture with 50 $g \cdot L^{-1}$ glucose addition (58.2 $g \cdot L^{-1}$) and after 10.5 h of cell culture with 80 $g \cdot L^{-1}$ glucose addition (99.1 $g \cdot L^{-1}$ reported). Another hypothesis is that the bacterial growth was affected by the accumulation of lactic acid (Ichinose *et al.*, 2020) and ethanol (van Bokhorst-van de Veen *et al.*, 2011) issued from the cell metabolism by itself (Adams and Hall, 1988; Schepers *et al.*, 2002). At the moment when the specific growth rate decreased, the lactic acid concentrations

were respectively equal to 5.0 and 5.2 $g \cdot L^{-1}$ for a 50 and 80 $g \cdot L^{-1}$ glucose addition, and the ethanol concentrations were respectively equal to 3.6 and 3.4 $g \cdot L^{-1}$. These values were however not so high to explain the shift observed on the maximum specific growth rate.

By considering the cell physiological state that was measured as the percentage of active and not permeabilized cells in the population at the beginning of the stationary phase (Table 3.1), no major difference was observed when glucose was added at 50 $g \cdot L^{-1}$ (83.0 % of active and not permeabilized cells) as compared to 80 $g \cdot L^{-1}$ (89.4 %). In addition, these percentages remained stable all along the culture. This difference was not significant as compared to the significant change applied on the initial glucose concentration. This result indicates that the glucose present for both conditions was enough to fulfill the cell requirements and that the metabolites produced during growth did not affect too much the cells, even at the highest concentrations observed when 80 $g \cdot L^{-1}$ glucose was added to the MRS medium (40.1 $g \cdot L^{-1}$ lactic acid and 18.3 $g \cdot L^{-1}$ ethanol). These percentages of active cells were higher than those reported for *L. reuteri* 55730 cultivated in MRS agar (Ayad *et al.*, 2020), which indicates that a strain effect has to be taken into account in addition to the medium composition.

From these results, it can be concluded that the supplementation of the MRS growth medium with 80 $g \cdot L^{-1}$ glucose improved mainly the production of metabolites, i.e. lactic acid, acetic acid and ethanol, but neither the biomass concentration nor the cell physiological state, as compared to a supplementation with 50 $g \cdot L^{-1}$ glucose. Moreover, it led to a longer duration of growth that consequently increased the global process duration. According to these considerations, the MRS supplementation with 50 $g \cdot L^{-1}$ glucose was selected as the “Plus” level of glucose addition in the first experimental design.

3.3.1.2 Preliminary tests about temperature

The optimum temperature for *L. reuteri* DSM 17938 growth was formerly demonstrated at being 37 °C (Hernández *et al.*, 2019) and this value was included in the Plackett and Burman design as the first level of this factor. Two other temperatures, namely 33 °C and 41 °C, were tested to verify that they could be included in the experimental design as the second level. They were chosen inside the range of growth temperatures of lactobacilli that is comprised between 30 °C and 45 °C (Van De Guchte *et al.*, 2002) and by considering a temperature difference of at least 4 °C from the optimum. Results obtained at these two temperatures, as well as at 37 °C, are given in Figure 3.3 and Table 3.2.

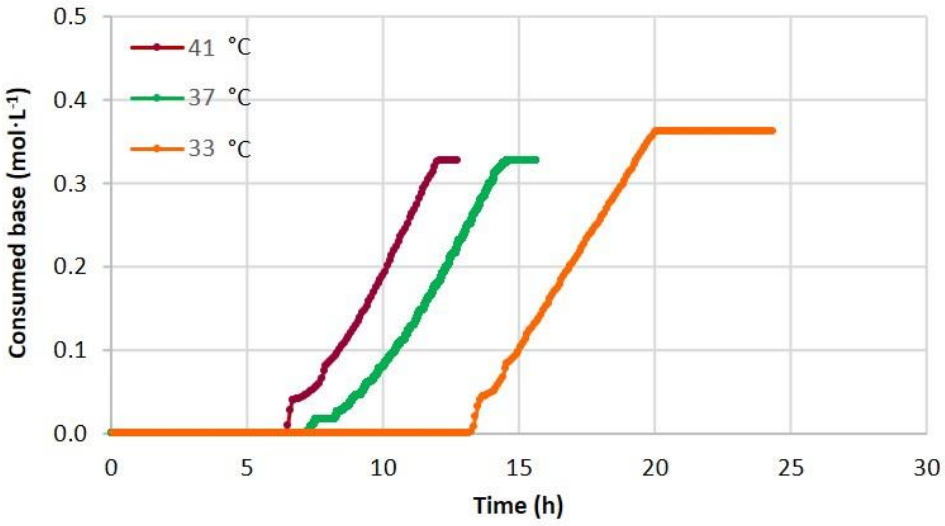

Figure 3.3. Base consumption during growth of *L. reuteri* DSM 17938 at various temperatures

From Figure 3.3, the time lag corresponding to the base consumption was longer at 33 °C (13.2 h) than at 37 °C (7.4 h) and 41 °C (6.4 h). In contrast, the duration of base consumption (6.2 h) was not significantly different, depending on the temperatures (between 5.8 h and 7.2 h). Additionally, no statistical difference was found in the maximum rate of base consumption between the three conditions, which revealed that it was not significantly affected in this range of temperatures (Table 3.2).

Table 3.2. Comparison of the growth performances of *L. reuteri* DSM 17938 during cultures performed at different temperatures

	Temperature (°C)		
	33	**37**	**41**
Cell concentration ($g_{CDW}·L^{-1}$)	4.41	4.34	4.38
Active cells and not permeabilized ($cell·mL^{-1}$)	$4.1·10^9$	$4.5·10^9$	nd
Consumed base ($mol·L^{-1}$)	0.36	0.30	0.31
Maximum base consumption rate ($mol·L^{-1}·h^{-1}$)	0.05	0.04	0.05
Lactic acid produced ($g·L^{-1}$)	23.0	17.4	16.9
Acetic acid produced ($g·L^{-1}$)	0.9	0.9	0.9
Ethanol produced ($g·L^{-1}$)	3.7	8.4	6.8

nd: not determined

From Table 3.2, the temperature of 33 °C led to a slightly higher base consumption than at 41°C or 37°C. This result was confirmed by the higher concentration of lactic acid at 33 °C whereas similar concentrations of acetic acid were obtained (Table 3.2). Conversely, the ethanol concentration was lower at 33 °C that at higher temperatures (Table 3.2). The molar ratio of the sum of [acetic acid + ethanol] to lactic acid was lower at 33 °C (0.37 $mol·mol^{-1}$) instead of 0.87 $mol·mol^{-1}$ at 41 °C.

As *L. reuteri* uses both PK and EMP pathways for glycolysis (Årsköld *et al.*, 2008), the result obtained in this set of experiments revealed that the part of glucose that entered the EMP pathway, as compared to that entering the PK pathway, was more important at 33 °C than at higher temperatures. The proportion of glucose entering the EMP pathway at 33 °C is 48 %, which is higher than at 41° C with 12 % of EMP pathway. The increase in lactic acid production observed at the low temperature is in agreement with the results of (Adamberg *et al.*, 2003) who explained that, by increasing the culture temperature at 41 °C, cells required more energy for maintenance, which could be due to the synthesis of heat shock proteins (Adamberg *et al.*, 2003).

However, despite the higher efficiency of EMP pathway for ATP synthesis as compared to PK pathway (Burgé *et al.*, 2015b), the growth of *L. reuteri* was not improved, as the final biomass concentrations were similar at the three temperatures (Table 3.2). This similarity was unfortunately not confirmed by the concentrations of active cells that were not measured at 41 °C (due to a technical problem). From these results, it can be hypothesized that the higher ATP quantity that may be produced at 33 °C may serve later, during the bioconversion step.

Finally, by considering that the final biomass concentration was similar at the two temperatures of 33 °C and 41 °C, and by taking into account that the condition of temperature of 33 °C led to a switch of a higher part of the central metabolism to EMP pathway, and then possibly to more ATP that can further help the cells to produce or to cope with more 3-HP, the temperature of 33 °C was retained to be included in the first experimental design as the second level.

3.3.1.3 Preliminary tests about controlled pH

As an important factor that impacts intracellular enzyme activity, the pH has already been studied. By considering *L. reuteri* sp., (Kandler *et al.*, 1980) indicated that pH 6.0 was an optimal value for the growth of this species. As it is used in many previous works (Dishisha *et al.*, 2014; Krauter *et al.*, 2012), it was then selected as the first level in the experimental design. Three other pH values were tested in order to evaluate the capacity of *L. reuteri* DSM 17938 to grow in non-optimal conditions: pH 5.0, pH 5.5 and pH 6.7. Results obtained at these four pH levels are given in Figure 3.4 and Table 3.3.

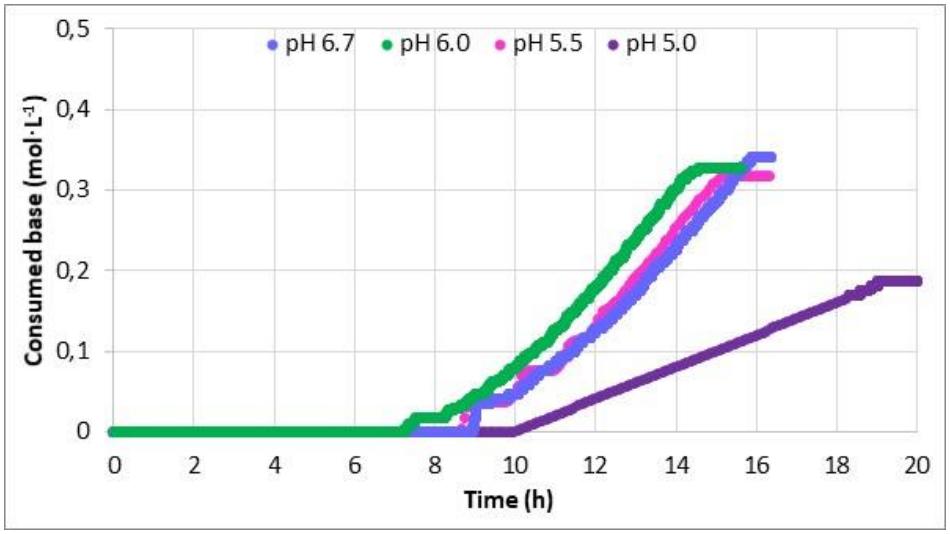

Figure 3.4. Base consumption during growth of *L. reuteri* DSM 17938 performed at various pH values

Figure 3.4 shows that the base consumption was only slightly different at pH 5.5 and 6.7, as compared to pH 6.0. However, it was negatively affected when the growth was conducted at pH 5.0. The rate of base consumption was similar at the three highest pH values, but was lower at pH 5.0. This information is confirmed by the results shown in Table 3.3. The concentration of base consumed was similar at pH 5.5, pH 6.0 and pH 6.7 but lower at pH 5.0, thus indicating that the final concentration of organic acids was lower in this latest condition.

Table 3.3. Comparison of the growth performances of *L. reuteri* DSM 17938 during cultures performed at different pH values

	pH			
	5.0	**5.5**	**6.0**	**6.7**
Cell concentration (g_{CDW}·L^{-1})	2.29	3.14	3.0	1.39
Active and not permeabilized cells (cell·mL^{-1})	$2.2 \cdot 10^9$	$2.9 \cdot 10^9$	$2.4 \cdot 10^9$	$1.0 \cdot 10^9$
Consumed base (mol·L^{-1})	0.19	0.33	0.35	0.35
Lactic acid produced (g·L^{-1})	16.5	15.9	15.1	16.1
Acetic acid produced (g·L^{-1})	0.7	0.8	0.9	0.6
Ethanol produced (g·L^{-1})	6.4	6.4	6.9	6.6

By considering the bacterial growth (Table 3.3), the final biomass concentration was higher at pH 5.5 and pH 6.0 than at pH 5.0 that was a deleterious condition for both growth and acidification (as indirectly determined from base consumption). Moreover, the maximum biomass concentration that was found at pH 5.5 that is in agreement with the results of (El-Ziney, 2018) and in accordance with the concentration of active and not permeabilized cells (Table 3.3). At pH 6.7, the growth was more affected, thus leading to lower concentrations, as measured in terms of cell dry weight and of physiologically-active cells.

The poor results obtained at pH 5.0 can be explained by considering the inhibiting effect of lactic acid and acetic acid on the bacterial growth (Schepers *et al.*, 2002), which differs according to the relative proportions of dissociated (A^-) and undissociated (AH) forms of these organic acids. From (Fu and Mathews, 1999), the relationship between dissociated and undissociated organic acid defines the pK_a of the molecule by the following equation:

$$pK_a = pH - \log([A^-]/[AH]) \qquad \text{Eq. 3.1}$$

With pK_a of lactic acid and acetic acid being equal to 3.86 and 4.76, respectively (Adams and Hall, 1988).

From Schepers *et al.* (2002), lactic acid affected much more the bacterial growth when the non-dissociated form was predominant. They showed that the specific growth rate of *L. helveticus* was not significantly affected at pH 6.0, i.e. when lactic acid was almost completely dissociated (> 99 % of the total), whereas it was strongly reduced at pH 4.5 where the undissociated form represented 19 % of the total lactic acid. In our study, at pH 5.5, the undissociated form of lactic and acetic acids represented 2 % and 15 %, respectively. At pH 5.0, they rose to 7 % and 37 %, respectively. These differences partly explained the poor results obtained at pH 5.0 compared to pH 5.5.

At pH 6.7, the final cell concentration was three times lower than at pH 5.5, whereas the lactic acid production remained similar. This indicates that the optimal pH for the production of lactic acid differed from that for cell growth, as previously indicated by (Palmfeldt and Hahn-Hägerdal, 2000) with *L. reuteri* ATCC 55730. The result is also in agreement with the range of pH comprised between 6.0 and 6.5 that maximized the lactic acid volumetric production rate from glucose and corn by *Lactobacillus amylophilus* (Mercier *et al.*, 1992).

Regarding these results, as the biomass production should be favored at the end of the growth phase, pH 5.5 was finally selected to be considered as the second level of pH in first the experimental design.

3.3.2 Kinetics of cell growth and glycerol bioconversion by *L. reuteri* DSM 17938 in the reference condition of the first experimental design

The Plackett and Burman experimental design recommends performing a duplicate of the reference condition. In our study, the experiments are complex because of the succession of two biotransformation steps in bioreactors: growth in batch mode to produce active cells, then bioconversion by resting cells in fed-batch mode, with a harvesting and concentration step between them. For this reason, we decided to triplicate the reference condition, in order to better characterize the repeatability of the experiments.

The nutritional and environmental characteristics during the growth phase of this reference condition were defined by an initial glucose concentration of 40 $g.L^{-1}$ (that corresponded to the concentration in MRS added with 20 $g.L^{-1}$), an initial yeast extract concentration of 5 $g.L^{-1}$

(contained in the MRS broth), no addition of any other component, a temperature of 37°C and a pH equal to 6.0 that was controlled with NH_4OH 14.8 M. The environmental characteristics during the bioconversion phase were fixed for all experiments: the pH at 6.0, the temperature at 37 °C and the glycerol feeding rate at 0.5 $g_{glycerol} \cdot h^{-1}$.

The results obtained in this reference condition during the growth phase are summarized in Figure 3.5 and in Table 3.4. The results obtained during the bioconversion phase are shown in Figure 3.6 and in Table 3.5.

From Figure 3.6, the glycerol consumption rate was constant all along the biotransformation, at 5.6 (± 0.13) $mmol.h^{-1}$ corresponding to 0.51 (± 0.01) $g_{glycerol}.h^{-1}$, in agreement with the delivered feeding rate. This indicates that substrate was totally consumed as and when it was supplied, which was confirmed by the residual glycerol concentration that was equal to zero during the whole bioconversion. The glycerol consumption ceased concomitantly to the base consumption (with a slight difference that was ascribed to the variability), which was confirmed from HPLC analyses. This indicated that the base profile was a relevant indicator of the cessation of the bioconversion.

3.3.2.1 Kinetics of *L. reuteri* DSM 17938 growth in the reference condition

Figure 3.5 shows the growth and the base consumption curves, measured during three repetitions in the reference condition, together with standard deviations.

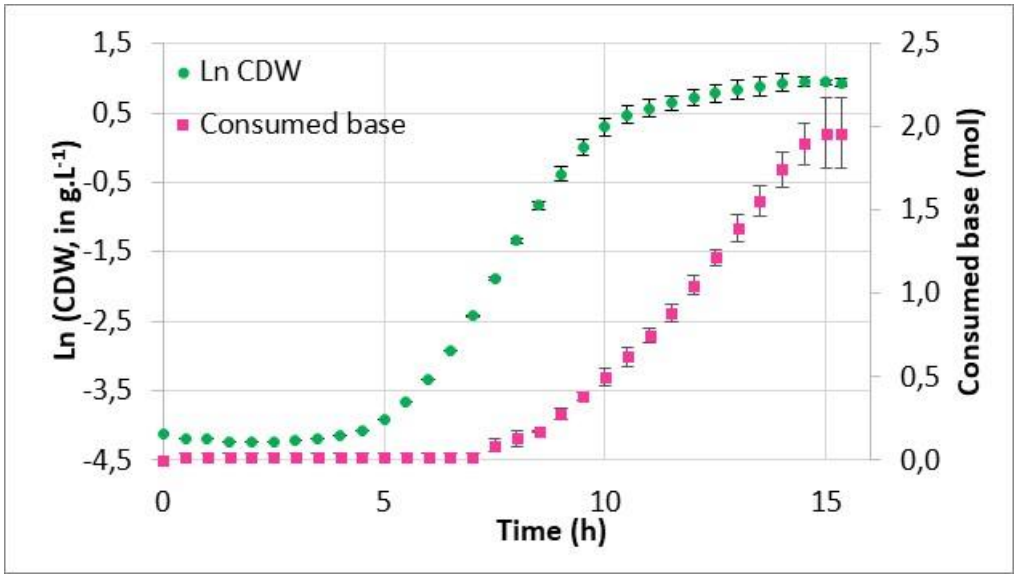

Figure 3.5. Kinetics of cell growth and base consumption of *L. reuteri* DSM 17938 in the reference condition (T1, 3 replicates) of the first experimental design (final volume 5.01 ± 0.00 L)

By considering the growth curve, it can be seen that the cell growth displayed a lag phase (4 h), two successive exponential phases (from 6.0 h to 9.0 h, and from 10.5 h to 14.0 h) and a stationary phase (after 14.5 h). The maximum specific growth rates corresponding to each

successive exponential phase were equal to 1.01 (± 0.01) h^{-1} and 0.13 (± 0.01) h^{-1}. This biphasic growth was not related to a substrate limitation, as the glucose concentration was equal to 8.5 (± 1.2) $g \cdot L^{-1}$ after 10 h of growth. In addition, the same biphasic kinetics was observed when cells were cultured at higher glucose concentrations (50 $g \cdot L^{-1}$ and 80 $g \cdot L^{-1}$) (Figure 3.2). This biphasic behavior might be caused by the lack of another nutrient than glucose that was not identified in this study, or by the accumulation of metabolic products which could inhibit cell growth. These products are lactic acid (Ichinose *et al.*, 2020), ethanol (van Bokhorst-van de Veen *et al.*, 2011) and acetic acid from the PK and EMP pathways. Their concentration reached 4.5 (± 0.7) $g \cdot L^{-1}$, 2.0 (± 0.3) $g \cdot L^{-1}$ and 0.2 (± 0.0) $g \cdot L^{-1}$, respectively, after 10 h of culture. Although these concentrations remained moderate and the inhibition due to organic acids was quite limited as they were mainly under the dissociated form at pH 6.0, they may contribute partly to the decrease of the specific growth rate. However, Schepers *et al.* (2002) indicated that, at pH 6.0, cell inhibition occurred at concentrations higher than 10 $g \cdot L^{-1}$ for *L. helveticus* R211.

At the end of the growth phase, a final cell concentration of 3.3 (± 0.3) $g_{CDW} \cdot L^{-1}$ was achieved (Table 3.4). This was similar to previous results obtained with the same *L. reuteri* strain at pH 5.5 (3.1 $g_{CDW} \cdot L^{-1}$) (Dishisha *et al.*, 2014). Concomitantly, 87.7 (± 5.6) % of cells were determined as being enzymatically-active and not permeabilized when the growth ended, before being harvested to be used for glycerol bioconversion. This result indicated that these reference conditions were favorable for *L. reuteri* DSM 17938 growth.

Table 3.4. Data characterizing the end of *L. reuteri* DSM 17938 growth phase in the reference condition (T1, 3 replicates) of the first experimental design

	Cell concentration at the end of growth ($g_{CDW} \cdot L^{-1}$)	**Lactic acid production yield ($g_{LA} \cdot g_{CDW}^{-1}$)**	**Ratio of [acetic acid + ethanol] to lactic acid ($mol \cdot mol^{-1}$)**
T1a	3.4	4.88	0.98
T1b	3.0	5.10	0.97
T1c	3.6	4.81	0.96

CDW: Cell dry weight

By considering the base consumption, a total quantity of 2.0 (± 0.02) mol was used during the growth to neutralize both lactic acid and acetic acid formation. Contrary to the growth, no inhibition of organic acids production was seen in Figure 3.5, as the base consumption stopped suddenly because of glucose starvation.

The growth phase stopped when all supplied glucose was consumed, which was confirmed by HPLC analyses. In other words, the cells entered the stationary phase when no more base solution was consumed as no more organic acids were produced due to the lack of glucose (

Figure 3.5). This behavior characterized a primary metabolism that could be explained by the low initial glucose concentration in the medium. Thus, in this reference condition, the glucose

availability might be a limitation for cell growth, which can be further improved by supplying more initial glucose.

In addition, the simultaneity of the end of growth and the end of base consumption in this reference condition allowed using the base profile to detect indirectly the growth cessation. In the case of a 50 $g \cdot L^{-1}$ glucose addition, the growth however stopped a few minutes before the end of base consumption (as seen in part 3.3.1.1 and Figure 3.2). But from a practical point of view, this on-line indicator was very useful to check that the growth was really stopped before harvesting, as the growth step occurred during night. It allowed defining the time at which bacterial cells have to be harvested and concentrated, and to start the bioconversion in the morning of the next day.

Final concentrations of metabolites were determined from HPLC analyses. After 14.6 (± 0.1) h of culture, the lactic acid, acetic acid and ethanol concentrations reached 16.4 (± 1.2) $g \cdot L^{-1}$, 3.7 (± 0.4) $g \cdot L^{-1}$ and 7.6 (± 0.8) $g \cdot L^{-1}$, respectively. The lactic acid production yield was determined as 4.93 (± 0.15) $g_{LA} \cdot g_{CDW}^{-1}$. This value was in agreement with previous results (Fu and Mathews, 1999).

The molar ratio between the sum of [acetic acid + ethanol] concentrations and lactic acid concentration was calculated at 0.97 (± 0.01) $mol \cdot mol^{-1}$ that is very close to 1 (Table 3.4). This revealed that the PK pathway of glycolysis was predominant, to the detriment of EMP pathway. This result obtained at controlled pH is consistent with previous studies done with *L. reuteri* ATCC 55730 at pH 5.5 (Årsköld *et al.*, 2008) and *L. reuteri* DSM 17938 grown at free-pH (Burgé *et al.*, 2015b). Regarding the efficiency of ATP synthesis, the domination of PK pathway is unfavorable compared to EMP pathway (production of 1 instead of 2 ATP per mole of glucose). Nevertheless, by producing less organic acids, the PK pathway reduced the bacterial cell inhibition by organic acids, thus improving their survival in an acidic environment (Burgé *et al.*, 2015b).

The molecular balances and the carbon mass balances were calculated during the growth step (see part 2.5.5 for the mode of calculation). They were equal to 98.97 (± 1.93) % and 106.71 (± 3.76) %, respectively. The difference between these two calculations is explained by the fact that the carbon mass balance takes into account the carbon included in the cellular biomass that was not the case of the molecular balance. The carbon mass balance was slightly higher than 100 %. This could be explained by the presence of other carbon sources in addition to glucose that could enter the glycolysis pathway. These other sources were identified as yeast extracts and peptones of MRS broth, whose elemental analysis revealed an organic carbon content of 40 % (Thompson *et al.*, 2017).

3.3.2.2 Kinetics of glycerol bioconversion by *L. reuteri* DSM 17938 in the reference condition

The second step of the bioprocess corresponds to glycerol bioconversion using resting cells of *L. reuteri* DSM 17938. This biotransformation allowed ATP production through the 3-HP

production and $NAD^+/NADH_2$ equilibrium thanks to the parallel oxidative and reductive branches of the Pdu pathway, together with 1,3-PDO synthesis. The glycerol was fed into the bioreactor at a constant rate of 0.5 $g_{glycerol} \cdot h^{-1}$ until the bioconversion stopped. The temperature was controlled at 37 °C and the pH was maintained at 6.0 with NH_4OH 1.48 mM. The partial dissolved oxygen pressure (pO_2) was measured without being controlled, to create and check microaerobic conditions. Kinetics of glycerol bioconversion into 3-HP and 1,3-PDO are illustrated in Figure 3.6, by considering the three repetitions of the reference condition.

