Universidade de Aveiro Departamento de Química
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MARIA JOÃO PINHO MOTA

FERMENTAÇÃO MICROBIANA DE GLICEROL PARA PRODUÇÃO DE COMPOSTOS DE VALOR-ACRESCENTADO SOB ALTA PRESSÃO

MICROBIAL FERMENTATION OF GLYCEROL FOR PRODUCTION OF VALUE-ADDED COMPOUNDS UNDER HIGH PRESSURE

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química Sustentável, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro, e da Professora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, pelo incansável apoio ao longo de todas as etapas da minha vida.

o júri

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palavras-chave Microrganismos, fermentação, glicerol, *stress*, adaptação, alta pressão.

resumo

A conversão microbiana de glicerol em compostos de valor-acrescentado constitui uma aplicação promissora para o excesso de glicerol gerado pela indústria do biodiesel. Uma possível estratégia para melhorar estes processos passa pelo uso de níveis sub-letais de Alta Pressão (AP) ao longo da fermentação, de modo a estimular o crescimento dos microrganismos fermentativos e/ou aumentar a velocidade e os rendimentos do processo. Assim, o presente trabalho teve como objetivo o estudo da aplicação de AP (entre 10 e 50 MPa) em dois processos fermentativos: um deles com a *Lactobacillus reuteri*, que produz 1,3-propanediol a partir de glicerol; e outro com a *Paracoccus denitrificans*, que produz polihidroxialcanoatos a partir de glicerol.

No caso da fermentação com a *L. reuteri*, a produção de 1,3-propanediol foi estimulada a 10 MPa, resultando em aumentos de rendimento e produtividade de ≈ 11 e 12 %, respetivamente, relativamente às amostras à pressão atmosférica. A adaptação da *L. reuteri* à pressão foi ainda avaliada durante quatro ciclos de fermentação consecutivos. Após o quarto ciclo a 10 MPa, a concentração de 1,3-propanediol aumentou 15 % relativamente ao respetivo ciclo à pressão atmosférica, e ainda mais (52 %) relativamente à “abordagem convencional”, i.e. sem os ciclos de fermentação, à pressão atmosférica. Estes resultados confirmam que diferentes estratégias de aplicação de AP podem aumentar a produção de 1,3-propanediol. De modo a compreender os efeitos dos ciclos de pressão no metabolismo da *L. reuteri*, realizou-se um estudo metabolómico comparativo entre amostras de fermentação a diferentes condições. Foram observados perfis metabólicos distintos de acordo com a pressão aplicada e com o ciclo fermentativo, sendo que o efeito adaptativo ao longo dos ciclos foi mais acentuado a 10 MPa. Estes resultados fornecem informação relevante relativa à adaptação de bactérias ácido-láticas a pressões sub-letais.

Numa fase inicial do estudo da fermentação de glicerol pela *P. denitrificans*, analisou-se o efeito da disponibilidade de ar no processo, uma vez que este corresponde a um parâmetro crítico que afeta o crescimento celular e o perfil metabólico deste microrganismo, e poderá ser limitado durante a fermentação sob pressão. De facto, amostras com elevada disponibilidade de ar mostraram crescimento celular considerável, mas ausência de produção de etanol ou ácidos orgânicos. Por outro lado, amostras sem ar mostraram reduzido crescimento celular, mas atividade metabólica ativa, com produção de etanol, succinato e acetato. Relativamente ao estudo do efeito da pressão, a *P. denitrificans* mostrou capacidade para crescer a 10, 25 e 35 MPa, ainda que não tão acentuadamente quanto à pressão atmosférica. O uso da AP promoveu alterações nos perfis fermentativos da *P. denitrificans*. Para além disso, a AP afetou a produção de polihidroxialcanoatos, resultando em concentrações de polímero mais baixas, mas conteúdos de polímero em biomassa (%) superiores, o que indica uma maior capacidade de acumular

palavras-chave Microrganismos, fermentação, glicerol, *stress*, adaptação, alta pressão.