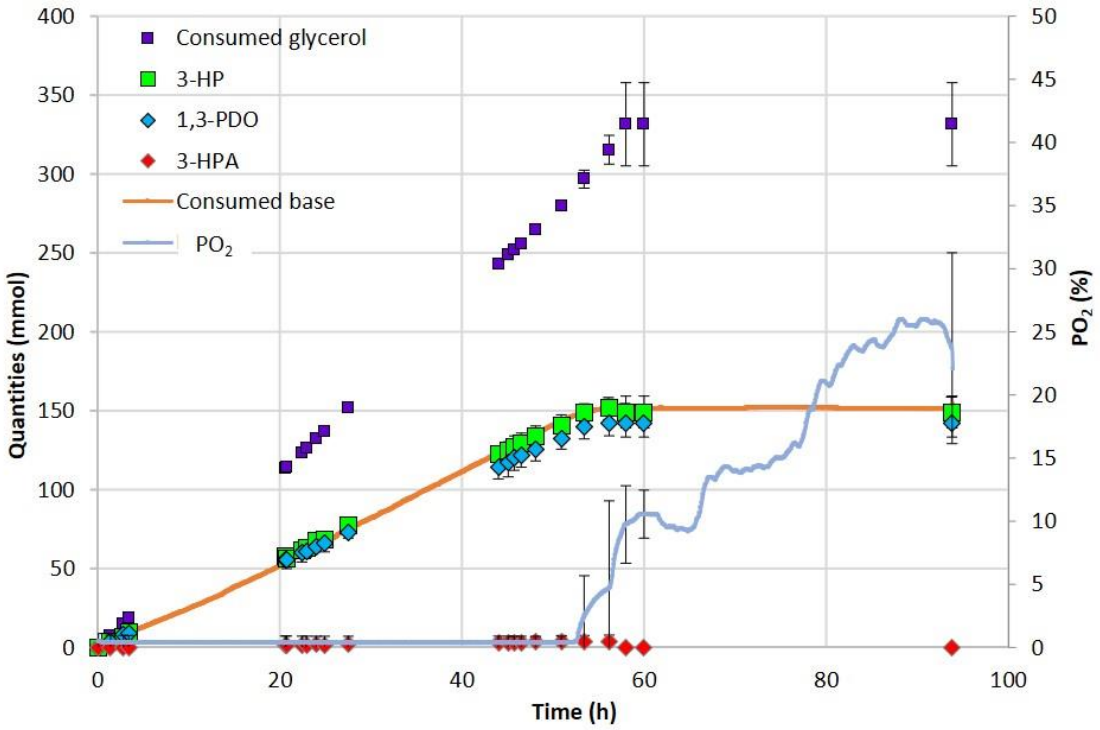

Figure 3.6. Kinetics of glycerol bioconversion into 3-HP, 3-HPA and 1,3-PDO by *L. reuteri* DSM 17938 in the reference condition (T1, 3 replicates) of the first experimental design (final volume 1.14 ± 0.25 L)

From Figure 3.6, the glycerol consumption rate was constant all along the biotransformation, at 5.6 (± 0.13) $mmol.h^{-1}$ corresponding to 0.51 (± 0.01) $g_{glycerol}.h^{-1}$, in agreement with the delivered glycerol feeding rate. This indicates that substrate was totally consumed as and when it was supplied, which was confirmed by the residual glycerol concentration that was equal to zero during the whole bioconversion. The glycerol consumption ceased concomitantly to the base consumption (with a slight difference that was ascribed to the variability), which was confirmed from HPLC analyses. This indicated that the base profile was a relevant indicator of the cessation of the bioconversion.

The data characterizing the bioconversion performances are shown in Table 3.5.

Table 3.5. Data characterizing the 3-HP bioproduction from glycerol by *L. reuteri* DSM 17938 in the reference condition (T1, 3 replicates) of the first experimental design (final volume 1.14 ± 0.25 L)

	Final 3-HP quantity (g)	Final 1,3-PDO quantity (g)	3-HP conversion yield ($g_{3\text{-HP}} \cdot g_{gly}^{-1}$)	3-HP production yield ($g_{3\text{-HP}} \cdot g_{CDW}^{-1}$)	Bioconversion duration (h)
T1a	12.4	10.4	0.43	0.76	57.7
T1b	13.7	10.4	0.51	0.90	53.4
T1c	14.1	11.5	0.51	0.68	56.2

Firstly, the carbon mass balances during bioconversion were verified at 100.12 (± 4.39) %. They were equivalent to the molecular balance, as no biomass was produced during the bioconversion step, and no complex components (such as yeast extracts or phytone peptones) were used during this second step of the bioprocess.

The 3-HP conversion yield was equal to 0.48 ± 0.04 $g_{3\text{-HP}} \cdot g_{glycerol}^{-1}$. This value was very close to the theoretical value of 0.49 $g_{3\text{-HP}}/g_{glycerol}^{-1}$ that corresponds to 0.5 $mol_{3\text{-HP}}/mol_{glycerol}^{-1}$.

After 55.8 (± 2.2) h of bioconversion, the two final products 3-HP and 1,3-PDO were obtained at final concentrations of 11.8 (± 0.5) $g \cdot L^{-1}$ and 9.5 (± 0.5) $g \cdot L^{-1}$, respectively. These concentrations corresponded to total amounts of 3-HP and 1,3-PDO produced at the end of the fed-batches that were respectively equal to 13.4 (± 0.9) $g_{3\text{-HP}}$ and 10.8 (± 0.7) $g_{1,3\text{-PDO}}$.

In comparison with the previous study of (Dishisha *et al.*, 2015) using *L. reuteri* DSM 20016, the duration of the bioconversion was similar (56 h), but the final 3-HP titers and quantities were 16 % lower in our study. This could be ascribed to the lower substrate feeding rate used in the present work (0.5 $g_{glycerol} \cdot h^{-1}$ instead of 0.75 $g_{glycerol} \cdot h^{-1}$ in Dishisha *et al.*, 2015).

The 3-HP production yield was equal to 0.78 (± 0.11) $g_{3\text{-HP}} . g_{CDW}^{-1}$, which was also lower than the value reported by (Dishisha *et al.*, 2015) (i.e., 1.65 $g_{3\text{-HP}} . g_{CDW}^{-1}$). All along the bioconversion, 3-HP was produced equimolarly to 1,3-PDO (Figure 3.6), as the molar ratio was equal to 1.04 (± 0.06) $mol \cdot mol^{-1}$. This indicated that the redox balance between NAD^+ and NADH was well maintained during the whole glycerol bioconversion. These results were also in accordance with those obtained by (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015) a molar ratio of 1 $mol \cdot mol^{-1}$.

As it can be seen from Figure 3.6, a small amount of 3-HPA was transiently accumulated when bioconversion stopped. The maximal 3-HPA concentration detected into the bioconversion broth was equal to 0.4 (± 0.1) $g \cdot L^{-1}$. 3-HPA is an intermediate product of glycerol bioconversion into 3-HP that is known to display an inhibitory effect against *L. reuteri* (Cleusix *et al.*, 2007). However, this value was about 10 times lower than the minimum inhibition concentration reported for *L. reuteri* DSM 20016 (2.2 – 3.7 $g \cdot L^{-1}$), thus limiting the detrimental effects of 3-HPA (Cleusix *et al.*, 2007). As 3-HPA was slightly accumulated at the precise moment when

bioconversion ceased, it might be hypothesized that the incriminated enzyme(s) is(are) involved in the second part of the Pdu pathway (i.e. between 3-HPA and 3-HP, see Figure 1.7).

The molar ratio between 3-HP and NH_4OH was equal to 1.08 ± 0.01 mol·mol^{-1}, which is a little bit higher than 1. As no other organic acid was detected from HPLC analyses, this small divergence could be explained by a slight volatilization of the ammonia solution during the bioconversion.

Micro-aerobic conditions were applied in this study to facilitate the glycerol metabolism toward 3-HP production (Zabed *et al.*, 2019). It means that neither air nor nitrogen was transferred to the bioconversion broth during the process. However, a slight oxygen transfer occurred thanks to the equilibrium of the gaseous phase with the liquid medium at the stirring rate of 100 rpm, thus creating microaerobic conditions. During the bioconversion, the partial dissolved oxygen pressure (pO_2) in the medium remained at a value lower than 0.5 % from the beginning of glycerol supply until 51 h (Figure 3.6). At that time, the pO_2 increased, which meant that dissolved oxygen was not consumed anymore by bacterial cells. At the same time, the base consumption started to decrease and 3-HP and 1,3-PDO production ceased, thus indicating that these variables were related. This link between the pO_2 increase and the cessation of bioconversion is consistent with recent works (Dishisha *et al.*, 2019; Zabed *et al.*, 2019). The pO_2 increase may be due to a reduction of the activity of the enzyme NAD(P)H oxidase that uses molecular oxygen as a substrate (Dishisha *et al.*, 2019). We may hypothesize that the stop of the bioconversion corresponded to a global dysfunction of the whole enzyme pool of bacterial cells. In that case, the catalytic work of the NAD(P)H oxidase stopped concomitantly with that of the enzymes of the Pdu pathway that drive the 3-HP bioproduction (notably the coenzyme-A dependent enzyme and phosphotransacylase). The decrease in oxygen consumption was thus observed simultaneously to the reduction in 3-HP bioproduction. Moreover, our results indicate that the pO_2 measurement may constitute an early *on-line* indicator of the cessation of bioconversion, which is an original approach for controlling the fed-batch bioconversion of glycerol into 3-HP.

The cell physiological state was evaluated by measuring the concentrations of enzymatically-active and not permeabilized cells in the medium all along the bioconversion process (Figure 3.7).

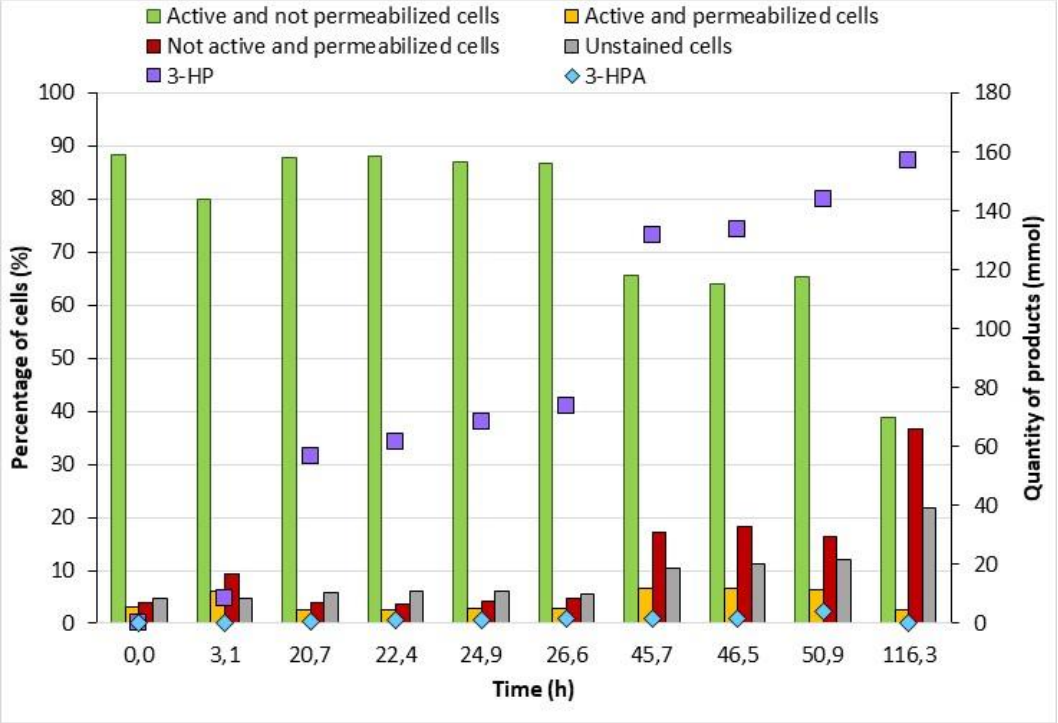

Figure 3.7. Evolution of the physiological state of *L. reuteri* DSM 17938 during 3-HP bioproduction from glycerol bioconversion in the reference condition (T1) of the first experimental design (final volume 1.15 L)

The initial percentage of active and not permeabilized cells was similar at the beginning of the bioconversion phase (83.5 ± 2.3 %) than at the end of the growth phase (87.7 ± 5.6 %). This indicates that the harvesting and concentration steps that were done between the growth and the bioconversion stages did not significantly affect the physiological state of *L. reuteri* DSM 17938 cells. During the bioconversion, the percentage of active and not permeabilized cells remained constant during the first 26 h, at 86.3 ± 3.2 %. Then, it decreased to reach 65 ± 0.9 % between 45 h and 51 h, and finally decreased to 38.9 ± 0.0 % at 116 h.

This behavior could be explained by the combination of three factors: the total absence of nutrients and energy sources in the medium during the bioconversion step; an inhibiting effect of 3-HP that reached 6.4 ± 0.2 $g \cdot L^{-1}$ at 26 h, as (Ramakrishnan *et al.*, 2015) indicated that a 3-HP titer of 3.15 $g \cdot L^{-1}$ was inhibiting for *L. reuteri* RPRB 3007; and perhaps an inhibiting effect of 3-HPA whose concentration was equal to 4.5 $mmol \cdot L^{-1}$ at 26 h, even if this concentration was lower than that indicated by (Sabet-Azad *et al.*, 2013) (8.1 $mmol \cdot L^{-1}$). Then, between about 27 h and 51 h, the ATP production continued but the higher 3-HP concentration (between 6.9 ± 0.1 $g \cdot L^{-1}$ and 10.6 ± 0.9 $g.L^{-1}$), together with the accumulation of 3-HPA (5.5 ± 1.7 $mmol \cdot L^{-1}$) and the absence of growth substrate might be detrimental to the cells, thus lowering their physiological state. At the end of the bioconversion, the high 3-HP concentration (9.2 ± 0.8 $g \cdot L^{-1}$ at 116 h) led to a stronger inhibition that blocked its own synthesis and the corresponding ATP production. These phenomena, together with the total absence of nutrients and energy sources in the medium during the bioconversion step,

combined to reduce the proportion of active and not permeabilized cells in the medium. Moreover, it has to be kept in mind that the apparent absence of 3-HPA observed at the end of the bioconversion was explained by its difficult quantification, due to the dilution of the bioconversion broth linked to the introduction of glycerol and base solutions.

3.3.2.3 Summary of information

From this information, the reference condition led to 3-HP bioproduction in fed-batch mode but in limited amounts as compared to results displayed in the bibliography. The cells were able to maintain an active physiological state for at least 2 days of bioconversion, and no 3-HPA significantly accumulated in the medium. However, a possible inhibiting effect of 3-HP was pointed out.

The variability of these experiments was considered as correct, regarding the complexity of the bioprocess that included a growth phase, a harvesting and concentration phase, and then a bioconversion phase. In addition to these results, the fed-batch process was shown to be controlled by considering both NH_4OH consumption and pO_2 variation.

Consequently, the implementation of an experimental design is of great interest, to circumvent these limitations and to define culture conditions able to increase 3-HP concentration, quantity and production rate.

3.3.3 Effect of growth culture conditions on growth performance of *L. reuteri* DSM 17938

According to the Plackett and Burman experimental design (Table 2.2), various growth medium recipes and environmental conditions were designed to screen, among 11 factors, the best combination to improve bacterial growth with the aim to enhance 3-HP production performance. The final biomass concentration ($g_{CDW}·L^{-1}$) at the end of growth was retained as the variable to characterize the bacterial growth performance. Indeed, as the growth occurred during night without possibility to monitor it by a biomass probe at that step of the thesis, the effect of the experimental factors on the specific growth rate could not be established. The lactic acid production yield was also used to characterize the metabolic activity of bacterial cells during their growth.

Before analyzing the results, the molecular balances and the carbon mass balances during the growth phase were verified for all the performed experiments. They were equal to 99.51 (± 2.54) % and 104.33 (± 3.82) %, respectively. The values exceeding 100 % in the case of the carbon mass balance could be ascribed to the utilization of other organic carbon from yeast extract, peptones and/or phytone peptone than only from glucose, as aforementioned. The results obtained from the 14 experiments (i.e., 3 repetitions of the reference condition and 11 experiments of the Plackett and Burman experimental design) are summarized in Table 3.6.

Table 3.6. Data characterizing bacterial growth of *L. reuteri* DSM 17938 in the experiments of the first experimental design

Experiment code	Cell concentration at the end of growth ($g_{CDW}·L^{-1}$)	Lactic acid production yield ($g_{LA}·g_{CDW}^{-1}$)	Ratio of [acetic acid + ethanol] to lactic acid ($mol·mol^{-1}$)
T1	3.3 ± 0.3	4.93 ± 0.15	0.97 ± 0.01
T2	3.0	8.69	0.91
T3	4.8	5.72	0.89
T4	3.2	3.51	0.93
T5	5.1	6.39	0.95
T6	5.7	4.63	0.89
T7	4.5	5.61	1.01
T8	3.0	4.72	0.93
T9	4.5	3.27	0.98
T10	2.4	6.68	0.92
T11	3.8	7.37	0.92
T12	3.5	3.51	1.05

T1: reference condition, including 3 replicates

These data were used for statistical analyses whose results on several response variables are presented in Table 3.7, with the aim to assess the effect of each factor on the selected response variables.

Table 3.7. Effect of growth conditions on growth performances of *L. reuteri* DSM 17938

	Cell concentration at the end of growth ($g_{CDW}·L^{-1}$)	Lactic acid production yield ($g_{LA}·g_{CDW}^{-1}$)	Ratio of [acetic acid + ethanol] to lactic acid ($mol·mol^{-1}$)
Constant	3.90	5.42	0.95
Additional glucose	0.58**	0.98**	-0.0175 **
Additional yeast extract	ns	-0.80**	ns
Phytone peptone	ns	ns	ns
Tween 80	ns	ns	ns
Vitamin B12	ns	ns	0.014**
1,2-propanediol	ns	-0.18**	-0.019**
Cysteine	ns	-0.24**	ns
Betaine and KCl	ns	0.29**	0.0175 **
Temperature	0.53**	-0.70**	0.029***
pH	ns	0.46**	ns
Base	ns	0.15*	ns
R^2	0.873	0.990	0.952

Confidence level: * 90 %; ** 95 %; *** 99% confidence level; ns: not significant;
R^2 regression coefficient adjusted for degree of freedom

This table summarizes the values of the parameters of the models that were obtained by analyzing the results of the 14 experiments in the different conditions, including the three repetitions of the reference condition. When the parameters were not significant at a confidence level of at least 90 %, they were not retained. This low confidence level was retained because of the complexity of the experiments. The significant parameters were then introduced in the models, whatever the confidence level (90 % and 95 %) to estimate the response variables.

3.3.3.1 Effect of growth conditions on the final cell concentration

From Table 3.7, only two factors displayed a significant effect on *L. reuteri* DSM 17938 cell concentration at the end of growth, namely the addition of glucose and the higher temperature, which positively influenced the final biomass concentration by 18 % and 15 %, respectively.

Results related to the glucose addition were confirmed by considering the concentration of active and non-permeabilized cells at the end of the culture, enumerated by flow cytometry. It was improved by 134 % in the presence of 50 $g \cdot L^{-1}$ additional glucose ($7.5 \cdot 10^9$ $cell \cdot mL^{-1}$) compared to a 20 $g \cdot L^{-1}$ addition ($5.6 \cdot 10^9$ $cell \cdot mL^{-1}$), which matched with the aforementioned result. This positive effect of glucose on *L. reuteri* growth was also consistent with early studies (Burgé *et al.*, 2015b; Couvreur *et al.*, 2017).

The temperature affected the biomass concentration at the end of growth that was higher at 37 °C than at 33 °C, which was also confirmed by flow cytometry measurements of cell concentrations ($5.6 \cdot 10^9$ $cell \cdot mL^{-1}$ instead of $4.5 \cdot 10^9$ $cell \cdot mL^{-1}$). This result was consistent with the temperature found in the digestive tract, the ecological niche of *L. reuteri* (Duar *et al.*, 2017).

The addition of yeast extract (25 $g \cdot L^{-1}$) or phytone peptone (25 $g \cdot L^{-1}$) to the MRS medium as nitrogen sources did not improved neither the biomass concentration nor the cellular state. This result is contrary to those obtained in previous studies (Atilola *et al.*, 2015; Couvreur *et al.*, 2017) and suggested that the growth limitation previously observed during the growth phase cannot be justified by a lack of these nutrients. The existence of interactions between the tested factors, which cannot be considered by the type of experimental design used in the present study, may however partly hinder this effect. This means that these components may perhaps influence positively the bacterial growth in one set of conditions, but negatively in another set of conditions, what was not possible to demonstrate with the adopted experimental strategy.

Tween 80 and cysteine are both growth factors for lactobacilli. However, the supplementation of the growth medium with 4 $g \cdot L^{-1}$ (in addition to the 1 $g \cdot L^{-1}$ already included in MRS broth) and 1 $g \cdot L^{-1}$ of these compounds, respectively, seemed not enough to impact the cell concentration.

The addition of vitamin B12 (0.1 mg·L^{-1}), 1,2-PDO (3 g·L^{-1}) and betaine (0.234 g·L^{-1}) together with KCl (7.455 g·L^{-1}) did not affect the growth, which was expected as they were added to act during the bioconversion stage.

Finally, the pH value (pH 5.5 or pH 6.0) and the base used for pH control (NH_4OH or NaOH) did not significantly modify the final cell concentration of *L. reuteri* DSM 17938. Therefore, they can be used equally to maintain the pH during the growth phase.

3.3.3.2 Effect of growth conditions on the lactic acid production yield

Some experimental factors of the experimental design were shown to improve the lactic acid production yield. They corresponded to the addition of 50 g·L^{-1} glucose to the MRS medium, the addition of betaine plus KCl, the pH control at 6.0 and the use of NaOH as the base for pH control (Table 3.7). Conversely, this variable was negatively affected by the addition of yeast extract, 1,2-PDO and cysteine, and at the higher temperature.

The initial glucose concentration showed the highest impact, corresponding to an increase of 18 % of the lactic acid production yield with the addition of 50 g·L^{-1} glucose to the MRS broth (6.40 g_{LA}·g_{CDW}^{-1}) as compared to 20 g·L^{-1} (5.42 g_{LA}·g_{CDW}^{-1}). By looking back to the results about cell concentration (Table 3.7), the higher initial glucose concentration was directed towards both lactic acid production and biomass production. Nevertheless, these results differed from that of the preliminary tests (see Section 3.3.1.1) showing that additional glucose of 80 g·L^{-1} led to more organic acid production without improving biomass concentration, compared to additional glucose of 50 g·L^{-1}. The uncoupling between lactic acid production and biomass production was then more pronounced at glucose concentrations higher than 50 g·L^{-1}.

A slight positive effect of betaine + KCl addition (0.234 g·L^{-1} + 7.455 g·L^{-1}) was detected, as these components improved the lactic acid production yield by 5 %. This might be ascribed to the osmoprotectant role of betaine in lactobacilli, as demonstrated with *L. buchneri* R1102 (Louesdon *et al.*, 2014). This result is in agreement with a previous study which reported that addition of 2 g·L^{-1} betaine led to a maximum lactic acid titer in *L. casei* ZW-63A (Zou *et al.*, 2013). These authors explained their results by an improvement of the lactate dehydrogenase activity in the presence of betaine (Zou *et al.*, 2013).

The pH 6.0 induced a slight increase (+ 8 %) in the lactic acid production yield, as compared to pH 5.5 (Table 3.7). The result is in agreement with those obtained by (El-Ziney, 2018) with *L. reuteri* DSM 12246 that increasing pH from 5.5 to 6.0 resulted in raising of lactic acid production yield by 13 %. Generally, lactic acid bacteria have acid-resistance systems to ensure intracellular pH homeostasis. However, the key mechanism for intracellular pH maintenance requires high ATP consumption for expulsion of dissociated acids and protons from the cytoplasm through H^+-ATPase (Cotter and Hill, 2003; Singhvi *et al.*, 2018). The less expenditure of energy at pH 6.0 to maintain the intracellular pH could be a reason to explain this result.

By considering the type of base employed for pH control, the use of sodium hydroxide 8.75 mol·L^{-1} slightly improved the lactic acid production yield (+ 3 % at a confident level of 90 %) as compared to ammonium hydroxide 14.8 mol·L^{-1}. Even if this difference is very low, it is consistent with the observation of (Vasiljevic *et al.*, 2005) who indicated that NH_4OH was better for the growth of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 than for the lactic acid production, as compared to NaOH.

The lactic acid production yield was negatively affected by the addition of yeast extract (25 g·L^{-1}), 1,2-PDO (3 g·L^{-1}) and cysteine (1 g·L^{-1}). It was significantly reduced by 15 % when the medium was supplemented with 25 g·L^{-1} yeast extract. As yeast extract contains some carbon source that constitutes 40 % (mol·mol^{-1}) of its composition (Thompson *et al.*, 2017), it could be hypothesized that this compound acts as a carbon source for cell growth, thus being in competition with glucose.

The addition of 1,2-PDO and cysteine in the medium was done to further improve glycerol bioconversion. 1,2-PDO might help the cells to prepare their dedicated enzymatic machinery for 3-HP bioproduction (Dishisha *et al.*, 2015). Cysteine was added to counteract the oxidative stress caused by 3-HPA that is a precursor of reuterin (Schaefer *et al.*, 2010). Their impact on the lactic acid production yield was however very modest (reduction by 3 % and 4 %, respectively).

Finally, a culture temperature of 37 °C induced a reduction by 13 % of the lactic acid production yield in comparison with 33 °C. As this higher temperature led to a significant increase in biomass concentration (see paragraph 3.3.3.1) as compared to 33 °C, and as the quantity of lactic acid produced (82.2 ± 5.9 g) was not affected by the temperature, the production yield decreased only because of the biomass increase.

3.3.3.3 Effect of growth conditions on the ratio between [acetic acid + ethanol] and lactic acid

The analysis of the molar ratio between the sum of [acetic acid + ethanol] and lactic acid informs the relative part of PK pathway and EMP pathway for glucose catabolism (Årsköld *et al.*, 2008). In this study, the constant characterizing this molar ratio was equal to 0.95 (Table 3.7). It was close to the value of 1.0 that indicates a dominant PK pathway in the glycolysis process, as it was already observed by (Burgé *et al.*, 2015b).

From Table 3.7, two factors negatively affected this variable, i.e. the addition of glucose 50 g·L^{-1} and 1,2-PDO, and three factors showed a positive influence. However, since their impact was always lower than 3 % compared to the constant, the PK pathway remained superior to the EMP pathway in all set of conditions tested. In contrast, addition of vitamin B12 and betaine + KCl as well as a high temperature slightly reduced the ratio, thus decreasing the part of PK pathway in the glycolysis reactions.

3.3.4 Effect of culture conditions on bioconversion performance of *L. reuteri* DSM 17938

The Plackett and Burman experimental design allowed identifying the growth medium composition and culture conditions that affected the ability of *L. reuteri* DSM 17938 to further perform glycerol bioconversion into 3-HP.

During all bioconversion experiments, no residual glycerol was detected in the bioconversion broth, which indicated that it was completely converted into 3-HP and 1,3-PDO as soon as it was supplied to the bioreactor.

The carbon mass balances of the bioconversion step were established at 100.12 ± 4.39 %, which allowed to be confident in the further analysis of the results. Table 3.8 summarizes the results obtained from the 14 different experiments (including three replicates of the reference condition), to explain the effects of the 11 factors on three variables that characterized the bioconversion step: final quantity of 3-HP produced, 3-HP production yield and bioconversion duration.

Table 3.8. Data characterizing glycerol bioconversion for 3-HP bioproduction by *L. reuteri* DSM 17938 in the experiments of the first experimental design

Experiment code	Final volume (L)	Total 3-HP produced (g)	3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)	Bioconversion duration (h)
T1	1.14 ± 0.25	13.4 ± 0.89	0.78 ± 0.11	55.6 ± 3
T2	1.13	12.4	0.60	51.4
T3	1.12	11.1	0.42	52.2
T4	1.21	13.3	0.88	63.2
T5	1.36	18.9	0.61	92.3
T6	1.27	16.1	0.50	75.6
T7	1.20	11.9	0.35	64.3
T8	1.33	19.6	1.99	88.6
T9	1.09	14.3	0.68	58.8
T10	1.09	11.0	0.83	44.1
T11	1.26	16.3	0.63	72.7
T12	1.14	11.1	0.68	50.1

T1: reference condition, including 3 replicates

These data allowed performing statistical analyses to reveal the factors that displayed significant effects on the variables characterizing the 3-HP bioproduction. The corresponding results are presented in Table 3.9.