resumo

este composto no interior das células. Sob determinados níveis de pressão, os polímeros formados apresentaram modificações na sua composição monomérica, relativamente aos obtidos à pressão atmosférica. É possível antever que estas alterações poderão afetar as propriedades físicas e mecânicas do polímero.
Em suma, o presente trabalho revelou diferentes caraterísticas metabólicas da *L. reuteri* e da *P. denitrificans*. Os resultados obtidos mostraram ainda que, quando aplicada em condições adequadas, a tecnologia de AP poderá apresentar-se como uma ferramenta útil para modificar e até melhorar processos de fermentação de glicerol, bem como os respetivos produtos. Este estudo abre também os horizontes para o uso desta tecnologia no estímulo de outros processos fermentativos que sejam relevantes para a indústria biotecnológica.

keywords Microorganisms, fermentation, glycerol, stress, adaptation, high pressure.

abstract

Microbial conversion of glycerol into value-added products constitutes a promising approach to dispose the excess glycerol generated by the biodiesel industry. A possible strategy to improve these processes is the performance of fermentations under sub-lethal high pressure (HP), which may stimulate cell growth and/or increase fermentation rates and yields. Therefore, the goal of the present work was to study the application of HP (in the range of 10 to 50 MPa) on two different microbial processes: one of them with *Lactobacillus reuteri*, which is able to produce 1,3-propanediol from glycerol; and the other with *Paracoccus denitrificans*, which is able to produce polyhydroxyalkanoates from glycerol.

In the case of *L. reuteri* fermentation, the production of 1,3-propanediol was stimulated at 10 MPa, resulting in yield and productivity improvements of ≈ 11 and 12 %, respectively, relatively to the same samples at atmospheric pressure. Adaptation of *L. reuteri* to pressure was assessed during four consecutive fermentation cycles. After the fourth cycle at 10 MPa, 1,3-propanediol titers increased 15 % relative to the respective cycle at atmospheric pressure, and even more (52 %) relative to the "conventional approach", i.e. without the fermentation cycles, at atmospheric pressure. These results confirm that different strategies of HP application may stimulate the production of 1,3-propanediol. To better understand the effects of these HP-cycles on the *L. reuteri* metabolism, a comparative metabolomic study between *L. reuteri* fermentation samples was performed. The results showed distinct metabolic profiles according to the pressure applied and the fermentation cycles. The adaptive effect throughout the cycles was considerably more accentuated at 10 MPa. These results unveil relevant information regarding the adaptation of lactic acid bacteria to sub-lethal HP.

Regarding glycerol fermentation by *P. denitrificans*, it was necessary to give special focus on air availability, since this is a critical parameter that affects cell growth and metabolic profile, and it may be limited during fermentation under HP conditions. Samples with higher air availability showed considerable cell growth, but no production of ethanol, acetate and succinate. On the other hand, samples without air had lower cell growth, but active metabolic activity (with the production of organic acids and ethanol). Regarding the experiments of fermentation under HP, *P. denitrificans* was able to grow at 10, 25 and 35 MPa, but at lower extent compared to atmospheric pressure. Application of HP promoted modifications in the *P. denitrificans* fermentative profiles at different pressure levels. In addition, HP was found to affect polyhydroxyalkanoate production, resulting in lower titers, but higher polymer content in cell dry mass (%), which indicates higher ability to accumulate these polymers in the cells. Some levels of HP also affected PHA monomeric composition, showing considerable differences relative to the ones obtained at atmospheric pressure. It is possible to foresee that these changes in polymer composition may also affect its physical and mechanical properties.

keywords Microorganisms, fermentation, glycerol, stress, adaptation, high pressure.

abstract Overall, the present work demonstrated new metabolic features of *L. reuteri* and *P. denitrificans*. The obtained results showed that HP technology (applied at specific levels) can be a useful tool to modify and possible improve glycerol fermentation processes and products. It also opens the possibility of using this technology to stimulate other fermentations relevant for the biotechnological field.

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LIST OF PUBLICATIONS

This thesis is based on the following scientific articles:

Fermentation at non-conventional conditions in food- and bio-sciences by application of advanced processing technologies
Mota, M. J.; Lopes, R. P.; Koubaa, M.; Roohinejad, S.; Barba, F. J.; Delgadillo, I.; Saraiva, J. A., Critical Reviews in Biotechnology 2018, 38, 122-140 (doi: 10.1080/07388551.2017.1312272).

***Lactobacillus reuteri* growth and fermentation under high pressure towards the production of 1,3-propanediol**
Mota, M.J., Lopes, R.P., Sousa, S., Gomes, A.M., Delgadillo, I., Saraiva, J.A., Food Research International 2018, 113, 424-432 (doi: 10.1016/j.foodres.2018.07.034).

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During my studies, I have also contributed to the following scientific articles that are not included in this thesis:

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LIST OF ABBREVIATIONS

1,3-PDO	1,3-propanediol
2,3-BDO	2,3-butanediol
3-HP	3-hydroxypropionic acid
3-HPA	3-hydroxypropionaldehyde
ATP	adenosine triphosphate
CDW	cell dry weight
DHA	dihydroxyacetone
DNA	deoxyribonucleic acid
EMP	Embden–Meyerhof–Parnas
FDR	false discovery rate
FID	free induction decay
GC	gas chromatography
GRAS	generally recognized as safe
HP	high pressure
HPLC	high-performance liquid chromatography
HSPs	heat-shock proteins
LAB	lactic acid bacteria
LC	liquid chromatography
LDH	lactate dehydrogenase
mcl-PHA	medium chain length polyhydroxyalkanoates
MEF	moderate electric fields
MS	mass spectrometry
NADH/NAD^+	nicotinamide adenine dinucleotide
NADPH/$NADP^+$	nicotinamide adenine dinucleotide phosphate
MRS	de Man, Rogosa and Sharpe
NMR	nuclear magnetic resonance
OH	ohmic heating
PCA	principal component analysis
pdu	propanediol-utilization operon
PEF	pulsed electric fields
PEP	phosphoenolpyruvate
PHA	polyhydroxyalkanoates
PLS-DA	partial least squares discriminant analysis

PTT	polytrimethylene terephthalate
RI	refraction index
scl-PHA	short chain length polyhydroxyalkanoates
TCA	tricarboxylic acid
tmRNA	transfer-messenger ribonucleic acid
TSP-*d4*	3-(trimethylsilyl)propionic-2,2,3,3-*d4* acid
US	ultrasound
UV	ultraviolet

CHAPTER I

Fermentation at non-conventional conditions and its application to food- and bio-sciences: A literature revision

Adapted from:

Mota, M.J., Lopes, R.P., Koubaa, M., Roohinejad, S., Barba, F.J.; Delgadillo, I., Saraiva, J.A., 2018. Fermentation at non-conventional conditions in food- and bio-sciences by application of advanced processing technologies. Crit. Rev. Biotechnol. 38, 122–140.

1.1. General overview

Microorganisms may show different reactions when exposed to stressful conditions. At more extreme stress conditions, cells are unable to withstand and adapt, leading to microbial destruction. Food pasteurization is based on this principle, since the main goal is to destroy vegetative microbial contaminants in order to assure food safety and further preservation. In contrast, when mild stress conditions are applied (at sub-lethal levels), the microorganisms may be able to survive due to the activation of specific stress response mechanisms, as well as possible adaptation to the new conditions (Huang et al., 2014; Lado and Yousef, 2002). Stress sensing systems and defenses are developed by microorganisms to allow them to withstand extreme conditions and sudden environmental changes. Bacterial stress responses rely on the coordinated expression of genes that alter different cellular processes (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.) to improve the stress tolerance (Storz and Hengge, 2000; van de Guchte et al., 2002). As a consequence, several changes in microbial morphology and metabolic reactions are also observed (Huang et al., 2014). Some of the stress-induced genes seem to be genuinely specific, while others are induced by a wide variety of stresses and are thought to be general stress response genes (De Angelis et al., 2001; Serrazanetti et al., 2009). Responses are different and vast and depend on the species, strain, and the type of stress applied (Mills et al., 2011; Serrazanetti et al., 2009; van de Guchte et al., 2002; Zamfir and Grosu-Tudor, 2014).