Table 3.9. Effect of growth conditions on glycerol bioconversion performances of *L. reuteri* DSM 17938 (Final volume 1.19 ± 0.09 L)

	Total 3-HP produced (g)	**3-HP production yield ($g_{3\text{-}HP}{\cdot}g_{CDW}^{-1}$)**	**Bioconversion duration (h)**
Constant	14.12	0.75	64.1
Additional glucose	ns	-0.23**	4.0**
Additional yeast extract	ns	ns	ns
Phytone peptone	0.94*	0.11*	5.7**
Tween 80	0.84*	0.10*	2.1*
Vitamin B12	-2.16**	-0.15**	-10.6***
1,2-propanediol	1.03*	0.093*	4.5**
Cysteine	ns	-0.10*	ns
Betaine and KCl	ns	0.10*	4.6**
Temperature	ns	-0.15**	2.1*
pH	ns	0.096*	ns
Base	ns	ns	ns
R^2	0.893	0.919	0.978

Confidence level: * 90 %; ** 95 %; *** 99 %; ns: not significant; R^2 adjusted for degree of freedom.

From Table 3.9, nine factors displayed a statistically significant effect on one or several variables. Only two factors did not show any significant effect on the characteristics of the 3-HP production step: the addition of yeast extract and the type of base used for pH control.

The three response variables were positively influenced by the addition of phytone peptone, Tween 80 and 1,2-PDO, but negatively by a vitamin B12 supplementation. The addition of betaine enhanced the 3-HP production yield and the bioconversion duration. A high glucose concentration and a high temperature positively affected the bioconversion duration but negatively the 3-HP production yield. Finally, the addition of cysteine and a low pH value negatively influenced the 3-HP production yield.

In addition to these three variables, the quantity of 1,3-PDO produced and the molar ratio between 3-HP and 1,3-PDO were also determined and will be analyzed when required.

3.3.4.1 Effect of glucose supplementation on 3-HP bioproduction

As explained above, the growth medium supplementation with a higher glucose concentration led to a higher quantity of harvested biomass and a higher percentage of active cells at the beginning of bioconversion. However, in the experimental conditions chosen during the bioconversion step, no increase in 3-HP production was observed. This result can be explained by the moderate glycerol feeding rate (0.5 $g_{glycerol}{\cdot}h^{-1}$) used in this study. As a consequence, the quantity of glycerol provided was insufficient to feed the higher cell quantity obtained in the presence of a higher initial glucose concentration. The specific

glycerol feeding rate was thus lower in this condition (18.1 ± 3.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) as compared to that observed with the lower level of glucose addition (32.9 ± 8.6 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$). This observation was confirmed by the decrease in the 3-HP production yield (- 30.7 %).

The longer duration of bioconversion (+ 6.2 %) noticed when the cells were previously cultivated in the presence of a higher glucose concentration might be explained by a better enzymatic activity of the bacterial population at the beginning of bioconversion stage, as confirmed by the concentrations of active cells ($4.3 \cdot 10^{10}$ ± $0.9 \cdot 10^{10}$ cells·mL^{-1} with 50 g·L^{-1} added glucose, instead of $1.9 \cdot 10^{10}$ ± $0.6 \cdot 10^{10}$ cells·mL^{-1} with 20 g·L^{-1} added glucose). This higher concentration of active cells allowed *L. reuteri* DSM 17938 total population to convert glycerol for a longer time.

3.3.4.2 Effect of yeast extract and phytone peptone addition on 3-HP bioproduction

Results showed that phytone peptone slightly promoted the 3-HP bioproduction whereas yeast extract did not (Table 3.9). As these two compounds act as amino acid sources, the positive effect of phytone peptone can be related to a specific amino acid composition that differs from that of yeast extract. From the information available from the suppliers, phytone peptone contained more arginine, histidine, tyrosine (+ 200 %) and glycine (+ 90 %) than yeast extract. Particularly, the amino acids arginine and glycine have been shown to be used for energy production and were linked to the survival of *Lactococcus lactis* under starvation (Stuart *et al.*, 1999) and *Lactobacillus sakei* under acidic conditions (Champomier Vergès *et al.*, 1999).

3.3.4.3 Effect of Tween 80 addition on 3-HP bioproduction

A positive effect of Tween 80 supplementation was observed on the cell ability to produce 3-HP (Table 3.9) that is consistent with (Couvreur *et al.*, 2017). This component is known to support cell division of lactobacilli by modifying the cell membrane fatty acid composition (Béal *et al.*, 2001). In addition, it induces a down-regulation of the *de novo* fatty acid synthesis that helps the cells to save intracellular energy (Reitermayer *et al.*, 2018) that could thus be better directed towards 3-HP bioproduction and excretion.

3.3.4.4 Effect of vitamin B12 addition on 3-HP bioproduction

A negative effect of the addition of 0.1 mg·L^{-1} vitamin B12 in the growth medium was observed on the quantity of 3-HP produced, the bioconversion duration and the 3-HP production yield (Table 3.9). This result was an unintended outcome because this compound is a required cofactor of the first enzyme of the Pdu metabolic pathway (Dishisha *et al.*, 2014). It was reported that a supplementation with 0.1 mg·L^{-1} vitamin B12 in the bioconversion medium showed a positive impact on 3-HP production by *L. reuteri* DSM 17938 (Couvreur *et al.*, 2017). At that step of the study, the question remains about the ability of *L. reuteri* to internalize vitamin B12 that is a large molecule of molecular weight of 1355.4 g.mol^{-1}

(PubChem, 2021). Another hypothesis is that the early addition of exogenous vitamin B12 in the growth medium (i.e. before the bioconversion step) may reduce the ability of bacterial cells to produce endogenous vitamin B12 during the subsequent step of bioconversion.

3.3.4.5 Effect of 1,2-PDO addition on 3-HP bioproduction

Addition of 3 $g \cdot L^{-1}$ 1,2-PDO in the growth medium significantly improved the three variables characterizing the bioconversion step Table 3.9, in agreement with (Dishisha *et al.*, 2015). Here we consider that, due to the structural similarity between glycerol and 1,2-PDO, the addition of the latter in the growth medium might help the cells to prepare their enzymatic machinery (Sriramulu *et al.*, 2008). More particularly, these authors showed that 1,2-PDO induced the synthesis of glycerol dehydratase, which was confirmed by our results.

3.3.4.6 Effect of cysteine addition on 3-HP bioproduction

The addition of cysteine was related to a reduction of the 3-HP production yield (Table 3.9). This observation can be explained by its negative effect on vitamin B12 biosynthesis by *L. reuteri*, as previously demonstrated by (Santos *et al.*, 2009). As another hypothesis, cysteine may counteract the oxygen utilization by the cells, as suggested by (Turner *et al.*, 1999).

3.3.4.7 Effect of the addition of betaine and KCl on 3-HP bioproduction

The addition of betaine (0.234 $g \cdot L^{-1}$) together with KCl (7.455 $g \cdot L^{-1}$) led to a longer bioconversion, together with a higher production yield (Table 3.9) that can be explained by the protective effect of betaine against osmotic stress (Kets and De Bont, 1994). This result can be linked to that of (Louesdon *et al.*, 2014) who related a higher intracellular betaine content to a more rigid membrane. This phenomenon led to the reduction of the exchanges between the intracellular and extracellular compartments in *L. buchneri* R1102, thus making the cells more resistant (Louesdon *et al.*, 2014).

3.3.4.8 Effect of growth temperature on 3-HP bioproduction

Regarding the influence of the growth temperature on the further bioconversion step, a higher 3-HP production yield but a shorter bioconversion duration was observed when the previous growth was conducted at 33 °C instead of 37 °C (Table 3.9). As the glycerol feeding rate was fixed at 0.5 $g_{glycerol} \cdot h^{-1}$ during bioconversion, this higher 3-HP production yield was the consequence of the lower cell concentration achieved at the end of the growth phase, as already observed by considering the effect of the glucose addition (see part 3.3.3.1).

3.3.4.9 Effect of growth pH and base used for pH control on 3-HP bioproduction

The pH value during growth had a very little effect on the bioconversion step. Only the 3-HP production yield decreased slightly when the pH was reduced from 6.0 to 5.5 (Table 3.9). This

difference could be ascribed to a small, even not significant, increase in the final biomass concentration when the growth was conducted at pH 5.5.

In addition, it was demonstrated that the type of base used (NH_4OH or NaOH) had no effect, thus allowing us to use them equally. However, as the base NH_4OH prevents the formation of salt in the medium, it might be preferred to facilitate further downstream processes.

3.3.5 Selected growth conditions that improve *L. reuteri* DSM 17938 ability to convert glycerol into 3-HP

In order to enhance the capacity of *L. reuteri* DSM 17938 to perform 3-HP bioproduction at a glycerol feeding rate of 0.5 $g_{glycerol} \cdot h^{-1}$, the growth conditions leading to the best 3-HP titer, bioconversion duration and 3-HP production yield have been identified. They consist in MRS medium added with glucose (20 $g \cdot L^{-1}$), phytone peptone (25 $g \cdot L^{-1}$), Tween 80 (4 $g \cdot L^{-1}$), 1,2-PDO (3 $g \cdot L^{-1}$), betaine (0.234 $g \cdot L^{-1}$) plus KCl (0.745 $g \cdot L^{-1}$), temperature (33 °C) and pH 6.0. No additional yeast extract, neither supplementation with vitamin B12 nor cysteine were required. The base used to control the pH was NH_4OH as it was likely unimportant.

This set of conditions has been implemented in an additional experiment to confirm its positive effects on the bioconversion performances. The conditions used during the bioconversion step were identical as in the experimental design. Results are shown in Figure 3.8.

In comparison to the reference growth condition, when the bioconversion was launched after a growth step performed with the selected conditions, a real improvement of the 3-HP bioproduction was observed.

The bioconversion lasted longer, from 55.8 ± 2.2 h to 88.6 h, and the total 3-HP produced increased from 148.9 ± 9.9 mmol to 211.8 mmol (Figure 3.8). The 3-HP titer was increased from 11.8 ± 0.5 $g \cdot L^{-1}$ to 14.7 $g \cdot L^{-1}$, and the 3-HP production yield from 0.8 ± 0.1 $g_{3\text{-HP}} \cdot g_{CDW}^{-1}$ to 2.0 $g_{3\text{-HP}} \cdot g_{CDW}^{-1}$. In addition, the specific 3-HP production rate was improved from 14.0 ± 2.5 $mg_{3\text{-HP}} \cdot g_{CDW}^{-1} \cdot h^{-1}$ to 22.5 $mg_{3\text{-HP}} \cdot g_{CDW}^{-1} \cdot h^{-1}$. The volumetric productivity measured at the moment when 3-HP production ceased was doubled from 0.24 $g \cdot L^{-1} \cdot h^{-1}$ to 0.48 $g \cdot L^{-1} \cdot h^{-1}$. Finally, no 3-HPA accumulation was found in this bioconversion (the 3-HPA concentration could not be detected by HLPC).

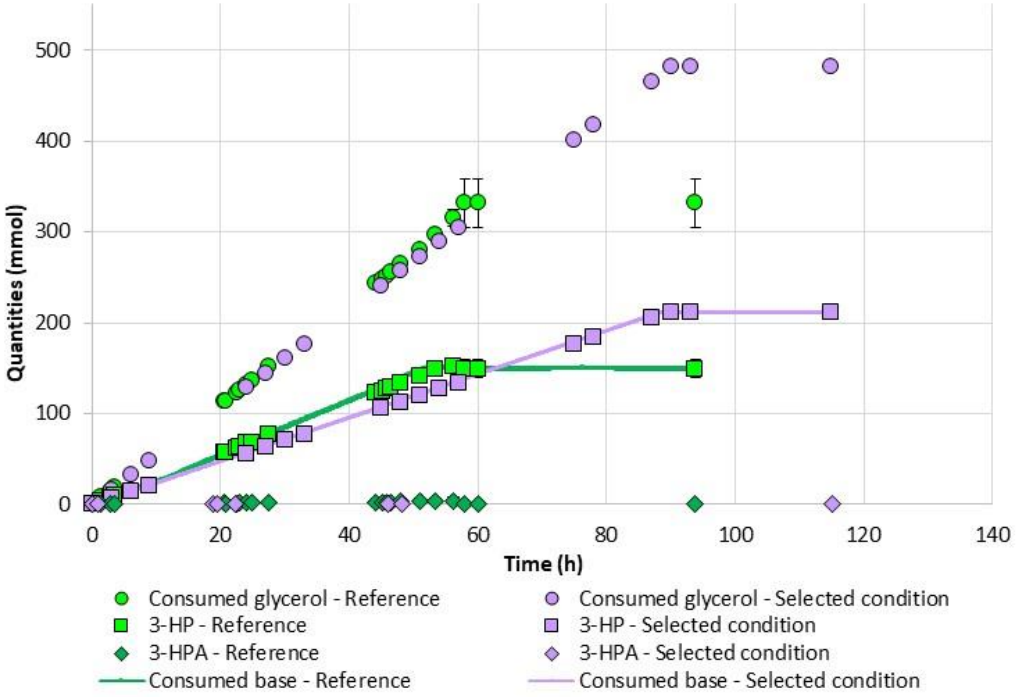

Figure 3.8. 3-HP bioproduction from glycerol by *L. reuteri* DSM 17938 in the selected conditions for the growth step (final volume 1.33 L) in comparison with the reference condition of the first experimental design (final volume 1.14 ± 0.25 L)

As the cell concentration at the end of the growth phase performed with this new set of conditions (3.0 $g_{CDW}{\cdot}L^{-1}$) was similar to that of the reference condition (3.3 ± 0.3 $g_{CDW}{\cdot}L^{-1}$), the observed improvement could be explained by a better ability of each cell to produce 3-HP from glycerol. This was supported by the higher ratio between 3-HP to enzymatically-active cells determined by flow cytometry, which increased from 0.98 ± 0.21 $g_{3\text{-}HP}.g_{cells}^{-1}$ in the reference condition to 2.05 $g_{3\text{-}HP}.g_{cells}^{-1}$ in the optimized recipe.

Finally, as the validation conditions allowed confirming the improvement of the 3-HP bioproduction performance, they were retained for the further steps of the study.

3.3.6 Effect of harvest time on the ability of *L. reuteri* DSM 17938 to produce 3-HP from glycerol

The physiological state of cells that will enter the bioconversion process as resting cells is of great importance. As this state is defined at the end of the growth phase, the harvesting time is an important factor, in addition to the other growth conditions, as previously demonstrated by (Rault *et al.*, 2008). It was thus of great importance to determine if the moment at which the cells of *L. reuteri* DSM 17938 were harvested, may influence, or not, their performances during bioconversion, and to determine the best harvesting time for 3-HP bioproduction.

Two harvesting times of *L. reuteri* DSM 17938 cells have been tested: the first one corresponded to the end of growth phase, i.e. to the very beginning of the stationary phase. It was detected from the cessation of base consumption at the end of the growth phase, as being equal to 14.6 ± 0.9 h from the three repeated experiments of the experimental design. The second harvesting time was delayed by 6 h from the first one, in order to recover the cells in more advanced stationary phase, i.e. at 20.6 h. The kinetics of the bioconversions performed with resting cells harvested in advanced stationary phase (i.e., 6 h after the base consumption stopped) were compared with those of the reference condition of the first experimental design. They are illustrated in Figure 3.9.

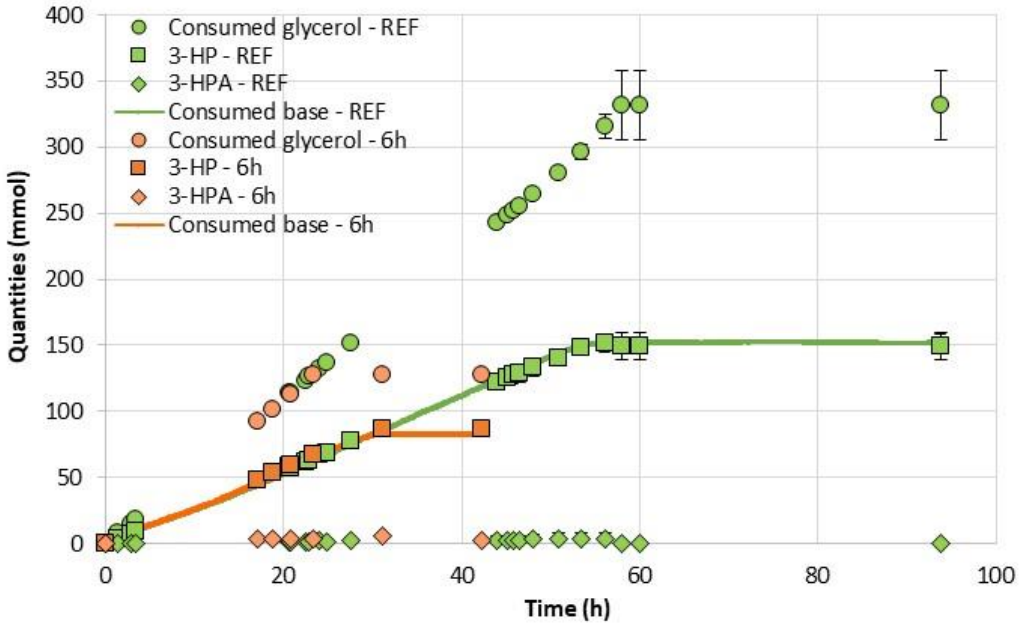

Figure 3.9. Comparison of kinetics of glycerol bioconversion by *L. reuteri* DSM 17938 cells harvested at the beginning of stationary phase (reference, in green) (final volume 1.14 ± 0.25 L) and in advanced stationary phase (after 6 h, in orange) (final volume 1 L)

The rates of base consumption and 3-HP bioproduction remained constant all along the bioconversion, whatever the harvesting time (Figure 3.9). They were slightly lower for cells harvested after 6 h in stationary phase. When no more 3-HP was produced, the base consumption stopped and the dissolved oxygen concentration started increasing (data not shown) as previously mentioned in Section 3.3.2.2.

By considering the other variables, strong differences were pointed out during the bioconversion phase, when the cells we harvested at the two different moments of the growth phase. The results obtained from these two experiments are summarized in Figure 3.9.

From Figure 3.10, the final biomass concentration measured at the moment when the cells were harvested slightly differed between the two harvest times. It was lower after 6 h in stationary phase (2.7 $g_{CDW} \cdot L^{-1}$) than at the beginning of stationary phase (3.3 ± 0.3 $g_{CDW} \cdot L^{-1}$),

by taking into account the standard variation. This result is in agreement with that of (Zabed *et al.*, 2019).

The 3-HP titer and 3-HP final quantity were lower when the cells were recovered in the advanced stationary phase. The titer was reduced by 34 % and the 3-HP quantity was decreased by 42 %. Consequently, the 3-HP production yield decreased by 30 % (Figure 3.10). These results can be linked to the reduction in the bioconversion duration that was lowered by 44 % when the cells were harvested in advanced stationary phase (Figure 3.9 and Figure 3.10). Meanwhile, the 3-HPA concentration was not significantly affected and remained below 0.5 $g_{3\text{-HPA}} \cdot L^{-1}$ in both cases.

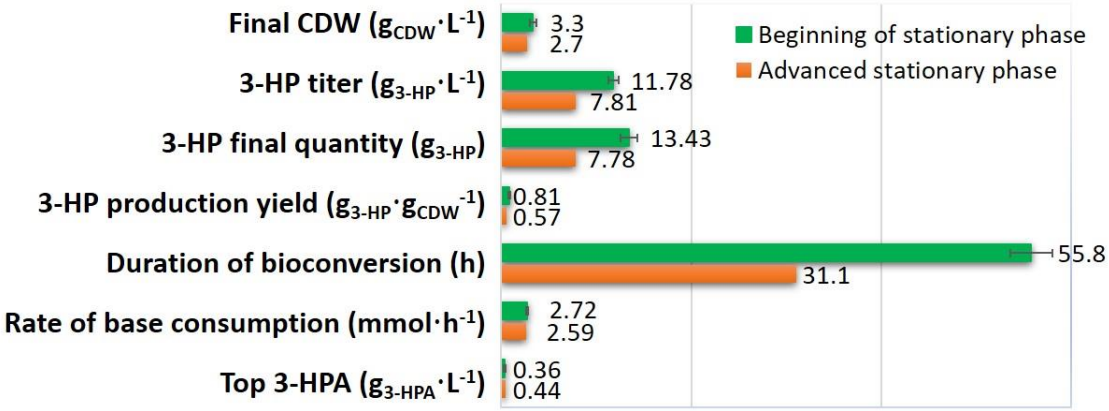

Figure 3.10. Effect of harvesting time on cell concentration and bioconversion performances of *L. reuteri* DSM 17938 cells harvested at the beginning of stationary phase (reference, in green) (final volume 1.14 ± 0.25 L) and in advanced stationary phase (after 6 h, in orange) (final volume 1 L)

A few authors demonstrated that cells of *L. reuteri* harvested in stationary phase exhibited a better 3-HP bioproduction than cells recovered in exponential phase. That was the case of *L. reuteri* ATCC 55730 (Ramakrishnan *et al.*, 2015) and *L. reuteri* FXZ014 (Zabed *et al.*, 2019). Results of this test thus contribute to enlarge the knowledge that recovering cells at the early stationary phase maximized their ability to further convert glycerol into 3-HP compared to cells harvested in more advanced stationary phase.

The influence of carbohydrate depletion in the up-regulation of some micro-compartmental genes in *L. reuteri* (Kim *et al.*, 2014; Ramakrishnan *et al.*, 2015; Sriramulu *et al.*, 2008) or in the reduction of the concentration of pyridine nucleotides (i.e., precursors of NAD^+/NADH cofactors (Nakamura *et al.*, 2012)) could be hypothesized to explain this effect of harvesting time on the 3-HP bioproduction.

From this analysis, harvesting the cells of *L. reuteri* DSM 17938 in advanced stationary phase showed a significant decrease in bioconversion performances in comparison to the beginning of stationary phase. Consequently, for the next experiments, cell will be harvested in this last condition.

3.4 Conclusion of Chapter 3

In conclusion, the influence of various growth conditions was assessed on the growth efficiency and on the ability of resting cells of *L. reuteri* DSM 17938 to produce 3-HP at a given glycerol feeding rate of 0.5 $g \cdot h^{-1}$. The implementation of a Plackett and Burman experimental design enabled 11 factors to be tested. At the end, glycerol bioconversion into 3-HP by resting cells was affected by nine growth conditions.

From the results obtained in the reference condition that was triplicated, a good repeatability was achieved, despite the complexity of the bioprocess that included two successive phases of growth and bioconversion, both performed in bioreactors. These reference conditions allowed to identify indicators of the end of the cultures, by considering the two steps. Within the used conditions, the base consumption that resulted from the neutralization of organic acids produced during growth was used as an indicator to define the end of growth. During the bioconversion step, two complementary indicators have been pointed out to define the end of the bioconversion: the base consumption that stopped when no more 3-HP was produced in the medium and the pO_2 that increased when the bioconversion ceased.

The results obtained from this first experimental design indicated that the supplementation of MRS medium with 50 $g \cdot L^{-1}$ glucose and the use of a temperature of 37 °C led to a greater cell quantity at the end of the growth phase. Meanwhile, the addition of phytone peptone, Tween 80, 1,2-PDO, betaine with KCl and the use of a suboptimal temperature, together with an optimal pH, were recognized as relevant options to improve the bioconversion duration and the 3-HP production yield at a given glycerol feeding rate of 0.5 $g \cdot h^{-1}$.

The best set of conditions has been identified and validated as it enhanced the 3-HP titer (+ 25 %), the 3-HP production yield (+ 150 %) and the specific 3-HP production rate (+ 61 %), as compared to the reference condition.

As cells recovered in advanced stationary phase showed a reduction of bioconversion performances compared to those harvested in early stationary phase, these last conditions will be retained for further studies.

With the aim of further improving the 3-HP bioproduction by *L. reuteri* DSM 17938, the environmental conditions during the bioconversion step should be optimized, together with avoiding 3-HPA accumulation. This part of the study will be presented in the following chapter 4.

Chapter 4. Effect of environmental conditions during bioconversion on 3-HP bioproduction by *L. reuteri* DSM 17938

4.1 Introduction

As described previously, the bioproduction of 3-hydroxypropionic acid by *L. reuteri* is performed through a two-step process, including a growth step performed in batch mode and a bioconversion step using bacteria as "whole-cell biocatalysts". Between these two steps, the cells are harvested and concentrated, before being used as resting cells that convert glycerol into 3-HP in fed-batch mode.

Some important environmental factors affect the performance of the glycerol bioconversion by *L. reuteri*. They include the composition of the bioconversion medium (Couvreur *et al.*, 2017; Doleyres *et al.*, 2005; Lüthi-Peng *et al.*, 2002), the bioconversion temperature and pH (Herlet *et al.*, 2017), the gaseous atmosphere (Doleyres *et al.*, 2005; Zabed *et al.*, 2019), and the glycerol feeding rate or the specific glycerol feeding rate during the fed-batch process (Dishisha *et al.*, 2014; Dishisha *et al.*, 2015; Doleyres *et al.*, 2005).

From (Doleyres *et al.*, 2005), the composition of bioconversion medium was shown to influence the bioproduction of the metabolic intermediate 3-HPA, notably the glycerol concentration. The authors obtained that 3-HPA bioproduction was proportional to the initial glycerol concentration, the highest concentration of 3-HPA was achieved with the highest concentration of glycerol (Doleyres *et al.*, 2005). A study performed in the lab indicated that *L. reuteri* DSM 17938 cells harvested then suspended in osmosis water showed a better physiological state than cells re-suspended in potassium phosphate buffer (Görge, 2016).

The temperature has been studied by several authors. Most of them agreed to define 37 °C as a convenient temperature since this level is the optimal condition for highest activity of the enzyme glycerol dehydratase in *L. reuteri* CG001 (Huiliang *et al.*, 2013) and for specific activity of the propanediol utilization protein PduP (Luo *et al.*, 2011). However a low temperature of 30 °C was noticed by (Doleyres *et al.*, 2005) as the optimum condition to maximize 3-HPA production in ATCC 53608. The hypothesis of testing a temperature lower than the optimum is that cells might undergo less stress in the bioconversion conditions and can possibly keep on producing for a longer period. In order to prevent the low rate of bioconversion at a too low temperature, a value of 32 °C was retained for the test.

The pH acts on 3-HP bioproduction as demonstrated by (Dishisha *et al.*, 2014). A higher pH (pH 7.0 instead of pH 5.0) was shown by these authors to improve the performance of the bioproduction. However, it is also important to consider this environmental factor in

relationship with the further downstream processes. Indeed, the extraction and separation of 3-HP can be achieved by various downstream processes, such as crystallization, ionic chromatography or liquid-liquid extraction (Sánchez-Castañeda *et al.*, 2020). Among these processes, liquid-liquid reactive membrane extraction using primary amines is an interesting process that was successfully applied to 3-HP (Sánchez-Castañeda *et al.*, 2020). By considering this process, a low pH is required to allow the reaction between primary amines and the undissociated form of 3-HP. Consequently, it could be interesting to test the effect of low pH during the bioconversion step and assess the bioproduction performance in this particular condition.

The preliminary tests regarding temperature and pH were done by fixing growth volume (5 L) and growth conditions (pH 6.0, temperature 37 °C) as in the reference of experimental design 1, to be able to evaluate the effect of new conditions of pH and temperature during bioconversion. The comparisons are presented in following sub-parts.

The effect of gaseous atmosphere has already been studied on the production of 3-HPA (Doleyres *et al.*, 2005) and 3-HP (Zabed *et al.*, 2019). The study of (Doleyres *et al.*, 2005) showed that anaerobic conditions are required for glycerol bioconversion into 3-HPA, which was explained by the sensitivity of the glycerol dehydratase enzyme to oxygen (Zhao *et al.*, 2015). Furthermore, the production by *L. reuteri* JCM1112 of vitamin B12, which is a required cofactor of glycerol dehydratase, increased by 30 % under strict anaerobic conditions (Santos *et al.*, 2009). Anaerobic conditions maintained by continuous bubbling of nitrogen gas was applied for 3-HP bioproduction from glycerol by *L. reuteri* DSM 20016 (Dishisha *et al.*, 2015). The recent study of (Zabed *et al.*, 2019) was dedicated to the definition of aeration conditions to obtain various targeted products of the Pdu pathway. These authors reported that microaerobic conditions were favorable to maximize 3-HP production by *L. reuteri* FXZ014. These microaerobic conditions were achieved by gently mixing the bioconversion medium without adding any gas during the bioconversion.

Finally, the glycerol feeding rate and the specific glycerol feeding rate were identified as important factors that influenced 3-HP bioproduction from glycerol. It has been demonstrated that increasing the glycerol feeding rate resulted in accelerating the 3-HP bioproduction performed by *L. reuteri* DSM 20016 (Dishisha *et al.*, 2014). Moreover, a specific glycerol feeding rate of 62.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ was determined as the maximum value to obtain 3-HP with this strain, without accumulation of toxic 3-HPA (Dishisha *et al.*, 2015). As the aim of this chapter is to improve the 3-HP bioproduction from glycerol by *L. reuteri* DSM 17938, specific glycerol feeding rate was retained as a factor for the CCRD.

From this short synthesis of the literature, the factors temperature, pH and specific glycerol feeding rate seem to be the most promising drivers to improve the bioproduction of 3-HP by *L. reuteri*. The objective of this part of the study was to characterize the ability of *L. reuteri* DSM 17938 to perform 3-HP bioproduction in different environmental conditions during the

bioconversion phase and to define optimized conditions by considering the pH and the specific glycerol feeding rate.

4.2 Experimental strategy

Some preliminary tests have first been performed, in order to define the conditions to be used during the second experimental design. As being summarized in Figure 4.1, these tests included:

- The freezing and storage of the frozen cells for one month or three months between the growth and the bioconversion steps,
- The implementation of two steps of centrifugation between the growth and the bioconversion steps,
- The use of a double cell concentration during the bioconversion step.

Using the conditions defined from these preliminary tests, another set of preliminary experiments was conducted in order to determine the second experimental factor to be tested in addition to the specific glycerol feeding rate. The effects of bioconversion temperature and pH were investigated, with the aim to determine if these factors may be retained and to define their limit that will be tested in the second experimental design (Figure 4.1).

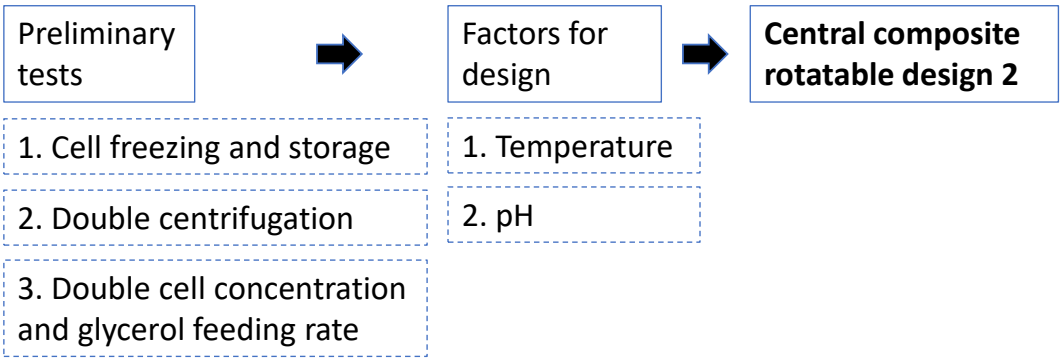

Figure 4.1. Overall methodology for the study of the effects of environmental conditions during the bioconversion step on the ability of *L. reuteri* DSM 17938 to perform glycerol bioconversion into 3-hydroxypropionic acid

Then, a CCRD (Box and Wilson, 1951) was established in order to define optimized conditions for the bioconversion step, by considering two important environmental and operational factors: the pH and the specific glycerol feeding rate. This kind of experimental design allowed us to determine the optimal values of these factors that maximize the characteristic variables of the bioconversion, including the 3-HP titer, 3-HP maximal quantity, 3-HP production yield, 3-HP production rate, 3-HP specific production rate, 3-HP volumetric productivity and specific glycerol consumption rate.

The conditions implemented during the growth and the bioconversion steps were defined for the whole experimental design. For the growth step, the conditions were selected from the results of the first experimental design. They consisted in using MRS medium added with glucose (20 $g \cdot L^{-1}$), phytone peptone (25 $g \cdot L^{-1}$), added Tween 80 (4 $g \cdot L^{-1}$), 1,2-PDO(3 $g \cdot L^{-1}$), and betaine (0.234 $g \cdot L^{-1}$) plus KCl (0.745 $g \cdot L^{-1}$). The addition of 20 $g \cdot L^{-1}$ glucose (and not 50 $g \cdot L^{-1}$) was chosen to avoid the slight downshift (– 7 %) of the 3-HP to 1,3-PDO molar ratio observed as a result of the first experimental design. The growth temperature was fixed at 37 °C, the pH was controlled at pH 6.0 with NH_4OH (14.8 $mol \cdot L^{-1}$), and no gas was added in the growth medium stirred at 100 rpm.

The conditions of the bioconversion step were fixed by considering the literature data or the results of preliminary experiments. The bioconversion medium was composed of osmosis water in which bacterial cells were re-suspended after harvesting (Dishisha *et al.*, 2014). The agitation rate was fixed at 100 rpm and the gaseous atmosphere was not controlled (no air nor nitrogen transfer), thus generating microaerobic conditions (Zabed *et al.*, 2019). The temperature was set at 37 °C from the results of preliminary tests displayed in Section 4.4.1.

4.3 Preliminary tests to define the conditions to be used before and during the bioconversion step

All preliminary tests were conducted with *L. reuteri* DSM 17938, in the growth conditions of the reference condition of the first experimental design, in order to allow a comparison of the results. These conditions are fixed and consisted in MRS medium added with components defined from the results of the first experimental design (i.e., 20 $g \cdot L^{-1}$ additional glucose, 25 $g \cdot L^{-1}$ phytone peptone, 4 $g \cdot L^{-1}$ additional Tween 80, 3 $g \cdot L^{-1}$ 1,2-PDO, 0.234 $g \cdot L^{-1}$ betaine plus 0.745 $g \cdot L^{-1}$ KCl), a temperature of 37 °C, a stirring rate of 100 rpm and a pH controlled at 6.0 with NH_4OH (14.8 $mol \cdot L^{-1}$). Only the test of a double centrifugation was done with a glucose concentration of 50 $g \cdot L^{-1}$. In that precise case, the aim was to obtain more lactic acid in order to better highlight the efficiency of eliminating this unexpected product for the subsequent bioconversion step. The bioconversions were carried out in the same conditions than those of the reference conditions of the experimental design 1 to allow comparison. The bioconversion temperature and pH were set at 37 °C and pH 6.0. The glycerol feeding rate was fixed at 0.5 $g_{glycerol} \cdot h^{-1}$. In the preliminary test to assess the effect of a double resting cell concentration and a double glycerol feeding rate, all other environmental conditions remained unchanged.

For all experiments, the biomass concentration at the beginning of the bioconversion step was similar at 18.47 ± 2.49 $g_{CDW} \cdot L^{-1}$, as it was the consequence of the growth phase that was conducted in identical conditions. In the case of the experiments to test the effects of a double centrifugation and a double cell concentration, the biomass concentration reached 44.5 $g_{CDW} \cdot L^{-1}$.

Concerning the bioconversion step, the carbon mass balances were verified before analyzing the results at 99 ± 3 %. As the 1,3-PDO was produced in equimolar proportion with 3-HP (i.e., molar ratio 0.9 ± 0.11 $mol_{1,3\text{-}PDO} \cdot mol_{3\text{-}HP}^{-1}$ for all the experiments), its concentration will not be analyzed in the next sub-chapters.

4.3.1 Effect of freezing frozen storage on glycerol bioconversion by *L. reuteri* DSM 17938

The objective of these experiments was to determine whether freezing and storing the concentrated cells at – 80 °C for one or three months may affect the ability of *L. reuteri* DSM 17938 to convert glycerol into 3-HP. The idea was to facilitate the further bioconversion studies, by producing large quantities of concentrated cells from large batches, and to use them for several bioconversion experiments.

From a methodologic point of view, the growth was performed in three bioreactors simultaneously, from the same inoculum and in the reference conditions of the first experimental design. At the end of growth, cells were harvested at the beginning of stationary phase, but not washed, which means that they were subjected to only one centrifugation. Cell pellets obtained from two bioreactors were rapidly frozen by immersion in liquid nitrogen and stored at – 80 °C for 1 and 3 months, respectively. Cell pellets from the third bioreactor were used directly (without freezing) as a control, by suspension in osmosis water to achieve a cell concentration of about 10^{10} $cell \cdot mL^{-1}$. All the three bioconversion experiments were conducted in the same conditions, i.e. at 37 °C, pH 6.0 and at a glycerol feeding rate of 0.5 $g_{glycerol} \cdot h^{-1}$.

Results corresponding to the final biomass concentrations at the end of the growth phase were comparable in the three bioreactors at 3.15 ± 0.28 $g_{CDW} \cdot L^{-1}$ and $4.0 \cdot 10^9 \pm 0.3 \cdot 10^9$ $cell \cdot mL^{-1}$. They were similar to that obtained in the reference experiments of the first experimental design (namely, 3.3 ± 0.3 $g_{CDW} \cdot L^{-1}$ and $4.2 \cdot 10^9 \pm 0.7 \cdot 10^9$ $cell \cdot mL^{-1}$). This good repeatability allowed us to study the effect of freezing and frozen storage on the ability of resting cells of *L. reuteri* DSM17938 to convert glycerol into 3-HP.

Comparison of bioconversion performances reached by unfrozen cells, frozen cells stored for one month and for three months are summed up in Table 4.1.

Table 4.1. Effect of freezing and frozen storage for 1 and 3 months on biomass concentration and performance of glycerol bioconversion into 3-HP by *L. reuteri* DSM17938

	Fresh cells	Cells frozen for 1 month	Cells frozen for 3 months
Concentration of active and not permeabilized cells at the beginning of bioconversion (cell·mL^{-1})	$1.4 \cdot 10^{10}$	$1.0 \cdot 10^{10}$	$1.2 \cdot 10^{10}$
3-HP titer (g·L^{-1})	11.2	13.7	11.2
3-HP final quantity (g)	12.4	15.3	12.7
3-HPA (g·L^{-1})	0.5	0.6	1.0
Specific glycerol consumption yield ($g_{glycerol} \cdot g_{CDW}^{-1}$)	1.98	2.22	1.92
Specific 3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)	0.73	0.86	0.88
Bioconversion duration (h)	57.7	62.1	50.9
Final volume (L)	1.11	1.11	1.14

After rapid freezing in liquid nitrogen and storage at very low temperature (- 80 °C), the percentage of active and not permeabilized cells on total cells was affected, as it decreased from 85 % by considering fresh cells to 63-66 % with frozen cells. This led to a lower concentration of active and not permeabilized cells entering the bioconversion phase (Table 4.1). However, the main effect was linked to losses of active cells due to centrifugation and handling (Streit, 2008), as the concentration of active and not permeabilized cells was only slightly higher for fresh cells.

The 3-HP titer and 3-HP final quantity at the end of bioconversion were not significantly affected by the cell freezing and the frozen storage, as they remained quite stable for the three conditions (Table 4.1). The obtained values were close to that reached in the reference condition of the first experimental design (11.8 ± 0.5 $g_{3\text{-}HP} \cdot L^{-1}$ and 13.4 ± 0.9 $g_{3\text{-}HP}$, respectively). Nevertheless, a higher accumulation of 3-HPA by the cells frozen and stored for three months was observed, as the 3-HPA titer was doubled (Table 4.1). Although this concentration remained lower than the 3-HPA inhibitory concentration (2.2-3.7 g·L^{-1}) (Cleusix *et al.*, 2007), it indicates that the balance between the enzymes activities was modified. In addition, this higher 3-HPA titer can explain the shorter duration of the bioconversion carried out with cells frozen and stored for 3 months.

With a glycerol feeding rate fixed at 0.5 $g_{glycerol} \cdot h^{-1}$, the specific glycerol consumption yield and the specific 3-HP production yield were similar whatever the state of the cells (Table 4.1). They were comparable to the values obtained for the reference condition of the first experimental design (2.3 ± 0.3 $g_{glycerol} \cdot g_{CDW}^{-1}$ and 0.83 ± 0.11 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$, respectively).

In summary, despite the good reproducibility of 3-HP bioproduction performed by frozen cells compared to fresh cells, the bioconversion process was slightly affected by the cell freezing, by considering the process duration, the 3-HPA accumulation and the enzymatic activity of bacterial cells. The consequences of these effects on the bioconversion performances

remained low because the specific glycerol feeding rate was moderate in this set of experiments (i.e., 33.3 ± 2.9 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$). But in the context of the next experimental design in which the specific substrate feeding rate will be varied, this negative impact may interfere more with the future results. Finally, even these tests indicated that *L. reuteri* DSM 17938 was quite resistant to freezing and frozen storage, this methodology was not retained for the next experiments.

4.3.2 Effect of two centrifugation steps on the ability of *L. reuteri* DSM 17938 to produce 3-HP from glycerol

The objective of these experiments was to better eliminate the remaining metabolites that originated from growth of *L. reuteri* DSM 17938, in order to reduce their possible inhibiting effect and to facilitate the further extraction processes. During the growth step, *L. reuteri* synthesizes lactic acid, acetic acid, ethanol and CO_2 as metabolites that originate from glycolysis. As demonstrated in Section 3.3.2.1, the concentrations of these products reached 16.4 (± 1.2) $g \cdot L^{-1}$, 7.6 (± 0.8) $g \cdot L^{-1}$ and 3.7 (± 0.4) $g \cdot L^{-1}$, respectively when the growth was performed in the reference conditions. As a reminder, CO_2 production was not measured but assumed to be produced equimolarly to ethanol. Before the process of 3-HP bioproduction begins, most of these products are removed during the concentration step by centrifugation and diluted in the bioconversion medium that corresponded to osmosis water. However, a remaining lactic acid concentration of 2.7 ± 1.5 $g \cdot L^{-1}$ was detected at the beginning of the bioconversion phase in the reference condition. When the growth was performed with a glucose supplementation of 50 $g \cdot L^{-1}$, the remaining lactic acid concentration was equal to 3.9 ± 1.0 $g \cdot L^{-1}$. This remaining lactic acid may negatively affect the ability of cells to convert glycerol into 3-HP. In addition, it could be an impediment to the future extraction process, as it will be extracted along with the 3-HP that will complicate the purification of the targeted molecule.

In order to better eliminate these remaining products, a washing step was added to the concentration step by performing two runs of centrifugation instead of one. The fermentation was done with extra glucose addition of 50 $g \cdot L^{-1}$ and was compared, at the same initial glucose concentration, with an experiment involving only one centrifugation. All other conditions were similar to those of the reference condition of the first experimental design. After each centrifugation, the cells were recovered in 0.8 L of sterile osmosis water. Results are presented in Table 4.2

Table 4.2. Effects of one or two centrifugation steps on the lactic acid and active cells concentrations

End of growth phase Before centrifugation	**Lactic acid ($g \cdot L^{-1}$)**	26.4	26.8
	Active cells ($cells \cdot mL^{-1}$)	$7.9 \cdot 10^{9}$	$4.6 \cdot 10^{9}$
Number of centrifugations		**1**	**2**
Beginning of bioconversion phase After centrifugation	**Lactic acid ($g \cdot L^{-1}$)**	3.3	0.4
	Active cells ($cells \cdot mL^{-1}$)	$3.8 \cdot 10^{10}$	$1.4 \cdot 10^{10}$
Bioconversion duration (h)		75.6	40.8
3-HP titer ($g \cdot L^{-1}$)		16.1	9.5
3-HP final quantity (g)		12.72	9.1
Final volume (L)		1.27	1.04

From Table 4.2, the concentrations of lactic acid and of enzymatically-active and not permeabilized cells at the end of growth phase were similar for the two experiments, by considering the standard deviations given in Chapter 3. As expected, the lactic acid concentration decreased by 88 % after one centrifugation and by 99 % after two centrifugations.

However, although the number of active cells was not significantly deteriorated after one centrifugation (2 % losses, from $4 \cdot 10^{13}$ to $3.9 \cdot 10^{13}$ cells), it was more affected after two centrifugations as it decreased by 39 % (from $2.3 \cdot 10^{13}$ to $1.4 \cdot 10^{13}$cells. This negative effect of centrifugation could be ascribed to losses of bacterial cells due to multiple cell handling and to losses of intracellular enzymatic activity and bacterial membrane integrity due to repeated centrifugations. This result is in agreement with that obtained by (Streit, 2008) who showed that centrifugation of *L. bulgaricus* was more deleterious than microfiltration, the latter being more adapted to maintain a good physiological state after concentration.

The negative impact of two centrifugation steps on the performance of 3-HP production (i.e., shorter bioconversion duration and lower 3-HP concentration and final quantity) was due to the lower quantity of active cells recovered in that case.

Thus, despite the advantage of better removing undesirable metabolites, a two-centrifugation step clearly showed a disadvantage for 3-HP bioproduction, by decreasing the quantity of enzymatically-active *L. reuteri* DSM 17938 cells. This methodological approach will then not be retained for the next steps of the study.

4.3.3 Effect of cell concentration and glycerol feeding rate on 3-HP bioproduction by *L. reuteri* DSM 17938

With the aim to enhance the efficiency of 3-HP bioproduction, increasing the glycerol feeding rate is a relevant strategy, as explained in Chapter 3. In fact, a limitation due to the glycerol feeding rate fixed at 0.5 $g \cdot h^{-1}$ was observed when growth conditions allowed increasing the quantity of active cells harvested at the end of growth (Section 3.3.4.1). In that case, increasing the feeding rate (in $g \cdot h^{-1}$) together with keeping the same specific glycerol feeding rate (in $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) could be interesting to enhance the efficiency of 3-HP production.

By considering *L. reuteri* DSM 17938, the aim of this part of the study was to quantify the consequences of doubling the glycerol feeding rate together with doubling the cell concentration during the bioconversion step, in order to reach the same specific glycerol feeding rate. The tests were done using the conditions of the reference cultures of experimental design 1. Detailed results are presented in Table 4.3.

Table 4.3. Effect of cell concentration and glycerol feeding rate on the performance of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938

	Reference condition	**Double cell concentration and glycerol feeding rate**
Cell concentration at the beginning of bioconversion ($g_{CDW} \cdot L^{-1}$)	21.9	44.5
Cell concentration at the beginning of bioconversion ($cell \cdot mL^{-1}$)	$1.7 \cdot 10^{10}$	$3.1 \cdot 10^{10}$
Concentration of active and not permeabilized cells ($cell \cdot mL^{-1}$)	$1.4 \cdot 10^{10}$	$1.9 \cdot 10^{10}$
3-HP titer ($g_{3\text{-}HP} \cdot L^{-1}$)	11.8	20.1
3-HP final quantity ($g_{3\text{-}HP}$)	13.4	19.4
Max 3-HPA ($g_{3\text{-}HPA} \cdot L^{-1}$)	0.27	0.45
Bioconversion duration (h)	55.8	38.2
3-HP production rate ($g_{3\text{-}HP} \cdot h^{-1}$)	0.24	0.51
Specific 3-HP production rate ($mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	13.7	14.3
Final volume (L)	1.14	0.97

The bioconversion was started with a double cell dry weight, which was confirmed by the quantification of the cell concentrations through flow cytometry measurements (Table 4.3). However, a double concentration of active and not permeabilized cells was not found (Table 4.3), which corresponded to a decrease in the percentage of active and not permeabilized cells from 84 % to 62 %. Since the glycerol feeding rate was doubled from 0.5 $g \cdot h^{-1}$ to 1.0 $g \cdot h^{-1}$, the 3-HP specific production rate was nearly maintained at about 14 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$. As a

consequence, the 3-HP production rate was doubled, which was consistent with the targeted conditions (Table 4.3).

Using these new conditions, the final 3-HP titer was enhanced by 70 % and the final 3-HP quantity produced increased by 45 % (the final volume was different due to the various bioconversion duration of the fed-batch process). These augmentations were however lower than expected from a double glycerol feeding rate. This disappointing performance could be explained by two reasons. First, the 3-HPA concentration was twice higher at the double glycerol feeding rate, reaching 6.2 $mmol \cdot L^{-1}$ that was close to the inhibiting value of 8.1 $mmol \cdot L^{-1}$ that affects the activity of the propionaldehyde dehydrogenase enzyme in the Pdu pathway (Sabet-Azad *et al.*, 2013). In addition, the duration of the bioconversion step was lowered by 31 %, which can be a consequence of the high cell concentration that could lead to transport limitation of glycerol and 3-HP. Indeed, (Zabed *et al.*, 2019) showed that the cell concentration giving the optimal result of glycerol biotransformation process in the case of *L. reuteri* CICC 6118 was around 12 $g_{CDW} \cdot L^{-1}$ (in a tested range comprised between 7.5 and 17.5 $g_{CDW} \cdot L^{-1}$). In the present case, the cell concentration reached for the test performed with a doubled cell concentration was much higher than 12 $g_{CDW} \cdot L^{-1}$, while the lower one obtained for the reference condition may limit this issue of transport limitation of substrate and metabolite.

From all these results, the use of higher values of cell concentration and glycerol feeding rate, by maintaining the specific glycerol feeding rate, allowed improving the 3-HP productivity from 0.21 to 0.37 $g_{3HP} \cdot L^{-1} \cdot h^{-1}$, i.e. by 76 %. However, this improvement was partly hidden by the double investment cost linked to biomass production. Finally, this strategy was not included in the next experimental design.

4.3.4 Summary of information

In summary, four sets of conditions have been tested preliminary to the second experimental design, in order to enhance 3-HP bioproduction. However, despite their own advantages, some of them were deleterious and were not retained for the next steps of the study.

When the cells were harvested in advanced stationary phase of growth instead of early stationary phase, the bioconversion step was shorter, the 3-HP titer was lower and 3-HPA slightly accumulated. Consequently, time at which the cells will be harvest was chosen at the early stationary phase.

Freezing and storing the cells at – 80 °C for one or three months allowed maintaining a high level of cell concentration, but generated higher amounts of 3-HPA that could hinder the bioconversion process. Accordingly, the next experiments will be done with fresh cells.

The interest of a better elimination of lactic acid (2-HP) remaining from growth in the medium surrounding the resting cells, by two centrifugations instead of one, was depleted by the higher decline in enzymatically-active cell concentration that affect the bioconversion

duration and consequently, the 3-HP production. Therefore, only one centrifugation step was retained for cell harvesting and concentration.

A test was performed by doubling both cell concentration and glycerol feeding rate. This approach allowed obtaining a double 3-HP production rate, but the final 3-HP concentration remained limited, as a possible of the increased 3-HPA production or of transport limitations. The next preliminary experiments will be conducted at the initial values of cell concentration and glycerol feeding rate, before studying the impact of the specific glycerol feeding rate as a factor of the central composite rotatable design.

These operational conditions were selected and retained to be implemented in the second experimental design.

4.4 Preliminary tests to define the factors to be retained in the second experimental design

Temperature and pH are two important environmental factors for the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938. As the selected experimental design should include only two factors, and as the specific glycerol feeding rate was already retained as the first factor, preparatory experiments were done in order to determine, among temperature and pH, which one might be kept and to define their lower limits.

4.4.1 Effect of the bioconversion temperature on the ability of *L. reuteri* DSM 17938 to produce 3-HP from glycerol

Temperature is a crucial environmental factor that affects the biological transformations, and was thus considered as a possible factor in the next experimental design. In order to evaluate the effect of temperature on the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938, two experiments were done at two different temperatures, 37 °C and 32 °C. All other conditions, during the growth and the bioconversion steps, were similar to those of the reference condition. At the end of growth phase, the cell concentrations obtained were equal to $3.6 \cdot 10^9$ cells·mL^{-1} and $3.2 \cdot 10^9$ cells·mL^{-1}, respectively. Results are summarized in Table 4.4.

Table 4.4. Effect of the bioconversion temperature on the performance of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938

	37 °C	**32 °C**
3-HP titer ($g_{3\text{-HP}}\cdot L^{-1}$)	11.8	9.3
3-HP final quantity ($g_{3\text{-HP}}$)	13.4	10.4
Max 3-HPA ($g_{3\text{-HPA}}\cdot L^{-1}$)	0.27	2.1
Bioconversion duration (h)	55.8	41.3
Concentration of active and not permeabilized cells after 24 h of bioconversion ($cell\cdot mL^{-1}$)	$1.5\cdot10^{10}$	$5.8\cdot10^{9}$
3-HP production rate ($g_{3\text{-HP}}\cdot h^{-1}$)	0.24	0.25
Specific 3-HP production rate ($g_{3\text{-HP}}\cdot g_{CDW}^{-1}\cdot h^{-1}$)	13.8	19.4
Final volume (L)	1.14	1.08

From Table 4.4, it can be seen that the 3-HP titer and the 3-HP final quantity were negatively affected, by about 21 %, when the bioconversion temperature was lower. This is linked to the reduction by 26 % of the bioconversion duration (Table 4.4). This shorter duration might be due to the strong increase in 3-HPA concentration at 32 °C (28.4 $mmol.L^{-1}$) that approached the inhibiting concentration demonstrated for *L. reuteri* DSM 20016 (Cleusix *et al.*, 2007), and that exceeded by three times the level affecting the activity of the propionaldehyde dehydrogenase enzyme in the Pdu pathway (8.1 $mmol\cdot L^{-1}$) reported by (Sabet-Azad *et al.*, 2013).