The implications and the involvement of the metabolic processes in the stress responses could affect fermentations, possibly resulting in improvements of bioprocesses and bioproducts relevant for different industries (Serrazanetti et al., 2009). As a result, the concept of performing fermentations at non-conventional conditions is arising, and it is based on the use of emerging processing technologies (typically applied for food pasteurization). Pulsed electric fields (PEF), moderate electric fields (MEF), ohmic heating (OH), ultrasound (US) and high pressure (HP) are therefore applied at sub-lethal levels, at some point during the fermentative processes, affecting the growth and metabolism of the microbial strains involved in fermentation, but without causing microbial inactivation.

Several microorganisms were tested under non-conventional conditions, including both bacteria and yeasts, and ultrasound was the most studied technology for this purpose. Considering the promising results obtained so far, we foresee that this subject will become a research trend within the next few years in microbial biotechnology. We believe that there is a great potential to explore, since several fermentative processes with a prominent role in different industries could benefit from this approach. These industrially relevant processes include not only food fermentations (e.g. for the production of dairy products, alcoholic beverages, and others), but also

the production of commodity biochemicals (such as acetate, citrate, and ethanol) and high-value bioproducts (such as vitamins, antibiotics, and biopolymers).

The following section focused on the application of HP to different fermentation processes, while providing a perspective regarding the strengths and limitations of the HP equipment currently available for fermentation processes. The use of electric fields and US in fermentation was already reviewed by Galván-D'Alessandro and Carciochi (2018), and it was not addressed in detail in the present work, since they were not studied here.

1.2. Fermentation under high pressure conditions

High pressure is an emerging technology with increasingly successful industrial application as a non-thermal food pasteurization method (Barba et al., 2015b, 2012). The mechanisms of HP on microbial cells are already well understood, with identification of several effects on metabolism, physiology and structural organization (Bartlett, 2002). With the increasing pressure, relevant cell structures and functions are successively compromised until it turns impossible to withstand the stress and survive at these hostile conditions (Mota et al., 2013). In terms of cell structure, different organelles show different sensitivity to high pressure. For instance, lipid membranes are particularly pressure sensitive, because of its high compressible potential. Thus, changes in membrane composition and fluidity are observed under HP, as well as weakening of important protein-lipid interactions (Winter and Jeworrek, 2009). High pressure treatments may also affect the structure of DNA, ribosomes and proteins (Abe, 2007; Macgregor Jr, 2002; Niven et al., 1999), possibly leading to inhibition of cell processes (such as replication, transcription and translation) and metabolic reactions essential for cell maintenance.

The magnitude of cell damage by HP is highly dependent on several parameters, which include the level and duration of the pressure treatments, the compression method and other environmental conditions (temperature, media composition, pH, etc.). In addition, each microbial strain has a specific degree of HP tolerance according to their intrinsic cellular characteristics. In general, prokaryotes are more HP-resistant than eukaryotes, Gram-positive bacteria are more HP-resistant Gram-negative bacteria, and cocci are more HP-resistant than bacilli (Huang et al., 2014). The cell growth stage was also found to affect the microbial tolerance to HP treatments, which is usually higher during the stationary phase than during the exponential phase. This can be explained by the lower stress tolerance of cells during the exponential phase, due to the continuous cell division and synthesis. In contrast, microorganisms in the stationary phase have complete cell structures, thus they are able to resist more severe stress levels (Huang et al., 2014; Patterson, 2005). Moreover, Hill et al. (2002) reported that the higher pressure resistance observed

during the stationary-phase is partially due to the synthesis of proteins that protect against a range of adverse conditions.

It is important to note that microorganisms are more likely to be stressed or injured than killed under HP, particularly when lower intensity treatments are applied (Huang et al., 2014). Several studies have found that microorganisms possess regulatory genes for environmental adaptation, generally involving the accumulation of heat-shock proteins (HSPs) within the cell to enhance the resistance to multiple environmental stresses (Lou and Yousef, 1997; Wemekamp-Kamphuis et al., 2004). Welch et al. (1993) reported that a HP treatment of 55 MPa induced a stress response by production of cold-shock proteins, heat-shock proteins, and other protective proteins. Hörmann et al. (2006) used a comparative proteome approach to characterize the HP effects on *Lactobacillus sanfranciscensis*, concluding that HP stress response uses subsets of other stress responses (such as cold and high salinity).