Moreover, it is notable that, at lower temperature, the concentration of enzymatically-active cells after 24 h decreased strongly by 62 % (Table 4.4). This resulted in raising the specific glycerol feeding rate related to active cells up to 104.5 $mg_{glycerol}\cdot g_{CDW}^{-1}\cdot h^{-1}$ (compared to a value of 47.3 $mg_{glycerol}\cdot g_{CDW}^{-1}\cdot h^{-1}$ for the bioconversion carried out at 37 °C). The value at 32 °C was much higher than the level of 62.5 $mg_{glycerol}\cdot g_{CDW}^{-1}\cdot h^{-1}$ reported by (Dishisha *et al.*, 2015) as the upper limit to avoid 3-HPA accumulation. It could explain the accumulation of this metabolic intermediate observed in this condition. The decrease in active cell concentration at 32 °C could be due to the difference of 5 °C with the optimum temperature of 37 °C for *L. reuteri* DSM 17938, resulting in a general impact on cell physiological state and on metabolic reaction rates.

The lower performance of 3-HP production reached at 32 °C may be explained by a lower rate of enzymes of the second part of the Pdu pathway. From previous studies, the temperature of 37 °C has been reported as the optimal temperature for activity of the enzyme glycerol dehydratase in *L. reuteri* CG001 (Huiliang *et al.*, 2013), of the propanediol utilization protein PduP (Luo *et al.*, 2011), and of the 1,3-PDO oxidoreductase in Pdu pathway (Chen *et al.*, 2011). Moreover, the optimum temperature for the reduction of 3-HPA into 1,3-PDO by *L. reuteri* CG001 resting cells was observed at 45 °C, that was explained by a probable higher enzyme stability than that at 37 °C (Chen *et al.*, 2011).

As shown in Table 4.4, the 3-HP production rate was not affected by the lower temperature of 32 °C in comparison to 37 °C. This can be explained by the glycerol feeding rate fixed at 0.5 $g \cdot h^{-1}$, which might be a limitation in the case of the bioconversion performed at 37 °C, as aforementioned. This result explained the increase in the specific 3-HP production rate when the bioconversion temperature was fixed at 32 °C compared to 37 °C, as cell concentration was lower at 32 °C (Table 4.4).

As a consequence of this result, due to the significant negative effect of temperature on active cell concentration and on the further bioconversion performances, the factor temperature was not the best option to be included in the second experimental design.

4.4.2 Effect of the bioconversion pH on the ability of *L. reuteri* DSM 17938 to produce 3-HP from glycerol

The pH at which the glycerol bioconversion into 3-HP is performed is a very important environmental factor (Dishisha *et al.*, 2014). It may act on intracellular pH, and thus on enzyme activities (Talarico *et al.*, 1990; Talarico and Dobrogosz, 1990) of *L. reuteri*. It may also influence the bioprocess by modifying the ratio between undissociated and dissociated forms of 3-HP, which can affect its inhibiting effect (Schepers *et al.*, 2002). In addition, the pH of the medium is an important factor by considering the further steps of downstream processing, especially when the separation is coupled to the bioconversion by using a liquid-liquid extraction process. Due to the pK_a of 3-HP equal to 4.51 (Wishart *et al.*, 2018), a pH close or lower to this level is required for its reactive extraction by tertiary amines (Sánchez-Castañeda *et al.*, 2020)

With the aim to study the influence of bioconversion pH on the ability of *L. reuteri* DSM 17938 to produce 3-HP, some preliminary tests have been programmed in order to determine the lower pH that was acceptable by the strain. Two experiments were performed, at pH 5.0 and pH 4.5. The growth conditions corresponded to those performed for the reference condition of the first experimental design. They allowed obtaining concentrations of active and not permeabilized bacterial cells at the beginning of the bioconversion step that were comprised between 1.8 and $2.3 \cdot 10^{10}$ $cells \cdot mL^{-1}$. During the bioconversion, the temperature was set at 37 °C and the glycerol feeding rate was fixed at 0.5 $g \cdot h^{-1}$. Results are presented in Table 4.5 and Figure 4.2.

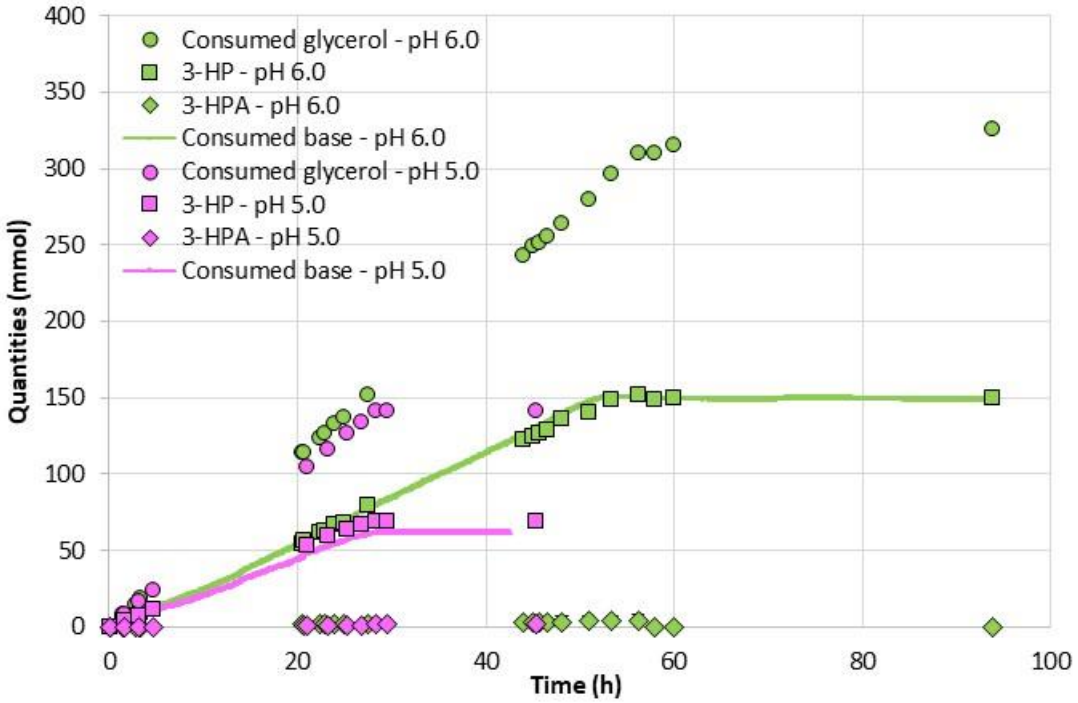

Figure 4.2. Comparison of kinetics of glycerol bioconversions by *L. reuteri* DSM 17938 cells performed at pH 6.0 (reference, in green, with the final volume 1.14 L) and pH 5.0 (in purple, final volume 0.94 L)

From Figure 4.2, the rates of glycerol consumption and 3-HP production were close for the two pH values. However, the bioconversion duration and the final concentrations were much lower at pH 5.0 than at pH 6.0.

Table 4.5. Effect of the bioconversion pH on the performances of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938

	pH 6.0	**pH 5.0**	**pH 4.5**
3-HP titer ($g_{3\text{-HP}}\cdot L^{-1}$)	11.8	7.2	nd
3-HP quantity ($g_{3\text{-HP}}$)	13.4	6.7	nd
Max 3-HPA ($_{3\text{-HP}}\cdot g\cdot L^{-1}$)	0.27	0.15	nd
Bioconversion duration (h)	55.8	33.3	7.4
3-HP production rate ($g_{3\text{-HP}}\cdot L^{-1}\cdot h^{-1}$)	0.24	0.20	nd
Specific 3-HP production rate ($mg_{3\text{-HP}}\cdot g_{CDW}^{-1}\cdot h^{-1}$)	13.75	11.38	nd
Final volume (L)	1.14	0.94	0.85

nd: not detected

From Figure 4.2, the rates of glycerol consumption and 3-HP production were close for the two pH values. However, the bioconversion duration and the final concentrations were much lower at pH 5.0 than at pH 6.0.

Table 4.5 shows that reducing the pH of the bioconversion medium induced a strong decrease in all measured variables during bioconversion. At pH 4.5, no significant quantities of 3-HP nor 3-HPA were detected in the medium. The bioconversion was very short and the rate of base consumption, that indirectly represents the rate of 3-HP production, was only 17 % of that measured at pH 6.0 (0.42 mmol·h^{-1} instead of 2.43 mmol·h^{-1}). The 3-HP titer and quantity obtained at pH 5.0 accounted for almost half of those obtained at pH 6.0. A limited concentration of 3-HPA was produced at pH 6.0 and pH 5.0. The 3-HP production rate and specific production rate was a little lower at pH 5.5, but the bioconversion duration was significantly reduced (- 40 %) at pH 5.0 compared to pH 6.0.

These results are consistent with those obtained by (Wu *et al.*, 2018) who indicated that an acidic stress due to lactate and low pH values acted at both proteome and transcriptome levels in *L. acidophilus*. It is also in agreement with the studies of (Talarico and Dobrogosz, 1990) and (Talarico *et al.*, 1990), which demonstrated that the optimum pH of the enzyme glycerol dehydratase, involved in 3-HPA synthesis, was pH 7.2 whereas those of propanediol oxidoreductase that is necessary for the cofactor balance for 3-HP biosynthesis, was lower and equal to pH 6.2.

Cell physiological state was also affected by the environmental pH as illustrated in Figure 4.3. It can be seen that, at a fixed pH value of 6.0, the percentage of active cells remained practically unchanged (86 – 88 %) after 26 hours of bioconversion. It then dropped and reached about 65 % after 45 h of bioconversion. This evolution was clearly different at pH 5.0, as the percentage of active cells was quite stable (85 – 87 %) only during the first 4 hours then dropped to 78 % after around 21 hours, and to 56 % after 43 h. At last, during the bioconversion performed at pH 4.5, active cells represented only 52 % of the total cell population at the beginning of bioconversion (after 10 min). This value then decreased to 37 % after 2 hours and became stable around 30 – 35 % thereafter. This explained why the glycerol metabolism proceeded only for few hours at pH 4.5. Consequently, the environmental pH of 5.0 and 4.5 were deleterious for the physiological state of bacterial cells.

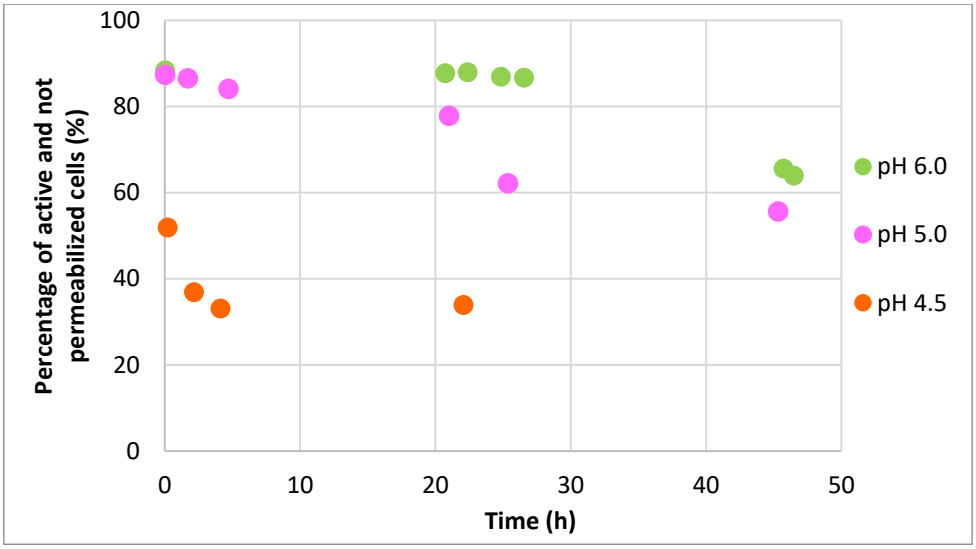

Figure 4.3. Evolution of the percentage of active and not permeabilized *L. reuteri* DSM 17938 cells during glycerol bioconversion performed at pH 6.0 (reference, in green), pH 5.0 (in purple) and pH 4.5 (in orange)

To conclude on this preliminary test concerning pH, the results demonstrated that the bioconversion was possible, even if affected, at pH 5.0. In contrast, at pH 4.5, the performance of the bioprocess was too much lowered, which made it impossible to be implemented. Consequently, the factor pH will be retained to be included in the second experimental design and the lower value that will be tested was chosen as pH 5.0.

4.4.3 Summary of information

The effect of a low temperature during bioconversion has been tested in order to decide if this factor could be, or not, included in the next experimental design. A bioconversion temperature of 32 °C was shown to permit 3-HP production at slightly lower concentration and quantity than a temperature of 37 °C. But the 3-HPA concentration was highly increased, which affected the bioconversion duration. This environmental factor was thus not retained to be included in the second experimental design.

By considering the bioconversion pH, preliminary tests indicated that this factor affected the bioconversion, but that the level pH 5.0 decreased only slightly the 3-HP production rate and specific 3-HP production rate, even if it shortened the bioproduction. However, the level pH 4.5 was too much deleterious.

Finally, the factor “pH” was retained to be included in a central composite design, together with the factor “specific glycerol feeding rate” that was considered as a decisive factor to optimize 3-HP production.

4.5 Optimization of 3-HP bioproduction from glycerol by *L. reuteri* DSM 17938

4.5.1 Experimental approach

To implement a two-factor CCRD, the central point and the boundary conditions have to be determined first.

By considering the pH, the central point was set at pH 6.0 that was the value employed during the first experimental design performed in this thesis, and that was already used in the literature (Burgé *et al.*, 2015c). According to the results obtained from the preliminary study on pH effect during the bioconversion phase, the lower limit for pH was chosen as pH 4.8, because the limitation was too strong at pH 4.5. As a consequence of these choices, the upper limit for pH was fixed at pH 7.2.

During the first experimental design, one experiment was performed at a low specific glycerol feeding rate of 20 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, as a result of the high biomass concentration obtained from the growth phase and of the fixed glycerol feeding rate (at 0.5 $g_{glycerol}.h^{-1}$). This value was then retained as the lower limit for the specific glycerol feeding rate. On the other hand, the upper limit can be defined by considering the results of (Dishisha *et al.*, 2015) who indicated that a maximum specific glycerol feeding rate of 62.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ could be achieved without detrimental 3-HPA accumulation. To include this value in the experimental design, the upper limit was then selected at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. Consequently, the central point was fixed at 50 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ and the other levels involved in the experimental design were deduced from these choices.

Eleven experiments were performed, including one duplicate at the central point. The growth phase that preceded the bioconversion phase was performed as defined in chapter 3. They corresponded to MRS broth supplemented with glucose 20 $g \cdot L^{-1}$ (to limit 1,3-PDO accumulation), phytone peptone 25 $g \cdot L^{-1}$, 1,2-PDO 3 $g \cdot L^{-1}$, betaine 0.234 $g \cdot L^{-1}$ plus KCl 7.455 $g \cdot L^{-1}$, Tween 80 4 $g \cdot L^{-1}$. During the bioconversion, the temperature was set at 37 °C, the agitation rate at 100 rpm and the pH was controlled by the addition of NH_4OH 1.48 $mol \cdot L^{-1}$.

At the end of the biomass production phase, the bacterial concentrations were equal to 3.1 ± 0.3 $g_{CDW}^{-1} \cdot L^{-1}$. These results were not significantly different from one biomass production to another, which indicated that the further bioconversions were started in similar conditions.

4.5.2 Results of the two-factors central composite rotatable design

The repeatability of the duplicate experiments corresponding to the conditions of the central point was satisfactory, as can be seen from the results illustrated in Figure 4.4 summarized in Table 4.6.

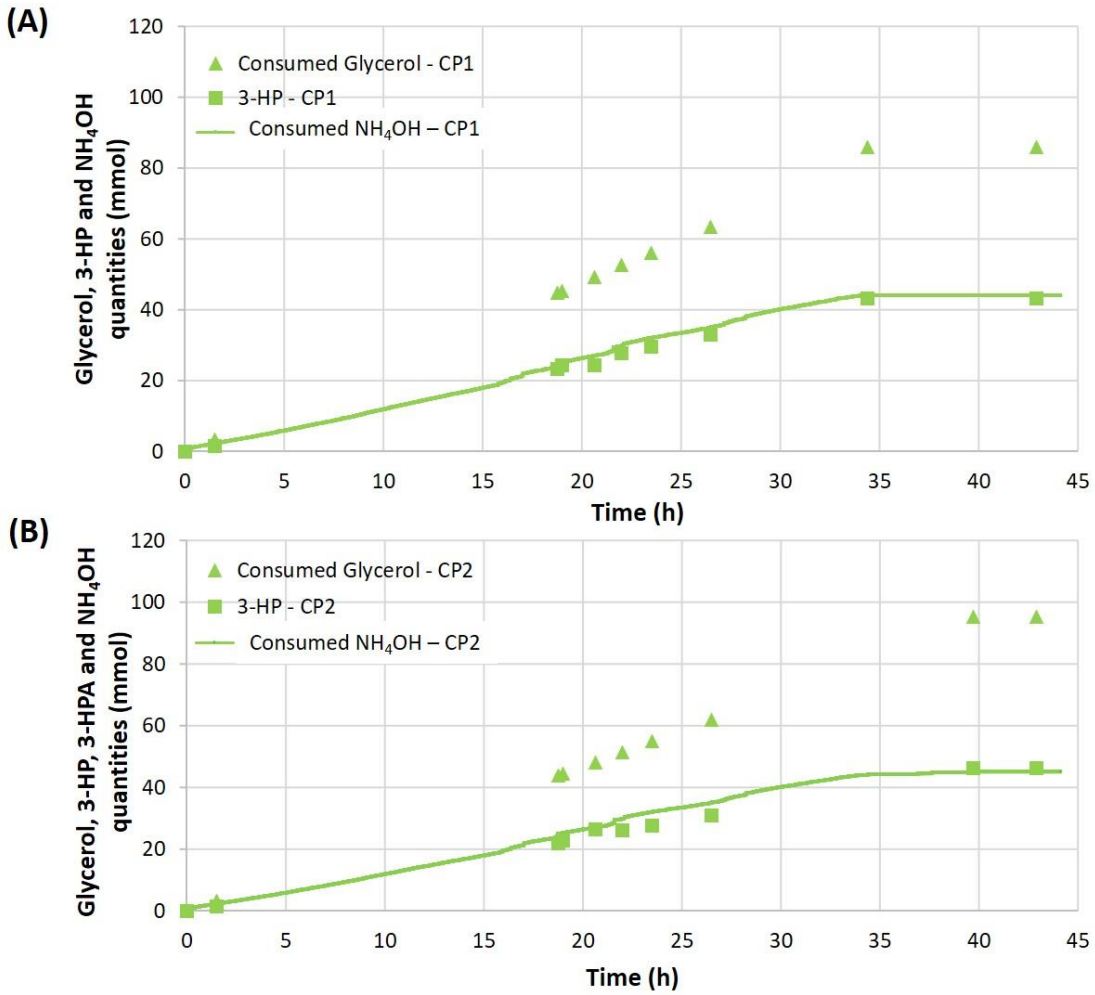

Figure 4.4. Kinetics of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 in the conditions of the central point of CCRD, i.e. pH 6.0 and specific glycerol feeding rate of 50 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, (A) central point 1 (CP1, final volume 0.36 L), (B) central point 2 (CP2, final volume 0.39 L)

Table 4.6. 3-HP bioproduction by *L. reuteri* DSM 17938 at the central point of the CCRD, i.e. pH 6.0 and specific glycerol production rate of 50 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$

Variables	Central point 1	Central point 2
Specific 3-HP production rate ($mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	30.6	29
3-HP titer ($g_{3HP} \cdot L^{-1}$)	10.9	10.7
3-HP final quantity (g)	3.9	4.2
3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)	1.1	1.2
3-HP production rate ($g_{3\text{-}HP} \cdot h^{-1}$)	0.1	0.1
3-HP volumetric productivity ($g \cdot L^{-1} \cdot h^{-1}$)	0.37	0.35
Final volume (L)	0.36	0.39

The influence of pH and specific glycerol feeding rate on glycerol bioconversion into 3-HP has been quantified on the following variables: glycerol specific consumption rate, maximal 3-HP

titer, 3-HP final quantity, 3-HP production yield, 3-HP production rate, specific 3-HP production rate and volumetric productivity of 3-HP. The final quantity of 3-HP was quantified by measuring the final volume inside the bioreactor at the end of each fed-batch experiment, in order to facilitate the comparison of the experiments. Because of the heaviness and the complexity of these experiments, is was not possible to characterize the physiological state of the cells during these experiments.

Before any further analyses, the carbon mass balances during the bioconversions have been verified at 97.2 ± 6.3 % which was considered as satisfactory. In addition, the molar ratios between 3-HP and 1,3-PDO were established for each set of conditions. There were equal to 1.0 ± 0.1 which confirmed that these two metabolites were equimolarly produced during the bioconversion, thus balancing the NAD^+/NADH cofactors (Dishisha *et al.*, 2014).

Multivariable second order linear regressions were carried out for each explained variable as a function of pH and specific glycerol feeding rate. The coefficients of the corresponding polynomials are summed up in Table 4.7. They are analyzed in following subparts.

From this table, the multiple correlation coefficients were comprised between 0.71 and 0.94. By considering the complexity of the experiments, which included two successive steps of growth and subsequent bioconversion with a harvesting and concentration step between them, it was considered that they were satisfactory, except for the 3-HP titer that displayed the lowest value.

Table 4.7. Coefficients of the polynomials describing the effects of pH and specific glycerol feeding rate on glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938

Polynomial parameters	**Variables characterizing the bioconversion performances**						
	Specific glycerol consumption rate ($mg_{glycerol}·g_{CDW}^{-1}·h^{-1}$)	**3-HP titer ($g_{3\text{-}HP}·L^{-1}$)**	**3-HP final quantity ($g_{3\text{-}HP}$)**	**3-HP production yield ($g_{3\text{-}HP}.g_{CDW}^{-1}$)**	**3-HP production rate ($g_{3\text{-}HP}·h^{-1}$)**	**Specific 3-HP production rate ($mg_{3\text{-}HP}·g_{CDW}^{-1}·h^{-1}$)**	**3-HP volumetric productivity ($g_{3\text{-}HP}·L^{-1}·h^{-1}$)**
a_0	54.999	10.805	4.015	1.100	0.105	29.755	0.36
a_1	2.500^{ns}	-0.036^{ns}	-0.145^{ns}	-0.041^{ns}	0.006^{ns}	0.695^{ns}	-0.015^{ns}
a_2	21.339^{***}	0.697^{*}	0.497^{***}	0.118^{**}	0.045^{***}	10.398^{***}	0.056^{***}
a_{11}	-5.625^{ns}	-1.248^{**}	-0.945^{***}	-0.329^{***}	0.011^{ns}	-1.611^{ns}	-0.025^{**}
a_{22}	-3.125^{ns}	-1.418^{**}	-0.700^{***}	-0.239^{***}	-0.001^{ns}	-2.531^{ns}	-0.033^{ns}
a_{12}	0.000^{ns}	-0.005^{ns}	-0.070^{ns}	-0.038^{ns}	0.008^{ns}	0.045^{ns}	-0.033^{ns}
STD	*0.010*	*0.762*	*0.213*	*0.073*	0.023	*3.955*	*0.03*
R^2	0.83	0.71	0.94	0.93	0.75	0.85	0.86

a_0: regression coefficient corresponding to the central point; a_1: linear coefficient for pH; a_2: linear coefficient for specific glycerol feeding rate; a_{11}: quadratic coefficient for pH; a_{22}: quadratic coefficient for specific glycerol feeding rate; a_{12} cross-product coefficient; STD: standard error of estimation of the variable; R^2: multiple correlation coefficient; * 90 %; ** 95 %; *** 99 %; ns: not significant at 90 % level.

4.5.3 Effect of pH and specific glycerol feeding rate on specific glycerol consumption rate

Figure 4.5 depicts the surface response of specific glycerol consumption rate as a function of specific glycerol feeding rate and pH. The glycerol consumption was well linked to the substrate feeding. This result is confirmed by the absence of glycerol accumulation in the medium during the bioconversion phase. It indicates that glycerol was adequately supplied to the cells that allowed fixing the rate of the limiting enzyme of Pdu pathway. Meanwhile, no significant effect of pH was displayed on this variable (Table 4.7).

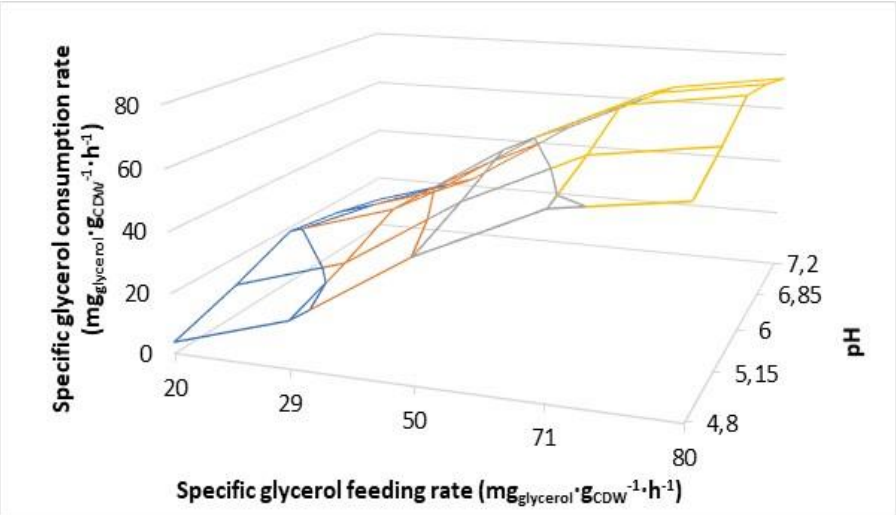

Figure 4.5. Effect of pH and specific glycerol feeding rate on the specific glycerol consumption rate of *L. reuteri* DSM 17938

4.5.4 Effect of pH and specific glycerol feeding rate on 3-HP titer, 3-HP final quantity and 3-HP production yield by *L. reuteri* DSM 17938

The coefficients of the polynomials shown in Table 4.7 indicated that pH and specific glycerol feeding rate both affected the 3-HP titer. The three-dimensional representation of these effects is displayed on Figure 4.6.

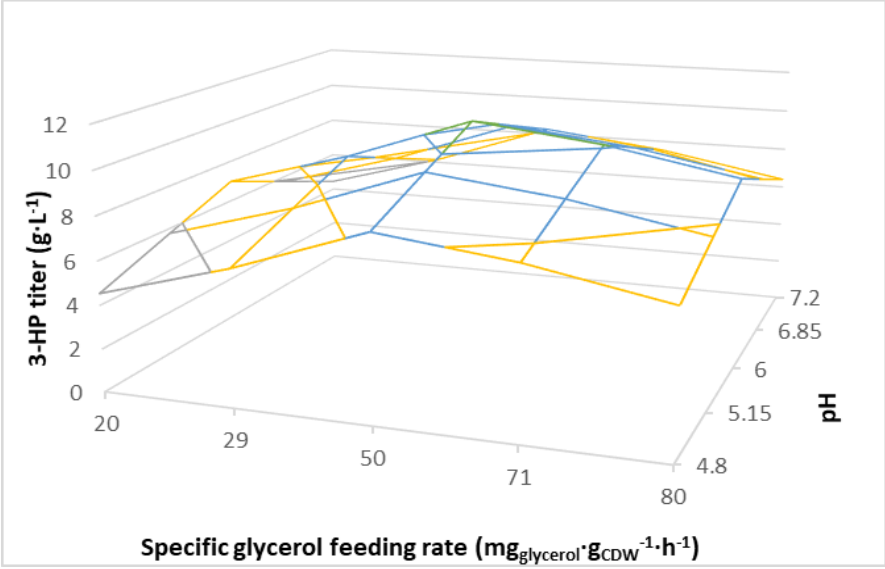

Figure 4.6. Effect of pH and specific glycerol feeding rate on the 3-HP titer of *L. reuteri* DSM 17938

From Figure 4.6, the effect of the two factors on 3-HP titer remained moderate, which is consistent with the quite low value of the multiple coefficient of the polynomial (71 %). This estimation was however not completely satisfactory.

As the biomass concentration at the beginning of the bioconversion step was similar for all experiments, and as the volume variation remained limited between 0.27 L and 0.39 L, the final 3-HP quantity and 3-HP production yield displayed similar behaviors than the 3-HP titer. From Table 4.7, these variables were affected by both pH and specific glycerol feeding rate, with a good accuracy (94 % and 93 %, respectively). The three-dimensional representations of the effects of the factors on these variables are displayed on Figure 4.7 and Figure 4.8.

No interaction between pH and specific glycerol feeding rate appeared on these variables.

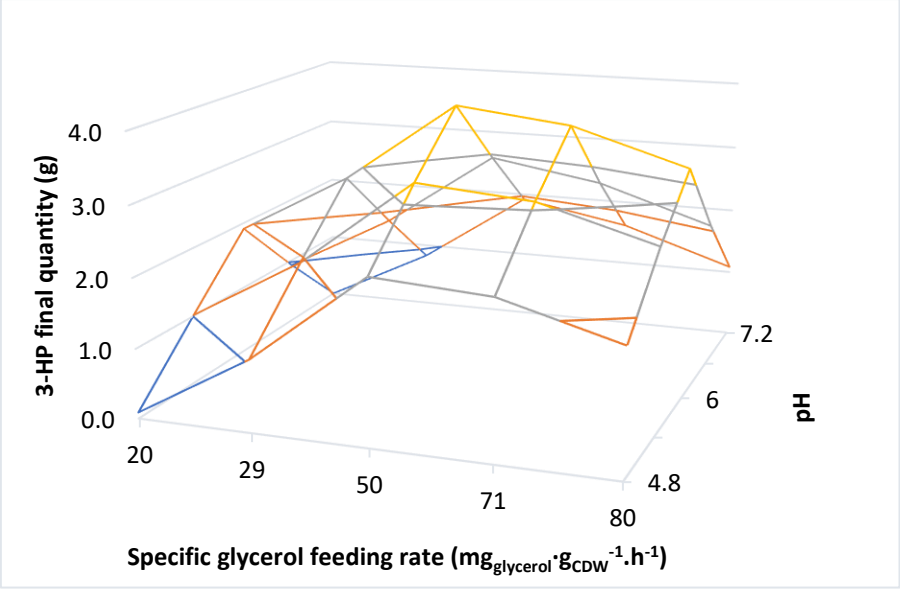

Figure 4.7. Effect of pH and specific glycerol feeding rate on the 3-HP final quantity produced by *L. reuteri* DSM 17938 (calculated with a final volume of 0.31 ± 0.05 L)

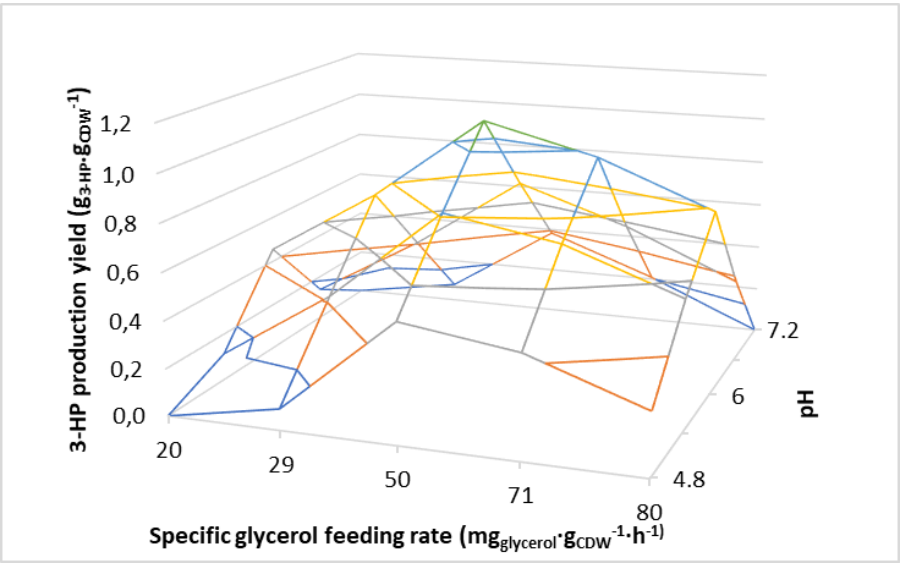

Figure 4.8. Effect of pH and specific glycerol feeding rate on the 3-HP production yield of *L. reuteri* DSM 17938

From Figure 4.7 and Figure 4.8, 3-HP final quantity and 3-HP production yield by *L. reuteri* DSM 17938 were dramatically low at low pH and low specific glycerol feeding rate. At the lowest value of two factors, very few 3-HP was produced (estimated value of 0.09 $g_{3\text{-}HP}$) and the yield was nearly zero. By increasing the specific glycerol feeding rate for the low pH values, these variables remained at very low values, for example, 1 g of produced 3-HP and 1.1 $g_{3\text{-}HP}$·g_{CDW}^{-1} at pH 4.8 with the feeding rate of 29 $mg_{glycerol}^{-1}$·g_{CDW}^{-1}·h^{-1}. This indicated that the resting cells of *L. reuteri* DSM 17938 were strongly stressed at pH 4.8, which is consistent with the results of the preliminary test performed at pH 4.5. These results are explained by the inhibitory effect of this low pH that is very far from the optimum pH for *L. reuteri* (Kandler *et*

al., 1980) and from that of the activity of the enzymes involved in the Pdu pathway as aforementioned (Talarico *et al.*, 1990; Talarico and Dobrogosz, 1990).

Another noticeable observation is that the final 3-HP quantity and 3-HP production yield obtained at pH 7.2 and a specific glycerol feeding rate of 20 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ were close to zero. This can be explained by the optimal pH of the glycerol dehydratase (pH 7.2) that catalyzes the dehydration of glycerol into 3-HPA (Talarico and Dobrogosz, 1990). As this enzyme is ten-times more rapid than the subsequent enzymes that oxidize 3-HPA into 3-HP (Dishisha *et al.*, 2014), an accumulation of 3-HPA occurred in that condition. This was confirmed experimentally by the quantification of the 3-HPA concentration that reached 0.4 $g{\cdot}L^{-1}$ in these conditions.

In addition to this analysis, pH 6.0 was shown to be the best condition for all levels of specific glycerol feeding rates tested. This value is close to pH 6.2, which was recommended as the optimal pH of the enzyme propanediol oxidoreductase involved in the reduction of 3-HPA into 1,3-PDO (Talarico *et al.*, 1990). However, it differed from the optimal pH of the enzyme glycerol dehydratase involved in the formation of the intermediate product 3-HPA in Pdu pathway, that was reported as pH 7.2 (Talarico and Dobrogosz, 1990). As pH 6.0 was recognized as the optimum for *L. reuteri* growth, this condition could help the cells to maintain a good physiological state. Moreover, this pH is unfavorable for detrimental 3-HPA accumulation (Talarico and Dobrogosz, 1990).

The specific glycerol feeding rate displayed both linear and quadratic effects on the three variables (Table 4.7). The 3-HP titer (Figure 4.6), the final 3-HP quantity (Figure 4.7) and the 3-HP production yield (Figure 4.8) values were maximum in the range of specific glycerol feeding rates comprised between 55 and 60 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$. This result is in accordance with the observation of (Dishisha *et al.*, 2014) about the maximum specific glycerol feeding rate.

For these three variables, since the effect of pH is only displayed by its quadratic coefficient, the higher values were reached at the central point of pH 6.0. On the other hand, as the effect of specific glycerol feeding rate was displayed by the linear and quadratic coefficients, a slight shift of the optimum of this factor from the central point was observed. Consequently, the optimal value of the specific glycerol feeding rate was calculated for the three variables. It was equal to 55.2 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ for 3-HP titer, 60 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ for 3-HP final quantity and 55.2 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ for 3-HP production yield. These values were close to each other and confirmed that these variables were affected similarly by the two tested factors.

The estimated maximal values of these variables were calculated for *L. reuteri* DSM 17938. The maximal 3-HP titer was estimated at 10.9 ± 0.8 g3-HP·L-1, the maximal 3-HP quantity at 4.1 ± 0.2 g3-HP, and the maximal 3-HP production yield at 1.11 ± 0.07 $g_{3\text{-}HP}.g_{CDW}^{-1}$. These estimations were consistent with the values obtained at the central point (i.e., pH 6.0 and 50 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$). However, they remained lower than those obtained with the selected

conditions of the first experimental design that could be ascribed to the higher temperature used during the growth phase of the current tests or to the existence of possible interactions that were not displayed by the kind of experimental design employed (Table 4.6).

Finally, the best conditions to improve these three variables were pH 6.0 and a specific glycerol feeding rate comprised between 55 and 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

4.5.5 Effect of pH and specific glycerol feeding rate on 3-HP production rate and specific 3-HP production rate of 3-HP by *L. reuteri* DSM 17938

The specific 3-HP production rate was calculated as the ratio of the 3-HP production rate to the biomass concentration. It represents the intrinsic capacity of the cells to produce 3-HP.

From the analysis of variance (Table 4.7), 3-HP production rate and specific 3-HP production rate were affected by the specific glycerol feeding rate ($p \leq 0.01$) but not by the pH. The multiple correlation coefficients (75 % and 85 %, respectively) indicated that the polynomials well represented the experimental results. In addition, no interaction between the two factors was pointed out.

Figure 4.9 and Figure 4.10 show the three-dimensional representations of the polynomials characterizing the 3-HP production rate and the specific 3-HP production rate, which enabled the response surfaces to be visualized, within the ranges of tested pH and specific glycerol feeding rates.

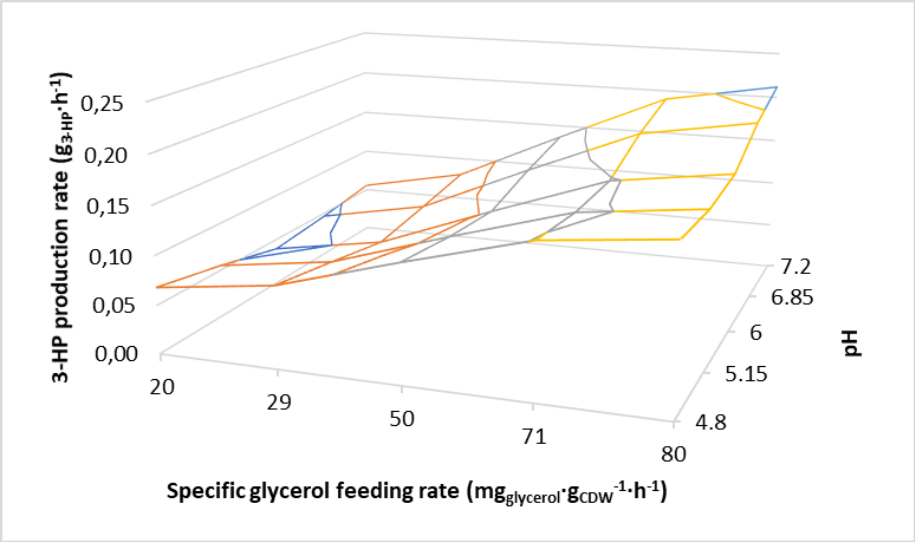

Figure 4.9. Effect of pH and specific glycerol feeding rate on the 3-HP production rate of *L. reuteri* DSM 17938

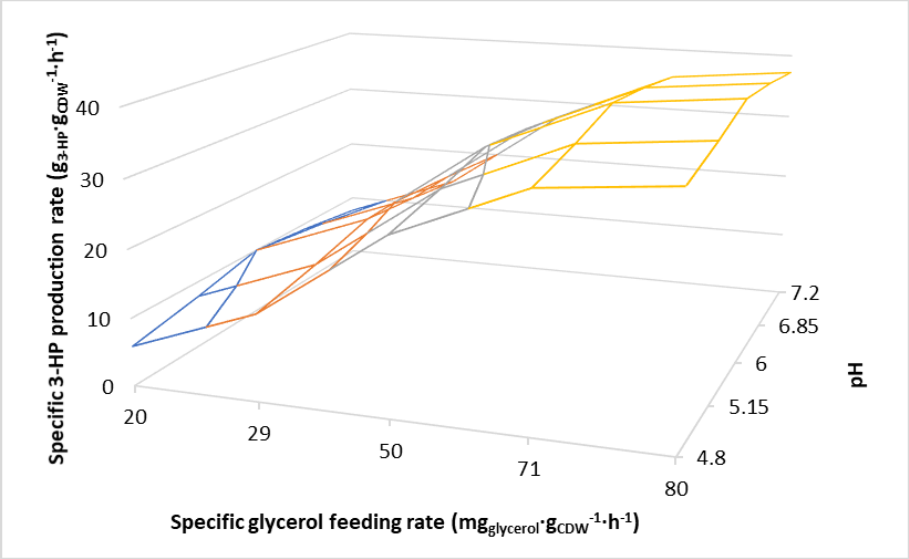

Figure 4.10. Effect of pH and specific glycerol feeding rate on the specific 3-HP production rate of *L. reuteri* DSM 17938

From these results, it can first be observed that the factor pH did not significantly affect the 3-HP production and specific production rates of *L. reuteri* DSM 17938. This result is in agreement with that of (Dishisha *et al.*, 2014) who demonstrated that no effect of pH was seen on the specific 3-HP production rate by *L. reuteri* DSM 20016. As these authors tested a lower range of pH (pH 5 and pH 7) and another strain, our results enlarge the validity of this observation.

At all pH levels, the 3-HP production rate and specific 3-HP production rate increased with increasing the specific glycerol feeding rate. This result indicated that the rates of 3-HP production were directly linked to the specific glycerol feeding rate that was thus the limiting factor during these experiments, as was hypothesized in chapter 3. The result is consistent with those reported by (Dishisha *et al.*, 2014) who indicated that, by enhancing the glycerol feeding rate from 0.6 $g \cdot h^{-1}$ to 1 $g \cdot h^{-1}$, irrespective of pH, the specific 3-HP production rate increased from 43 - 49 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$ to 62.4 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

A slowdown of the production rate increase was observed between 70 and 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, which could be explained by the accumulation of the inhibitory intermediate 3-HPA. Indeed, 3-HPA accumulation was not significant in all experiments, excepted at the specific glycerol feeding rate of 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ where it reached 0.6 $g \cdot L^{-1}$. This concentration is close to the minimum 3-HPA level (8.11 mM, 0.60 $g \cdot L^{-1}$) that inhibits the activity of the enzyme propionaldehyde dehydrogenase, which transforms 3-HPA into 3-hydroxypropionyl CoA (Sabet-Azad *et al.*, 2013). However, this 3-HPA accumulation was lower than that observed by (Dishisha *et al.*, 2014), who detected concentrations comprised between 25.9 mM and 29.0 mM (1.9 to 2.1 $g \cdot L^{-1}$).

From these observations, the optimal conditions that maximized the specific 3-HP production rate of *L. reuteri* DSM 17938 were determined from the parameters of the equation given in

Table 4.7. No optimal pH was highlighted by the polynomial. This indicates that the bioconversion could be done either at pH 4.8 or at pH 7.2 without affecting the specific and the global 3-HP production rate. This result was interesting as it indicated that, whatever the chosen pH, the bioconversion rate will remain stable during the time of bioconversion, even if low final 3-HP concentrations may be obtained at low or high pH. The optimal specific glycerol feeding rate could not be calculated as well, but for another reason. From the polynomial, its value should be higher than 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ that is out of the range tested. This expected value will be however higher than that recommended by (Dishisha *et al.*, 2015) at 62.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ for *L. reuteri* DSM 20016. However, as 3-HPA accumulated at high specific glycerol feeding rate, it can be hypothesized that the optimum will be not so far from 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

4.5.6 Effect of pH and specific glycerol feeding rate on 3-HP volumetric productivity of *L. reuteri* DSM 17938

The volumetric productivity of 3-HP was calculated after 24 h of bioconversion, in order to better compare the different conditions.

From Figure 4.11, a positive effect of the specific glycerol feeding rate on the 3-HP volumetric productivity was observed, with a linear coefficient only. Because the quadratic coefficient was the only significant one, the pH displayed an optimal value at pH 6.0. The polynomial described the experimental data with a quite good accuracy as the multiple coefficient of determination was equal to 86 %. The three-dimensional representation of the effects of these factors is given in Figure 4.11.

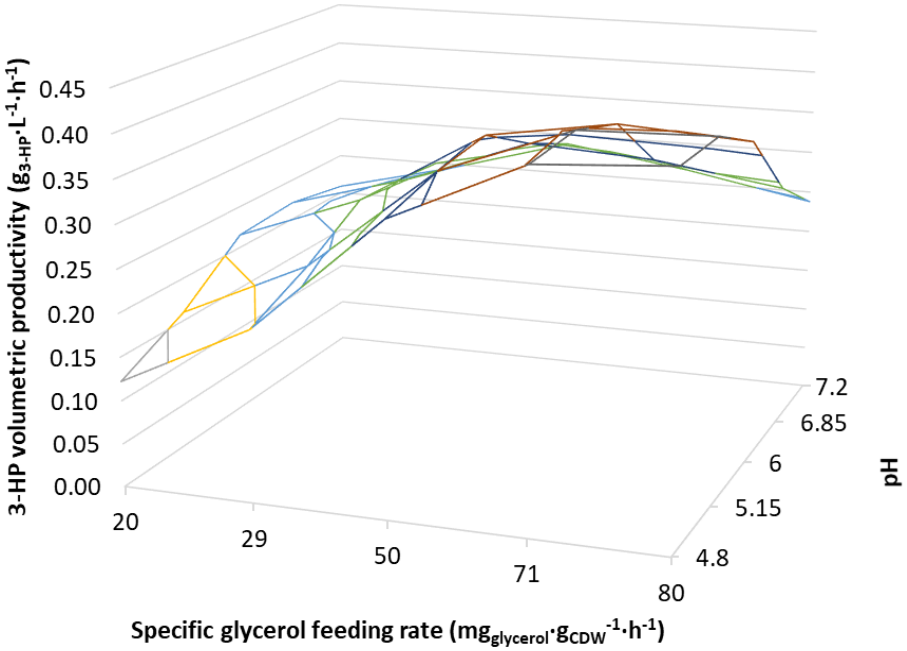

Figure 4.11. Effect of pH and specific glycerol feeding rate on the 3-HP volumetric productivity of *L. reuteri* DSM 17938

From these results, pH 6.0 was recognized as the most convenient pH for maximizing the 3-HP volumetric productivity. As for the production rates, the optimal specific glycerol feeding rate could not be estimated by the model, as it was out of the range of tested values, on the upper side. However, as a slowdown was observed at high specific glycerol feeding rates, the optimum should be close to 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. Although the coefficient of the interaction between pH and specific glycerol feeding rate was not significant, an interaction could be seen on Figure 4.11. It indicated that the range of pH comprised between 6.0 and 4.8 together with a range of specific glycerol feeding rates comprised between 55 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ and 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ displayed the best 3-HP volumetric productivity.

From the polynomial, the maximum 3-HP volumetric productivity could be estimated at 0.38 $g_{3\text{-}HP} \cdot L^{-1} \cdot h^{-1}$, at pH 6.0 and glycerol feeding rate at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. This value was higher than the 3-HP volumetric productivity obtained at the beginning of the thesis, i.e. 0.25 $g \cdot L^{-1} \cdot h^{-1}$ in the reference condition of the first experimental design. It was also higher than the result obtained by (Dishisha *et al.*, 2015) at 0.24 $g \cdot L^{-1} \cdot h^{-1}$.

4.5.7 Summary of the information

The effects of pH and specific glycerol feeding rate on seven variables characterizing the 3-HP bioproduction from glycerol bioconversion by *L. reuteri* DSM 17938 were established and analyzed. The results displayed two sets of conditions that maximized these variables.

By considering the variables 3-HP titer, 3-HP final quantity and 3-HP production yield, the optimal conditions were found to be at pH 6.0 and at a specific glycerol feeding rate in the range of 55 to 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. These conditions were explained by the optimal pH of the enzymes involved in the pathway for 3-HP biosynthesis from glycerol, by the good physiological state of the cells at this pH value and by the low accumulation of detrimental 3-HPA.

In order to validate these results, i.e. to maximize these variables, a validation experiment should be planned. The conditions of this validation experiment will be set at pH 6.0 and at a specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

By considering the specific glycerol consumption rate, the 3-HP production rate, the specific 3-HP production rate and the 3-HP volumetric productivity, the optimal conditions were found to be at a specific glycerol feeding rate higher than 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, irrespective of the pH in the range of pH 4.8 and pH 7.2. These results demonstrated that the specific glycerol feeding rate was the main limiting factor for this biotransformation. However, as 3-HPA accumulated at high specific glycerol feeding rates, too high values of this factor should be avoided to prevent inhibition.

With the aim to further validate these results, pH 6.0 was retained in order to meet the optimal pH found for the previous variables. A specific glycerol feeding rate of 80

$mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ was selected to maximize the rates of the bioprocess, because the experimental design cannot be extrapolated, and to avoid 3-HPA accumulation.

Finally, two sets of experimental conditions were selected and will be used, in the next sub-chapter, to validate the estimations given by the models, with the aim to maximize the 3-HP production by *L. reuteri* DSM 17938. The results are discussed in the following subparts.

4.6 Validation experiments for the optimization of 3-HP bioproduction by *L. reuteri* DSM 17938

4.6.1 Experimental approach

Two validation experiments were performed to improve the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938. The growth phase was performed in the same growth conditions of all experiment in CCRD, as described in Section 4.4.1. At the end of the biomass production phase, the bacterial concentration was equal to 2.9 ± 0.0 $g_{CDW} \cdot L^{-1}$ that was in agreement with previous results. During the bioconversion phase, the temperature was set at 37 °C, the agitation at 100 rpm and the pH was controlled at pH 6.0 by the addition NH_4OH 1.48 $mol \cdot L^{-1}$.

Before analyzing the results, the molecular balance and carbon mass balance during the bioconversion phase were checked. They were equal to 104.4 ± 2.5 % that was satisfying. In addition, the redox balance was well maintained in the two conditions as the molar ratios between 3-HP and its co-product 1,3-PDO were equal to 1.01 $mol_{3\text{-}HP} \cdot mol_{1,3\text{-}PDO}^{-1}$ and 0.99 $mol_{3\text{-}HP} \cdot mol_{1,3\text{-}PDO}^{-1}$ at the end of the two bioprocesses.

4.6.2 Kinetics of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 at pH 6.0 and specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$

Figure 4.12 displays the kinetics of glycerol consumption, NH_4OH consumption and 3-HP production during the bioconversion phase of the validation experiment performed at pH 6.0 and at a specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. The results obtained at the central point of the experimental design are also represented for comparison.

In both cases, no glycerol remained in the medium during the bioconversion, thus indicating that the substrate was consumed as soon as it was supplied into the bioreactor. The specific glycerol consumption rate was close to the specific glycerol feeding rate, at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

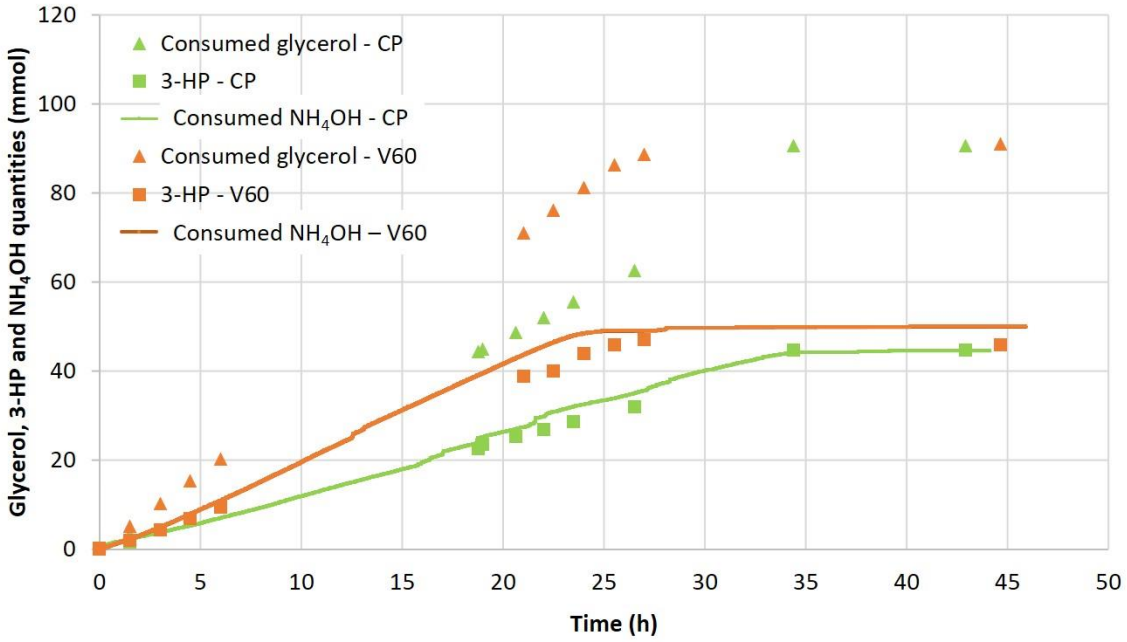

Figure 4.12. Kinetics of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 in the validation conditions (V60) fixed at pH 6.0 and specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ (final volume 0.36 L), in comparison to the conditions of the central point (CP) of the CCRD (final volume 0.37 ± 0.02 L)

The validation experiments done at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ showed a shorter duration of bioconversion than that at the central point (26.5 h instead of 37 ± 4 h).