Application of sub-lethal HP treatments leads to possible acquisition of new desirable characteristics, obtained by inhibition or even suppression of some metabolic pathways and/or utilization of new ones (Mota et al., 2013). This concept is gaining relevance over the last years, since the piezo-tolerant strains may have numerous interesting applications in different fields (Aertsen et al., 2009; Hörmann et al., 2006). The main studies regarding HP application of microbial fermentation processes are indicated in Table 1.1.

Most studies regarding the effects of HP on microbial growth and metabolism were performed with *Saccharomyces cerevisiae*. Picard et al. (2007) observed that at 10 MPa, the fermentation of glucose to ethanol by *S. cerevisiae* proceeded 3-fold faster than control fermentation. In addition, at 5 and 10 MPa the fermentation yield was enhanced by 6 % and 5 %, respectively, compared to the control. However, at pressures above 20 MPa fermentation was slowed down, and the authors estimated that alcoholic fermentation was interrupted at 87 ± 7 MPa, possibly due to loss of activity by one or more enzymes involved in the glycolytic pathway. Similarly, Bravim et al. (2012) observed that pre-treatment of *S. cerevisiae* with HP led to an increase in ethanol content upon fermentation. A global transcriptional analysis revealed the over-expression of several genes related to cell recovery and stress tolerance induced by HP. The most relevant case was the gene SYM1, which was related to enhancement of ethanol production and increase of stress tolerance upon fermentation.

Trehalose and glutathione are two major stress-induced metabolites with industrial value, which could be produced under HP conditions. *S. cerevisiae* CICC1339 growing at 0.5 MPa showed an increase of 58.7% in glutathione concentration in comparison with the control cells at atmospheric pressure (Qiao et al., 2006a). Similarly, application of HP at 1 MPa on *S. cerevisiae* resulted in increasing the yield of trehalose by 82.9 % (Dong et al., 2007). These two products are usually present at low concentrations in microorganisms (Bachhawat et al., 2013), but their

production is stimulated under stressful conditions (such as HP), possibly as a mechanism for protection (Dong and Jiang, 2016).

Table 1.1. General effects of high pressure on microbial cell growth and fermentation.

Microorganism	Main effects	References
Saccharomyces cerevisiae	- Acceleration of alcoholic fermentation (up to 3-fold) - Increased ethanol yields (5-6 %) - Over expression of several genes related to cell recovery and stress tolerance, including the gene SYM1 - Increased glutathione concentration (58.7 %) - Increased trehalose yield (82.9 %)	Bravim et al. (Bravim et al., 2012); Dong et al. (2007); Picard et al. (2007); Qiao et al. (2006b)
Clostridium thermocellum	- Metabolic shift, with increased ethanol production and decreased acetate (by-product) production - Higher ethanol:acetate ratio	Bothun et al. (2004)
Gluconacetobacter xylinus	- Decreased cell growth - Decreased production of bacterial cellulose - Cellulose ribbons with profound morphological differences	Kato et al. (2007)
Streptococcus thermophilus, Lactobacillus bulgaricus, and Bifidobacterium lactis	- Decreased fermentation rate - At higher pressures, microorganisms metabolically inhibited - Bacterial strains still viable at lower pressures, with ability to produce yogurt	Mota et al. (2015)
Oenococcus oeni	- Fermentation with *O. oeni* during and after HP-stresses - Decreased concentrations of L-lactate - Increased concentrations of D-lactate	Neto et al. (2016)

Thermophilic bacterium *Clostridium thermocellum* efficiently converts cellobiose to biofuels and other chemicals, but also synthesizes several undesired co-products, such as organic acids (acetate, lactate) and gaseous end-products (H_2, CO_2), limiting the commercialization of biofuel produced by this microorganism (Béguin and Aubert, 1994; Bothun et al., 2004; Herrero et al., 1985; Wiegel, 1980). Application of pressure of 7 and 17 MPa on *C. thermocellum* redirected the fermentative metabolism from the production of organic acids (such as acetate) to ethanol, leading to a 60-fold increase in the ratio ethanol:acetate under HP compared to

atmospheric pressure. The authors explained these shifts with the increased concentration of dissolved gases at HP and the consequent modification of the metabolic pathways involving these product gases (Bothun et al., 2004).