The 3-HP concentration at the end of the bioconversion performed in the validation conditions was equal to 11.4 $g_{3\text{-}HP} \cdot L^{-1}$, which is consistent with the predicted value of 10.9 ± 0.8 $g_{3\text{-}HP} \cdot L^{-1}$, by taking into account the standard variation. The 3-HP final quantity was similar at the end of the validation experiment (4.2 g) and in the central point conditions (4.0 ± 0.2 g) and it was close to the quantity estimated with the model (4.1 ± 0.2 g). These similarities can be explained by the small difference between the specific glycerol feeding rates in the central point (50 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) and in this experiment (60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$). As described previously, the bioconversion stopped when no base was consumed anymore. At that time, 3-HPA started to slightly accumulate, to reach 0.21 $g \cdot L^{-1}$ in the validation conditions instead of 0.11 ± 0.03 $g \cdot L^{-1}$ in the central point conditions. These values remained however lower than the inhibitory concentration of 0.6 $g \cdot L^{-1}$ that affects the enzymatic activities linked to the production of 3-HP (Sabet-Azad *et al.*, 2013). The 3-HP production yield was equal to 0.9 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$ that was lower than that of the central point (1.10 ± 0.07 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$) and of the predicted value (1.10 ± 0.07 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$).

The 3-HP production rate and the specific 3-HP production rate were respectively established at 0.16 $g_{3\text{-}HP} \cdot h^{-1}$ and 36.9 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$ for the validation condition. These values agreed with the predicted values by the model (0.14 ± 0.02 $g_{3\text{-}HP} \cdot h^{-1}$ and 35.9 ± 4.0 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$). The 3-HP production rate was 52 % higher than in the central point conditions (0.11 ± 0.01 $g_{3\text{-}}$

$_{HP}$·h^{-1}) and the specific 3-HP production rate was 24 % higher as compared to the central point conditions (29.8 ± 1.1 mg$_{3\text{-HP}}$·g$_{CDW}^{-1}$·h^{-1}). This specific 3-HP production rate was also higher than the best performance of 3-HP bioproduction that was previously obtained by (Dishisha *et al.*, 2015) at 28.4 g$_{3\text{-HP}}$·g$_{CDW}^{-1}$·h^{-1}.

To better compare this validation condition with the previous conditions, the 3-HP volumetric productivity at 24 h of bioconversion was calculated. It was equal to 0.47 g$_{3\text{-HP}}$·L^{-1}·h^{-1}, which was significantly higher than that obtained in the central point conditions (0.36 ± 0.01 g$_{3\text{-HP}}$·L^{-1}·h^{-1}). In addition, it was also higher than the 3-HP volumetric productivity that was determined in the reference condition of chapter 3, at the very beginning of the thesis work (0.25 g$_{3\text{-HP}}$·L^{-1}·h^{-1}), and than that obtained by (Dishisha *et al.*, 2015) at 0.24 g·L^{-1}·h^{-1}. However, it was slightly higher than the value estimated by the model (0.38 ± 0.03 g$_{3\text{-HP}}$·L^{-1}·h^{-1}).

4.6.3 Validation of the glycerol into 3-HP bioconversion kinetics by *L. reuteri* DSM 17938 at pH 6.0 and specific glycerol feeding rate of 80 mg$_{glycerol}$·g$_{CDW}^{-1}$·h^{-1}

Figure 4.13 presents the kinetics of glycerol consumption, 3-HP and NH_4OH consumption during the bioconversion phase of the validation experiment performed at pH 6.0 and at a specific glycerol feeding rate of 80 mg$_{glycerol}$·g$_{CDW}^{-1}$·h^{-1}, together with the kinetics obtained in the central point conditions.

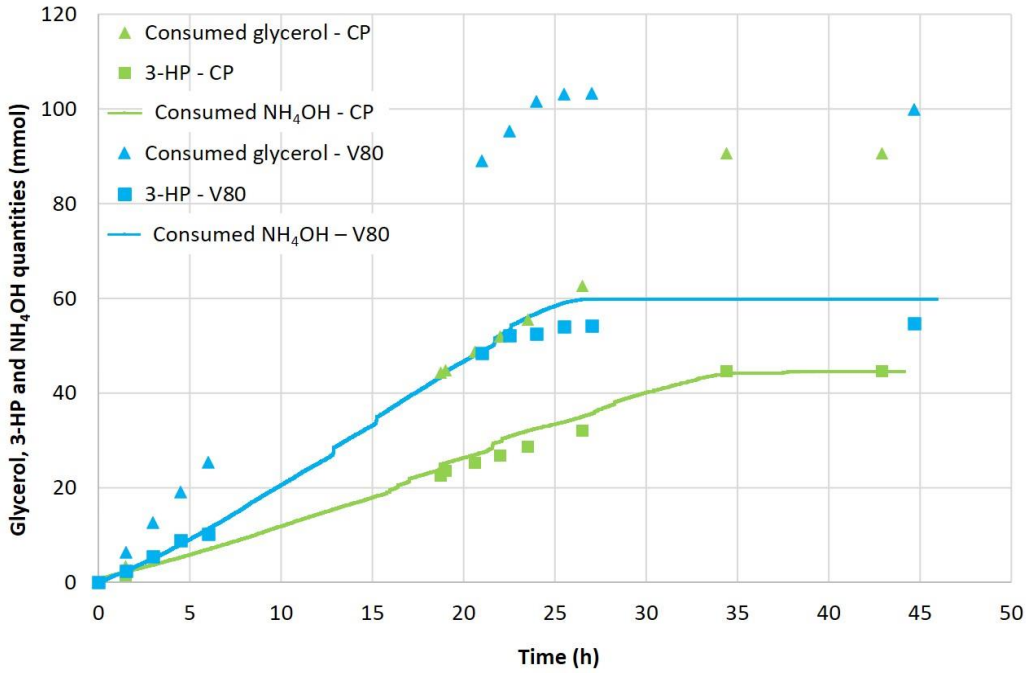

Figure 4.13. Kinetics of glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 in the validation conditions (V80) fixed at pH 6.0 and specific glycerol feeding of 80 mg$_{glycerol}$·gC$_{DW}^{-1}$·h^{-1} (final volume 0.40 L), in comparison to the conditions of the central point (CP) of CCRD (final volume 0.37 ± 0.02 L)

Bioconversion duration was lower at the higher specific glycerol feeding rate, as it was equal to 24.8 h instead of 37.0 ± 4.0 h in the central point conditions. The glycerol consumption rate was strongly enhanced and the specific glycerol consumption rate was calculated as 80 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ that equal to the specific glycerol feeding rate value.

More 3-HP was produced in these validation conditions, as the final concentration reached 12.2 $g{\cdot}L^{-1}$ and the final quantity attained 4.9 g, that was higher than 4.0 g as measured in the central point conditions. These values are higher with those predicted by the model (9.1 ± 0.8 $g_{3\text{-}HP}{\cdot}L^{-1}$ and 3.4 ± 0.2 $g_{3\text{-}HP}$). 3-HPA accumulated at a final concentration of 0.31 $g{\cdot}L^{-1}$, which remained lower than its inhibitory concentration (Sabet-Azad *et al.*, 2013) but higher than in the previous validation condition. Within these conditions, the 3-HP production yield was equal to 1.0 $g_{3\text{-}HP}.g_{CDW}^{-1}$. This value was lower than that of the central point (1.10 ± 0.07 $g_{3\text{-}HP}{\cdot}g_{CDW}^{-1}$) but higher than the predicted value (0.81 ± 0.07 $g_{3\text{-}HP}{\cdot}g_{CDW}^{-1}$).

The 3-HP production rate was higher in the validation condition (0.20 $g_{3\text{-}HP}.h^{-1}$) than in the central point conditions (0.11 ± 0.01 $g_{3\text{-}HP}.h^{-1}$). The specific 3-HP production rate was calculated as 40.0 $mg_{3\text{-}HP}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ which is remarkably higher than the specific 3-HP production rate at the central point (29.8 ± 1.1 $mg_{3\text{-}HP}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$). By comparison with the predicted values (0.17 ± 0.02 $g_{3\text{-}HP}{\cdot}h^{-1}$ and 39.4 ± 4.0 $mg_{3\text{-}HP}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$), the experimental results were well fixed to the estimation of specific 3-HP production rate but slightly higher than the prediction of 3-HP production rate.

Finally, the 3-HP volumetric productivity significantly increased from 0.36 ± 0.01 $g_{3\text{-}HP}{\cdot}L^{-1}{\cdot}h^{-1}$ in the central point conditions to 0.50 $g_{3\text{-}HP}{\cdot}L^{-1}{\cdot}h^{-1}$ in the validation conditions. This increase by 39 % was remarkable and confirmed that this validation condition improved a lot the performance of 3-HP bioproduction.

In the conditions of this validation experiment, the results of 3-HP specific production rate was 41 % higher than those reported by (Dishisha *et al.*, 2015). Moreover, the volumetric productivity was twice higher than that reported by (Dishisha *et al.*, 2015) at 0.24 $g{\cdot}L^{-1}{\cdot}h^{-1}$ in different glycerol feeding conditions, thus confirming that the conditions identified in our study were relevant to improve the 3-HP bioproduction.

4.6.4 Summary of the information

Two validation experiments were performed to validate the optimal conditions of pH and specific glycerol feeding rate that have been identified. From the results obtained with the experimental design CCRD, they corresponded to a pH value of 6.0 and a specific glycerol feeding rate of 60 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ to enhance the 3-HP titer, 3-HP final quantity and 3-HP production yield, and to pH 6.0 and a specific glycerol feeding rate equal to 80 $mg_{glycerol}{\cdot}g_{CDW}^{-1}{\cdot}h^{-1}$ to maximize the 3-HP production rate, specific 3-HP production rate and 3-HP volumetric productivity.

The results showed first that the experimental results fitted well with the estimated results of the models. The 3-HP titer and 3-HP final quantity were similar to those obtained in the reference condition, which was expected as the optimal pH and specific glycerol feeding rate were close too. The 3-HP production yield was however not improved. The 3-HP production rate and specific 3-HP production rate were significantly improved with the new conditions. As a final result, the 3-HP volumetric productivity was enhanced 198 % at a specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ and 208 % at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, as compared to (Dishisha *et al.*, 2015).

4.7 Assessment of intracellular pH and intracellular energy level during glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938

In order to understand the reasons why glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 stopped, while glycerol was still fed into the bioreactor and was thus potentially available for the cells to be converted into 3-HP and 1,3-PDO, some specific characteristics of cell physiological state were investigated.

4.7.1 Experimental approach

The intracellular pH (pHi) and the intracellular energy level that was linked to the measurement of DeltaIF20 were quantified during the bioconversion phase of the validation experiments described in Section 2.5.3. These two physiological characteristics have been identified as fully informative to understand the microbial behavior and cellular state throughout a bioprocess (Rault, 2009; Rault *et al.*, 2008).

The intracellular pH was measured by using a calibration curve after staining the cells with the fluorescent dye cFDA-SE (Rault *et al.*, 2009). The method that was previously developed in our lab has been adapted in this work for *L. reuteri* (Section 2.5.3).

The intracellular energy was quantified using the method proposed by (Rault *et al.*, 2008) and modified as described in Section 2.5.2. It was measured by staining the bacterial cells with cFDA, then incubating them for 20 min at 40 °C, and by quantifying the fluorescence intensity by flow cytometry before and after this incubation period. The difference in fluorescence intensities (DeltaIF20) was related to the active excretion of the fluorescent dye cF that required intracellular energy under the form of ATP. The higher the excretion level, the higher the intracellular energy of bacterial cells. Thanks to this method, the intracellular energy was expressed as DeltaIF20 (in %) that corresponded to the loss of intracellular fluorescence intensity after 20 min of incubation.

Because these methods were developed recently for *L reuteri* DSM 17938 (i.e., at the end of the experimental work of the thesis), they have been applied during the bioconversion step of the two validation experiments, performed at pH 6.0 and at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ or 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

4.7.2 Results and discussion

The data obtained during the two validation experiments are shown in Figure 4.14 for the bioconversion performed at pH 6.0 and 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ and in Figure 4.15 for the bioconversion at pH 6.0 and 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

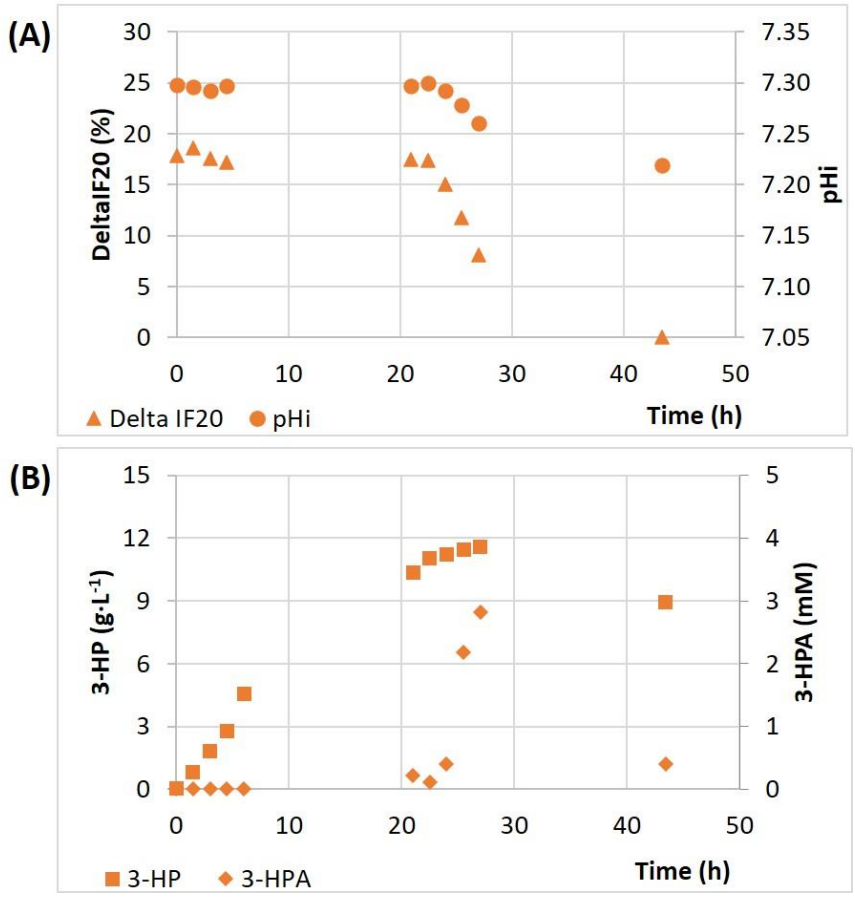

Figure 4.14. Variation of (A) intracellular pH (pHi) and intracellular energy level (DeltaIF20), (B) 3-HP titer and 3-HPA concentration during the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 performed at pH 6.0 and 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$

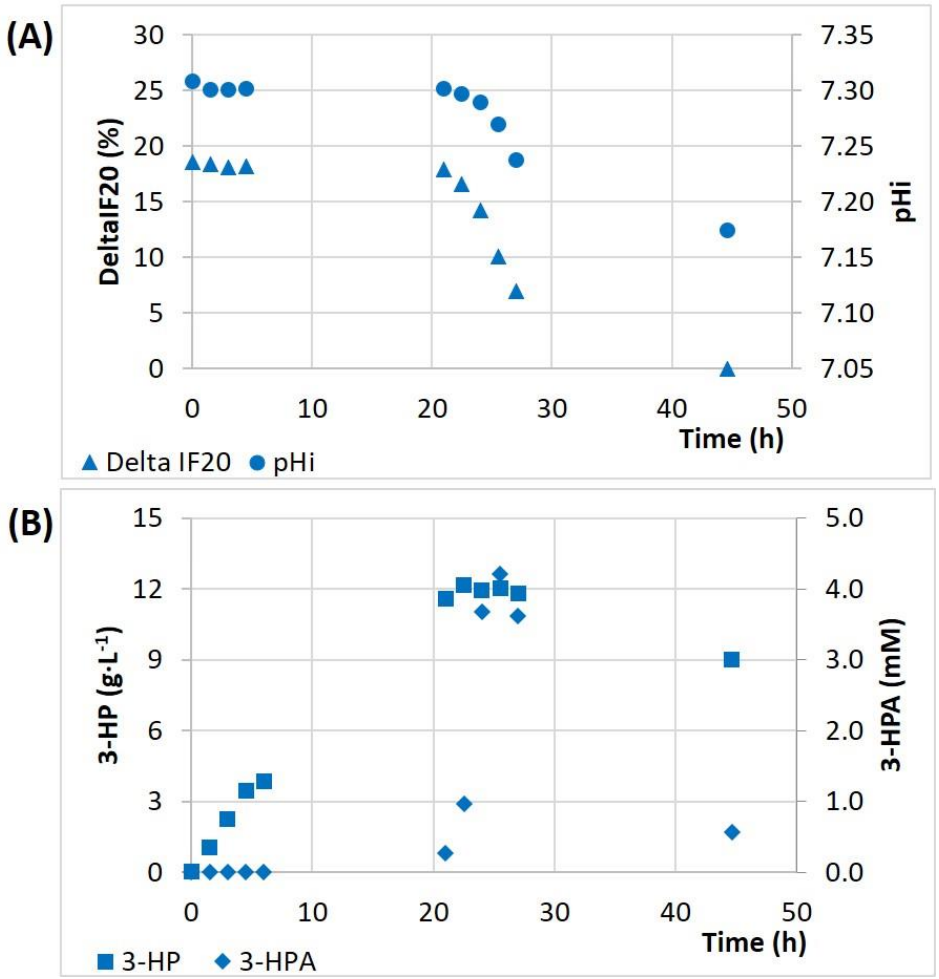

Figure 4.15. Variation of (A) intracellular pH (pHi) and intracellular energy level (DeltaIF20), (B) 3-HP titer and 3-HPA concentration during the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 performed at pH 6.0 and 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$

From Figure 4.14 and Figure 4.15, the pHi and the intracellular energy level of the bacterial cells remained constant during the first 21-23 hours of glycerol bioconversion into 3-HP. Then, they started decreasing simultaneously and these variations were also related to the cessation of 3-HP bioproduction and to the increase in 3-HPA concentration in the medium. If the two experiments displayed similar behaviors, a difference was observed about the moment at which these events occurred. They happened earlier during the culture performed at specific glycerol feeding rate of 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ than at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

During the first 22.8 h (at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) or 21 h (at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$), the intracellular pH was maintained between pH 7.29 and 7.31. Then a decrease in pHi occurred, to reach pH 7.26 after 27 h and 25.5 h, respectively of bioconversion. At that moment, the cells stopped converting glycerol into 3-HP and the 3-HPA concentration attained 2.8 to 3.7 $mmol \cdot L^{-1}$. Finally, at 44 h of bioconversion, the intracellular pH dropped to pH about 7.20.

Even if these experiments were not duplicated, and even if the pH differences were low at the moment the 3-HP production stopped, the similar behavior of the bacterial cells between

the two experiments indicated that the intracellular pH could be a key parameter to explain why 3-HP bioproduction was affected. In fact, the pHi is a very important property for bacteria as it affects the proton gradients of the cells (Rault *et al.*, 2008), modifies the rates of many biological reactions such as nutrient uptakes, protein and nucleic acid synthesis (Ai *et al.*, 2017), and affects the membrane permeability (Mieszkin *et al.*, 2017). Particularly, a low limit of intracellular pH was identified at 7.26 for *L. reuteri* DSM 17938, threshold below which the performance of 3-HP bioproduction began to be affected. This pH value was however slightly higher than the optimal pH of the enzymes involved in the Pdu pathway (Talarico *et al.*, 1990; Talarico and Dobrogosz, 1990). Consequently, it could be hypothesized that the most detrimental factor for 3-HP bioproduction was linked to a modification of the proton gradients, and thus of the intracellular energy level of the bacterial cells (Rault *et al.*, 2009; van Maris *et al.*, 2004)

This hypothesis was supported by considering the variation of DeltaIF20, as a measurement of intracellular energy level. From Figure 4.14 and Figure 4.15, DeltaIF20 remained unchanged during the first 22.8 h (at 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) or 21 h (at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$), at 17.8 ± 0.6 %, for both conditions. Then the value decreased to reach 14.7 ± 0.6 % after about 24 h, which corresponded to the end of 3-HP synthesis and to the moment when the 3-HPA concentration increased. After the cessation of glycerol bioconversion into 3-HP, the intracellular energy level continued to decrease, to reach about 7 % after 27 h and was equal to 0 % after 44 h. As no energy was supplied within the medium, the only ATP synthesis was achieved from 3-HP bioproduction (through the oxidative branch of the bioconversion pathway), with a molar ratio of 1 mole of ATP produced per mole of synthesized 3-HP. Consequently, it was consistent to observe that when the 3-HP production ceased, the intracellular energy level started to decrease.

These experiments have unfortunately not been duplicated, which restrict the scope of the information provided. In addition, due to their complexity and heaviness, it was not possible to measure, simultaneously, the variation of the concentrations of the subpopulations of active, not active, permeabilized and not permeabilized cells. It will then be useful to relate the actual findings to the level of permeabilization of the cells and to their enzymatic activity level. As a very important prospect, one has to confirm this information in the same conditions as well as in other bioconversion conditions and with other *L. reuteri* strains to enlarge the range of behaviors and to deepen the understanding of the phenomena explaining the bioconversion cessation for this species.

4.7.3 Summary of information

The analysis of intracellular pH and intracellular energy level of bacterial cells during glycerol bioconversion into 3-HP provided deeper understanding about the limitation of 3-HP bioproduction despite the continuity of glycerol supply during the fed-batch process. The decrease in intracellular pH below a limit value of 7.26, together with a decrease in DeltaIF20

that occurred simultaneously, were related to the cessation of 3-HP bioproduction process by *L. reuteri* DSM 17938. These new insights have however to be confirmed by complementary experiments.

4.8 Conclusion of chapter 4

The bioconversion of glycerol into 3-HP by *L. reuteri* DSM 17938 has been improved by implementing a central composite rotatable design. Five preliminary experiments were first performed to define some key conditions to be used during the experimental design. From the obtained results, the number of centrifugations between the growth and the bioconversion phases was fixed at one step. The cells have to be used under fresh form, without being frozen and stored. A double glycerol feeding rate associated to a double cell concentration was shown to be possible but not sufficient to double the 3-HP bioproduction. The temperature was shown to be not sufficiently discriminating to be added in the experimental design. Finally, the pH was retained as a key factor to be studied and the low and high limits of pH were identified to be introduced in the experimental design.

The central composite rotatable design allowed quantifying the effects of the two factors “bioconversion pH” and “specific glycerol feeding rate” during the bioconversion phase, with a growth phase previously performed in identical conditions, as defined from chapter 3. Statistical analyses allowed identifying optimal conditions of these two factors, by considering seven variables: specific glycerol consumption rate, 3-HP titer, 3-HP final quantity, 3-HP production yield, 3-HP production rate, 3-HP specific production rate and volumetric productivity of 3-HP.

The pH 6.0 was identified as the optimum pH for the bioprocess, by considering the 3-HP titer, 3-HP final quantity and 3-HP production yield. In addition, these variables were maximized for a specific glycerol feeding rate comprised between 55 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ and 60 $mg_{glycerol} \cdot g_{CDW} \cdot h^{-1}$. The specific glycerol consumption rate, 3-HP production rate, 3-HP specific production rate and 3-HP volumetric productivity were maximized at the upper limit the experimental design that was 80 $mg_{glycerol} \cdot g_{CDW} \cdot h^{-1}$. But they were not statistically affected by the pH that could be chosen equally in the range of pH 4.8 to pH 7.2.

These results were then validated by two additional experiments that allowed the 3-HP bioproduction process to be significantly improved. Particularly, the higher volumetric productivity was achieved at pH 6.0 and at a specific glycerol feeding rate of 80 $mg_{glycerol} \cdot g_{CDW} \cdot h^{-1}$. It was 138 % higher than in the reference experiment of the first experimental design. However, it was not possible to continue the experiments beyond a certain limit of time, as the 3-HP production stopped even if glycerol was still fed into the bioreactor.

To better understand this limitation, the intracellular pH and the level of intracellular energy of bacterial cells were quantified during two bioconversion processes. The results showed

that a strong energetic limitation occurred, that was observed when the intracellular pH dropped below a limit of pH 7.26 for *L. reuteri* DSM 17938. These results have unfortunately not been confirmed, which constitutes a weakness that shall be corrected in the future.

5 Conclusion and prospects

The production of “building block” molecules is a major industrial issue in a context of increasing use of renewable resources as substitutes to fossil resources and of reduction in environmental footprint of human activities. 3-Hydroxypropionic acid is recognized as a key platform molecule since it is the precursor of a large variety of chemicals for multipurpose applications. One way for its biological production involves the bacterium *Lactobacillus reuteri* according to a two-step process: a growth phase in batch mode followed by a bioconversion phase to transform glycerol into 3-HP in fed-batch mode. However, the performance of these processes are hindered by the inhibitory effect of 3-HP itself and of its metabolic intermediate 3-hydroxyprionaldehyde (3-HPA). In parallel of the approach dedicated to the genetic improvement of producing cells, efforts must be focused on improving the bioprocess to properly prepare bacterial cells and optimize their use as “whole-cell biocatalyst”.

The objective of the thesis was to increase the performance of 3-HP bioproduction by glycerol bioconversion performed by *L. reuteri* DSM 17938 and to gain some insights into understanding the physiological behavior of the cells. A first approach allowed studying the effect of 11 nutritional and environmental factors encountered by cells during their growth on the performances of growth and subsequent bioconversion, using an experimental design. The best conditions for growth were selected and carried out, through a second experimental design, to optimize the 3-HP bioproduction by considering the effects of pH and specific glycerol feeding rate. In addition, the physiological state of the bacterial cells was characterized in order to better understand the reasons why the bioconversion stopped even if the substrate was still fed. These experimental approaches aimed as answering the three scientific questions raised at the beginning of the study.

To answer the first scientific question of the thesis, the nutritional and environmental conditions implemented during the growth phase and that affect the 3-HP bioproduction during the further step of bioconversion have been determined.

By using a Plackett and Burman experimental design, this first part of the study quantified the effects of 11 nutritional and environmental factors of growth phase on the 3-HP bioproduction performance of *L. reuteri* DSM 17938. Previously, some preliminary experiments were performed to define the limit values of temperature, pH and glucose concentration that could be tested in the experimental design, as well as the best moment at which the cells have to be harvested at the end of growth. The performance of the bioprocess has been evaluated by the total quantity of 3-HP produced, the bioconversion duration, the 3-HP production yield, and the 3-HP volumetric productivity. As the bioprocess involved a

growth step followed by the bioconversion, some growth characteristics have also been quantified, such as the final cell concentration, the lactic acid production yield and the ratio between [acetic acid + ethanol] to [lactic acid]. The two-step bioprocess has been performed in bioreactors, using controlled conditions of temperature and pH, and under micro-aerobiosis conditions, the dissolved oxygen partial pressure (in percentage) being followed by a pO_2 probe. The conditions of the growth phase were defined according to the Plackett and Burman experimental design, on the basis of a MRS medium, and the conditions of the bioconversion phase were fixed for all experiments (temperature, pH and glycerol feeding rate). Each bioproduction lasted around one week, during which samples were taken and analyzed. The method to quantify accurately the 3-HP titer by HPLC has been improved during the thesis.

A statistical analysis allowed identifying the factors that significantly acted on the bioprocess performances and quantifying their effects. Because of the kind of experimental design selected, the interactions between factors were however not characterized. The results demonstrated that supplementing the growth medium with glucose (20 $g \cdot L^{-1}$), phytone peptone (25 $g \cdot L^{-1}$), Tween 80 (4 $g \cdot L^{-1}$), 1,2-propanediol (3 $g \cdot L^{-1}$) and betaine + KCl (0.234 $g \cdot L^{-1}$+ 0.745 $g \cdot L^{-1}$), together with controlling the pH at 6.0, enhanced the subsequent production of 3-HP and the bioconversion duration. The addition of yeast extract and the type of neutralizer used for pH control showed no significant effect, whereas the addition of vitamin B12 and cysteine displayed a negative effect on 3-HP bioproduction. A positive effect of a low temperature (33 °C) was demonstrated on 3-HP production yield. But it was linked to the fixed value of glycerol feeding rate (i.e., 0.5 $g \cdot h^{-1}$) implemented during the bioconversion phase, that was limiting for the larger quantity of cells obtained at 37 °C. Indeed, the cell concentration at the end of growth was improved at a temperature of 37 °C and with a supplementation of MRS medium with 50 $g \cdot L^{-1}$ glucose.

From these results, the best conditions were selected to design a validation experiment at the same glycerol feeding rate of 0.5 $g \cdot h^{-1}$. The MRS medium was supplemented with 20 $g \cdot L^{-1}$ glucose, 25 $g \cdot L^{-1}$ phytone peptone, 4 $g \cdot L^{-1}$ of additional Tween 80, 3 $g \cdot L^{-1}$ 1,2-PDO and betaine with KCl (0.234 $g \cdot L^{-1}$+ 0.745 $g \cdot L^{-1}$). The pH was controlled at pH 6.0 with NH_4OH and the temperature was set at 33 °C. This validation experiment confirmed that this set of conditions led to an improvement of the 3-HP titer (+ 25 %), the 3-HP quantity (+ 46 %), the specific 3-HP production rate (+ 61 %) and the 3-HP production yield (+ 150 %), compared to the reference conditions. The 3-HP titer was slightly higher than those obtained by the two previous works performed in fed-batch mode (Dishisha *et al.*, 2014); (Dishisha *et al.*, 2015). Nevertheless, no detrimental accumulation of 3-HPA was noticed under the conditions determined by the present work, in contrary to (Dishisha *et al.*, 2014). However, the volumetric productivity remained lower than values reported from literature (Table 5.1), as a consequence of the low glycerol feeding rate applied. The bioconversion duration was much longer in our work (+ 53 %) compared to the best results published to date, that was explained by the lower specific 3-HP production rate in our conditions compared to the literature.

Finally, even if some performance of the bioprocess has been improved as compared to those of the initial reference conditions of the thesis, this improvement remained limited due to the experimental conditions used during the bioconversion phase. Based on these works, the next steps of the study deal with the bioconversion conditions with the aim to further improve the bioprocess.

To answer the second scientific question of the thesis, the relevant conditions that act during the bioconversion step to improve the performance of 3-HP bioproduction have been defined and optimized.

In the second part of the study, the effects of two environmental conditions during the bioconversion phase were established with the aim to optimize the 3-HP bioproduction. In order to circumvent the limitation of substrate availability that was seen in the first part of the study and thanks to preliminary experiments, the specific glycerol feeding rate and the bioconversion pH were identified as two key factors of the bioconversion phase. They have been introduced in a central composite rotatable design, which aimed at defining optimal values for these two factors in order to maximize the 3-HP bioproduction performance. This second experimental design benefited from the results of the first one, as the growth conditions were selected identically to the validation experiment described above, except for the temperature defined at 37 ^{o}C, and fixed for all experiments. It also profited from the results of preliminary experiments that provided information about the number of centrifugations for cell harvesting, the necessity to use not frozen cells, the temperature during the bioconversion step and the range of pH values that could be tested. It was established that the bioconversion could be monitored *on-line* by using the base consumption that stopped with 3-HP bioproduction, and the pO_2 variation that strongly increased when glycerol bioconversion stopped.

Table 5.1. Comparison of the performance of glycerol bioconversion into 3-HP by *L. reuteri*

***L. reuteri* strain**	**DSM 17938**	**DSM 17938**	**DSM 20016**	**DSM 20016**	**DSM 17938**	**DSM 17938**
Bioproduction mode	**Batch mode**	**Batch mode**	**Batch then multi-step fed-batch mode**	**Batch then fed-batch mode**	**Fed-batch mode**	**Fed-batch mode**
Temperature (°C)	37	37	37	37	37	37
pH	6.0	Free pH	7.0	5.0	6.0	6.0
Specific glycerol feeding rate ($mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	nd	nd	190.5	62.1	50.9	80.0
Duration (h)	1	3	21.0	58.0	88.6	24.9
3-HP titer ($g_{3\text{-}HP} \cdot L^{-1}$)	0.9	3.0	10.6	14.0	14.7	12.2
3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)	nd	nd	2.53	1.65	1.99	1.00
3-HP production rate ($mg_{3\text{-}HP} \cdot h^{-1}$)	2.25	1.98	0.72	0.34	0.22	0.20
Specific 3-HP production rate ($mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$)	nd	nd	120.4	28.4	22.5	39.97
3-HP productivity ($g_{3\text{-}HP} \cdot L^{-1} \cdot h^{-1}$)	0.90	0.99	0.50	0.24	0.17	0.49
3-HPA ($mmol \cdot L^{-1}$)	75.7	13.5	18.38	0.00	0.00	4.22
Reference	(Burgé *et al.*, 2015c)	(Couvreur *et al.*, 2017)	(Dishisha *et al.*, 2014)	(Dishisha *et al.*, 2015)	**Chapter 3**	**Chapter 4**

nd: not determined

By establishing response surfaces, the designed study aimed at maximizing 3-HP bioproduction, by considering 3-HP titer, final quantity, production yield, production rate, specific production rate and volumetric productivity. Optimal conditions have identified for the 3-HP titer, 3-HP final quantity and 3-HP production yield. They corresponded to pH 6.0 and a specific glycerol feeding rate of 60 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. These results agree with the best conditions described in Table 5.1. However, the maximum values of 3-HP production rate, specific 3-HP production rate and 3-HP volumetric productivity were obtained at a higher specific glycerol feeding rate of 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$, whatever the pH value between pH 4.8 and pH 7.2. But in this second case, the optimal specific glycerol feeding rate could not be identified within the range of values tested in the experimental design. From our results, the production of 3-HPA slightly increased with the specific glycerol feeding rate, which was linked to the slowdown of the 3-HP production rates between 71 and 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$. It was thus hypothesized that the optimal specific glycerol feeding rate was close to 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ for *L. reuteri* DSM 17938.

Based on these results, two validation experiments have been designed to confirm the improvement in 3-HP bioproduction. They were both performed at pH 6.0, and either at 60 or at 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$.

The results showed first that the experimental results fitted well with the estimated results of the models. The 3-HP titer and 3-HP final quantity were similar to those obtained in the reference condition, which was expected as the optimal pH and specific glycerol feeding rate were close too. The 3-HP production yield was however not improved compared to the reference experiment. The 3-HP production rate and the specific 3-HP production rate were respectively increased by 80 % and 34 % for a specific feeding rate of 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ compared to the central point. The 3-HP volumetric productivity was improved by 39 % as compared to the central point of the second experimental design, and by 138 % in comparison with the initial experimental results obtained at the beginning of this study. Moreover, the specific 3-HP production rate and the volumetric productivity were respectively 1.6- and 2-times higher than those obtained in the previous study carried out at a constant glycerol feeding rate (Table 5.1). At last, the small 3-HPA accumulation observed for a specific glycerol feeding rate of 80 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$ showed that this conditions was close to the optimum for *L. reuteri* DSM 17938. Consequently, the combination of growth conditions that enhanced the cell ability to produce 3-HP and of optimized conditions during the bioconversion stage led to a significant improvement of the 3-HP bioproduction performance, while preventing deleterious 3-HPA formation during the bioprocess.

However, despite the improvement obtained for the 3-HP bioproduction process, it remained hindered by the sudden cessation of the glycerol bioconversion into 3-HP. By improving the conditions during both the growth and bioconversion steps, this cessation raised either later or at higher 3-HP concentration, but it always happened. In order to better understand why this phenomenon took place, some complementary experiments were performed to explore

what happened inside bacterial cells, by characterizing some of their physiological characteristics.

To answer the third scientific question of the thesis, some physiological characteristics at the intracellular level that could drive the bioconversion process have been identified.

As the bioconversion process stopped whatever the pH and specific glycerol feeding rate used, while glycerol was continuously fed into the bioreactor, the question arose about the physiological reasons that caused this sudden stop.

One main reason could be assigned to the inhibitory effect of 3-HP and 3-HPA that are detrimental to *L. reuteri* metabolism (Cleusix *et al.*, 2007; Sabet-Azad *et al.*, 2013). However, the 3-HPA titer remained lower than its inhibitory concentration on *L. reuteri* (8.1 mmol·L^{-1}), and as the pH was controlled at pH 6.0, the concentration of the non-dissociated form of 3-HP that is the most inhibitory remained low (3 % of the total 3-HP produced).

Another explanation could be ascribed to a lack of intracellular energy that occurred suddenly. In fact, as long as 3-HP was produced, ATP was also produced (Årsköld *et al.*, 2008) thus helping bacterial cells to ensure their maintenance in non-growing medium. Then, it could be hypothesized that the cell maintenance could not be ensured anymore, thus leading to the cessation of bioproduction. To gain more insights about this hypothesis, some physiological characteristics of the cells were studied in order to understand why the bioconversion process stopped. By adapting methods based on the use of fluorescent dyes and flow cytometry, the intracellular pH (pHi) and the intracellular energy level (DeltaIF20) have been measured during the bioconversion step of two experiments. It was demonstrated that these physiological characteristics were affected at the moment when the 3-HP production ceased. After 24 h, when the bioconversion was running and just before it stopped, the pHi decreased by 0.05 pH units and the DeltaIF20 was reduced by a value of 3.1 %. Later on, after 44 h, the reduction was stronger, with a total decrease of 0.1 pH units for pHi and a final value of intracellular energy that was close to 0 %. These observations were however evidenced at the end of the thesis, and only in the course of two experiments. It is thus of first priority to confirm these findings by assessing their reproducibility in order to gain insight into the understanding of bacterial physiology during the metabolic process of glycerol bioconversion.

From these results, some prospects may be proposed to strengthen the results and enlarge the research approach

As a first prospect of this study, because some experiments could not be reproduced, priority needs to be given to the repetition of these experiments, to definitely confirm the results.

Secondly, the second experimental design allowed identifying optimal conditions for the specific glycerol feeding rate that were higher than the highest limit of the tested values. It is

thus essential to conduct other experiments at higher specific glycerol feeding rates, together with various pH, to further improve the 3-HP bioproduction. In addition, the effect of the temperature could not be tested within this experimental design, but this factor is another potential lever to increase the 3-HP bioproduction.

Thirdly, the experiments devoted to the characterization of the physiological events that could explain or be a consequence of the cessation of glycerol bioconversion into 3-HP have to be done again, with the same strain and the same conditions, and then also by carrying out the bioconversion in other conditions and with other strains.

Another more in-depth analysis of the cellular responses during bioconversion may be programmed, mainly by using proteomic and transcriptomic approaches. More particularly, it will be of great interest to detect whether *L. reuteri* cells express some specific genes or synthesize some stress proteins to cope with the stresses encountered as a result of 3-HP and 3-HPA accumulation or due to their non-growing environment. The determination of 3-HP minimum inhibitory concentration that affect the bioconversion is another physiological characteristic that has to be explored.

Finally, other modes of cultivation can also be searched by maintaining a low glycerol concentration in the medium to avoid 3-HPA accumulation, and by extracting 3-HP as soon as it is produced, both with the aim to reduce bacterial inhibition. As the present study is included in a larger research program, it will be followed by the integration of the bioconversion process to an appropriate extraction process. More specifically, the coupling with in-stream liquid-liquid reactive extraction will be considered, in order to extract 3-HP as soon as it is produced. In that context, cells may face some new challenges such as the toxicity of the extraction phase made of solvents towards microorganisms. A recent study already described a first approach of the concomitant production and extraction of 3-HP by *L. reuteri* DSM 17938 (Sánchez-Castañeda *et al.*, 2020) that should be further broaden, by using a continuous mode of operation. A modeling approach could also be considered, by associating simultaneously, in a single model, the production and the extraction operations.

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Appendix

Appendix 1
DNA extraction of *Lactobacillus reuteri*

1. Principle of the method

The extraction of the DNA is performed by cell lysis, firstly by enzymatic lysis, then by mechanical lysis. Then the DNA is separated from the cellular components by the action of phenol and chloroform and the residual RNA is removed. The DNA is finally purified and concentrated using a column purification kit.

2. Chemicals

Product	Provider
Guanidine thiocyanate	Sigma-Aldrich
N-Lauryl-Sarcosine	Sigma-Aldrich
SDS (Sodium dodecyl sulfate)	Sigma-Aldrich
Tris-HCl 1M pH 7,8	Sigma-Aldrich
Sucrose	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Lyticase	Sigma-Aldrich
Proteinase K	VWR
Water BPC Grade	Sigma-Aldrich
Chloroform	VWR
Phenol chloroform pH 8	VWR
Ribonuclease A	Sigma-Aldrich

3. Materials

- Tubes PCR grade (Sarstedt)
- Eppendorf tubes
- Grinding beads zirconium 0.1 mm (BioSpec)
- Grinding beads zirconium 0.5 mm (BioSpec)
- Vortex
- Water bath
- Crusher (Precellys Evolution, Bertin Ozyme)
- Dry-bath (Thermostat Plus, Eppendorf)
- Centrifuge (5415R Eppendorf)

– Kit Genomic DNA Clean & Concentrator-10" Zymo Research

4. Preparation of solutions

4.1. Preparation of guanidine thiocyanate 4M, Tris-HCl, pH 7.8 0.1 M

- Tris-HCl solution
  Tris-HCl 1 M pH 7.8 was diluted 1/10 in MilliQ water.
- Guanidine thiocyanate 4M, Tris-HCl, pH 7.8, 0.1 M
  47.44 g of guanidine thiocyanate were dissolved in sterile Tris-HCl, 0.1 M to reach 100 mL.

4.2 Preparation of N-Lauryl sarcosine 10%

2 g of N-Lauryl sarcosine were dissolved in MilliQ water to reach 20 g.

4.3 Preparation of the lysozyme-lyticase-TES solution

- Preparation of TES (Tris 50 mM – EDTA 1 mM – Sucrose 6.7 %- pH 8)
  0.030 g EDTA, 0.606 g Tris and 6.7 g sucrose were dissolved in Water BPC Grade to reach 100 mL. The solution was stored at room temperature.
- Preparation of lyticase solution
  The lyticase solution was prepared at 5 000 U/mL by diluting 10 times the commercial solution in TES.
- Preparation of lysozyme-lyticase-TES solution
  600 mg of lysozyme and 4 mL of lyticase solution at 5 000 U/mL were mixed with TES to reach 15 mL. After homogenization, the solution was aliquoted in Eppendorf tubes and stored at -20 °C.

4.4 Preparation of SDS 20%

5 g of SDS were weighted and dissolved in Water BPC Grade to reach 25 g. The solution was stored at room temperature.

4.5 Preparation of proteinase K 14 mg/mL

140 mg of proteinase K were dissolved in 10 mL of Milli-Q water. Aliquots of 1 mL were prepared in Eppendorf tubes and stored at – 20 °C.

4.6 Preparation of SP buffer

- Preparation of solution $Na_2HPO_4.12H_2O$ 0.2M solution
  2.8 g of $Na_2HPO_4.12H_2O$ were dissolved in MilliQ water to reach 100 mL.
- Preparation of $NaH_2PO_4.H_2O$ 0.2M solution
  2.76 g of $NaH_2PO_4.H_2O$ 0.2 M were dissolved in MilliQ water to reach 100 mL.
- Preparation of SP buffer
  47.35 mL of $Na_2HPO_4.12H_2O$ 0.2M solution were mixed with 2.65 mL of $NaH_2PO_4.H_2O$ 0.2M solution and added with 50 mL MilliQ water.

4.7 Preparation AE buffer

0.68 g of sodium acetate 50 mM and 0.37 g of EDTA 10 mM were dissolved in MilliQ water to reach 100 mL.

4.8 Preparation of RNaseA 20 mg/mL

In an Eppendorf tube, 20 mg of ribonuclease A were dissolved in 1 mL of TE (1X). The solution was aliquoted by 200 µL in Eppendorf tubes and stored at -20°C.

5. Operating mode

The DNA extraction method was adapted from the method described by (Monnet *et al.*, 2006).

5.1 Sample preparation

After 10 min of centrifugation at 10 000 rpm (Eppendorf centrifuge 5415R), the cell pellets were re-suspended into 250 µL of guanidine thiocyanate (4 M) in Tris-HCl (pH 7.8, 0.1 M) and 40 µL of sodium lauryl sarcosine (100 g/L). They were transferred into a 2-mL bead-beating tube that contained 500 mg of zirconium beads (mixture at 50/50 of 0.1 and 0.5 mm beads). The samples were homogenized using a vortex.

5.2 Enzymatic lysis

After the addition of 75 µL of lysozyme 3 mg – lyticase (20 µl of 5000 U/mL solution) in TES buffer (55 µL), the samples was vortexed and incubated in a water-bath à 37°C during 30 min. Then 40 µL of proteinase K (14 mg/mL) and 100 µL of sodium dodecyl sulfate (200 g/L) were added and the tubes were incubated for 30 min in a water bath at 55°C.

5.3 DNA separation

The tubes were added with 200 µL of SP buffer (phosphate buffer), 200 µL of AE buffer (sodium acetate and EDTA buffer) and 500 µL of phenol-chloroform (beforehand balanced at pH8). They were shaken in the bead beater (Precellys Evolution) for a 45-s mixing sequence at a speed of 10,000 rpm. After incubation at 55 °C for 2 minutes (dry bath Thermostat Plus Eppendorf) they were cooled on ice. The mixing sequence was repeated, incubated at 70 °C for 2 minutes (dry bath Thermostat Plus Eppendorf) and cooled on ice for 2 min. The tubes were centrifuged at 13,000 rpm for 30 minutes at 20°C (Eppendorf centrifuge 5415R). The aqueous (upper) phase was transferred to a 2 mL Phase Lock Gel tube to improve separation between the aqueous and organic phases. After addition of 500 µL of phenol-chloroform and gently mixing, the tubes were centrifuged at 13,000 rpm for 5 minutes at 20°C (Eppendorf centrifuge 5415R). The previous operation was repeated with 500 µL of chloroform. The aqueous phase (500 µL) was collected and transferred to a 2 ml Eppendorf tube.

5.4 Ribonuclease

The samples were added with 2 µL of RNase A (20 mg/ml) then incubated 30 min à 37°C (dry bath Thermostat Plus Eppendorf).

5.5 Column purification “Genomic DNA Clean & Concentrator-10” Zymo research

First 96 mL of 100% ethanol (or 104 mL of 96 % ethanol) were added to 24 mL of DNA Wash Buffer.

In the tube containing the aqueous phase, 2 volumes of ChIP DNA Binding Buffer were added to 1 volume of genomic DNA. After homogenization, the mix was transferred in the Zymo-Spin IC-XL column placed in a collecting tube. It was centrifuged for 30 sec and the eluate was eliminated. This operation is repeated until the entire volume of mix DNA/Buffer was passed through the column. 200 µL of DNA Wash Buffer was introduced in the column, then centrifuged for 1 min. This step was repeated for a second time. Then, 40 µL of Water BPC Grade was added directly in the background of the column that was maintained for 1 min at room temperature. The column was transferred in an Eppendorf tube and centrifuged for 20 seconds to eluate the DNA.
The DNA was then quantified using a Nanodrop spectrophotometer or a Qubit fluorimeter.

Appendix 2

Elemental analysis of *L. reuteri* DSM 17938 and complex media

1. MRS medium

Element	Measure (%)
Carbon	37.87
Hydrogen	6.26
Nitrogen	6.40
Oxygen	41.55
Sulfur	0.83

General formula: $CH_{1.98}O_{0.82}N_{0.14}S_{0.01}$

2. Yeast extract

Element	Measure (%)
Carbon	38.76
Hydrogen	6.51
Nitrogen	10.93
Oxygen	34.79
Sulfur	0.55

General formula: $CH_{2.02}O_{0.67}N_{0.24}S_{0.01}$

3. Phytone peptone

Element	Measure (%)
Carbon	39.17
Hydrogen	6.03
Nitrogen	10.02
Oxygen	37.24
Sulfur	2.12

General formula: $CH_{1.85}O_{0.71}N_{0.22}S_{0.02}$

4. *L. reuteri* DSM 17938

Element	Measure (%)
Carbon	40.28
Hydrogen	6.36
Nitrogen	11.03
Oxygen	33.18
Sulfur	0.48

General formula: $CH_{1.89}O_{0.62}N_{0.24}$

Appendix 3

Production of 3-HPA for HPLC analysis

As 3-HPA is not commercially available, it was synthesized through a one-step reaction from 1,2,4-butanetriol (Sigma-Aldrich) at URD ABI-AgroParisTech (Pomacle, France). The method was adapted from that of (Burgé *et al.*, 2015a).

The substrate 1,2,4-butanetriol (1.0 g) was dissolved in tetrahydrofuran or 1,4-dioxane (0.05 M, Sigma-Aldrich) in acetone (190 mL). The mixture was then added with 4.7 mL water and 8.0 g sodium periodate (Sigma-Aldrich) and magnetically stirred at 1,100 rpm at room temperature. The reaction was followed by thin layer chromatography with ethyl acetate/methanol (9:1, v/v) until completion. The crude mixture obtained was then filtered on Celite® (VWR), then washed with 1,4-dioxane. The solvents were then removed by vacuum evaporation for about 2 h. Subsequently, purification of the crude chemical was performed by flash chromatography in 100 % ethyl acetate that allowed obtaining a colorless viscous liquid that was checked as pure 3-HPA.

The synthesized 3-HPA was stored in dark vials at room temperature.

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

Titre : Optimisation de la bioconversion du glycérol en acide 3-hydroxypropionique (3-HP) par *Lactobacillus reuteri* en bioréacteur

Mots clés : Acide 3-hydroxypropionique, *Lactobacillus reuteri*, plan d'expériences, bioconversion du glycérol, fed-batch

Résumé : L'acide-hydroxypropionique (3-HP) est une molécule "plateforme" dont la production par voie biologique implique la bactérie *Lactobacillus reuteri*, selon un procédé en deux étapes : une croissance sur glucose en mode "batch" et une bioconversion du glycérol en 3-HP en mode "fed-batch". Dans le but d'améliorer la production de 3-HP, la thèse a pour objectifs (1) d'identifier les conditions nutritionnelles et environnementales mises en œuvre lors de la croissance permettant d'améliorer la production ultérieure du 3-HP et (2) de rechercher les conditions opératoires de la phase de bioconversion permettant d'augmenter les performances de production. Les conditions de croissance affectant la production de la biomolécule ont été déterminées grâce à un plan d'expériences de Plackett et Burman impliquant 11 facteurs. La supplémentation du milieu de croissance avec de la vitamine B12 et de la cystéine a montré un effet négatif sur la production ultérieure de 3-HP. L'addition de glucose, phytone peptone, Tween 80, 1,2-propanediol et bétaïne dans le milieu de culture, associée à un pH optimal de 6,0 pendant la croissance ont permis d'accroître la production de 3-HP et la durée de la bioconversion. L'ajout d'extrait de levure et le type de neutralisant utilisé n'ont démontré aucun effet. Néanmoins, les résultats ayant été obtenus à un débit d'alimentation en glycérol fixé à 0,5 $g \cdot h^{-1}$, l'amélioration s'est trouvée limitée. Les conditions de la bioconversion ont alors été étudiées selon un plan composite centré isovariant par rotation qui a permis de tester différents niveaux de pH et de vitesse spécifique d'alimentation en glycérol. Par l'établissement de surfaces de réponses, des conditions optimales, différentes selon la variable considérée (concentrations, rendements, vitesses), ont été identifiées. Des essais de validation ont permis de vérifier la pertinence des conditions retenues, avec une amélioration significative de la productivité du bioprocédé de 96 %. Finalement, le pH et le niveau d'énergie intracellulaires sont proposés comme des marqueurs de la dégradation des capacités de production de 3-HP.

Title : Optimization of glycerol bioconversion into 3-hydroxypropionic acid (3-HP) by *Lactobacillus reuteri* in bioreactor

Keywords : 3-Hydroxypropionic acid, *Lactobacillus reuteri*, experimental designs, glycerol bioconversion, fed-batch

Abstract : 3-Hydroxypropionic acid (3-HP) is a platform molecule whose biological production involves the bacterium *Lactobacillus reuteri*, according to a two-step process: a growth phase in batch mode on glucose, then a glycerol bioconversion into 3-HP in fed-batch mode. With the objective of improving the 3-HP bioproduction, the thesis aims at (1) identifying the nutritional and environmental conditions implemented during the growth phase to improve the subsequent production of 3-HP and (2) searching the operating conditions during the bioconversion phase that increase the 3-HP bioproduction performance. Growth conditions that influence the subsequent 3-HP production were determined using a Plackett and Burman experimental design involving 11 factors. Supplementing the growth medium with vitamin B12 and cysteine showed a negative effect on this subsequent production. The addition of glucose, phytone peptone, Tween 80, 1,2-propanediol and betaine to the culture medium, combined with an optimal pH of 6 during growth, enhanced the subsequent production of 3-HP and the bioconversion duration. The addition of yeast extract and the type of neutralizer showed no significant effect. However, since these results were obtained at a glycerol feeding rate value set at 0.5 $g\ h^{-1}$, the process improvement was limited. The bioconversion conditions were then studied according to a central composite rotatable design that allowed testing various pH levels and specific glycerol feeding rates. By establishing response surfaces, optimal conditions have been identified, that were different depending on the considered variable (concentrations, yields, rates). Additional validation experiments made it possible to verify the relevance of the selected conditions, with a significant improvement of the bioprocess productivity by 96 %. Finally, intracellular pH and energy level were proposed to explain the degradation of 3-HP production ability.

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