High pressure might also be used to alter the properties of biopolymers produced by microorganisms (Aertsen et al., 2009). *Gluconacetobacter xylinus* has been used as a model organism for bacterial cellulose biosynthesis (Ross et al., 1991). It was demonstrated that *G. xylinus* cells remained viable and retained the ability to produce cellulose under HP (30, 60 and 100 MPa). However, a decrease in the count of viable cells was observed at 100 MPa, leading to a decrease in the amount of cellulose produced. Cellulose ribbons had profound morphological differences depending on the applied pressure conditions, since the cellulose fibers produced under HP had significantly higher density compared with the cellulose produced at atmospheric pressure (Kato et al., 2007). Nevertheless, the result of these morphological changes on the functional properties of the polymer still needs further investigation.

Recently, our research group evaluated the effect of HP (5-100 MPa) on lactate fermentation that occurs during probiotic yogurt production (Mota et al., 2015). We observed that HP reduced the fermentation rate, possibly due to the inhibitory effect of pressure on the growth of the microorganisms relevant to the process (*Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium lactis*). However, extension of the fermentation time at 5 MPa (used as a case study) yielded a typical pH for probiotic yogurt, showing that lactic acid fermentation can be performed at this pressure without compromising the viability of the bacterial strains (including the probiotic one) and their ability to produce yogurt. However, more studies are needed to understand the effects of HP on the nutritional and organoleptic properties of yogurt produced at these conditions.

It is also possible to apply HP-stresses only in the beginning of fermentation, with the remaining time taking place at atmospheric pressure. Neto et al. (2016) used this approach when evaluating the effect of different HP-stresses (50 MPa, 8 h; 100 MPa, 8 h; 300 MPa, 0.5 h) on *Oenococcus oeni* metabolism. This is a lactic acid bacterium employed by wine industry to perform malolactic fermentation. *Oenococcus oeni* was able to perform fermentation during and after HP-stresses of 50 and 100 MPa, although with some metabolic changes. Particularly, the HP-stress of 100 MPa lead to lower concentrations of L-lactate and higher concentrations of D-lactate, compared to the control. The HP-stress of 300 MPa, 0.5 h resulted in complete inactivation of *O. oeni*, but malolactic fermentation was still observed at some extent, showing that malolactic enzyme maintained some residual activity at these conditions.

All in all, the studies in literature reveal that HP could not only be applied intermittently, but can also be maintained during the whole fermentation time, without serious cell loss and no heating effect. Since there is no need of refrigeration, the energetic costs of the fermentative

process are lower, and the application of HP is simpler. This versatility can represent an advantage to HP-assisted fermentation, since it allows to adapt the application mode according to the specificities and requirements of each bioprocess.

1.2.1. Technological equipment suitable for fermentation processes

In this section, the most relevant aspects regarding HP-equipment for microbial growth and fermentation are briefly described. Since the 1990s, a number of equipment manufacturers have entered the HP industry, most of them starting from parallel sectors (such as water jet cutting and diamond manufacturing) with their experience. HP equipment and services industry have been expanded and have spread globally. At present, companies like HIPERBARIC and AVURE Technologies are dominating the market of industrial HP systems (Elamin et al., 2015).

It is difficult to compare different types of HP equipment because each one offers unique characteristics based on the operation parameters, such as range of operating pressure, temperature control, volume of the vessel or layout of system (Bermúdez-Aguirre and Barbosa-Cánovas, 2011). High pressure systems are typically made up of high strength steel alloys with high fracture toughness and corrosion resistance. The most suitable equipment for fermentation (Figure 1.1) includes a fermenter in a pressure vessel (thick-wall cylinder), in some cases with agitation, aeration, and temperature control.

The desired pressure in the pressure vessel is achieved through compression of pressure-transmitting fluid, using the combined action of a pump and an intensifier. The most commonly used pressure-transmitting fluid is water, but glycol, glycol and water, silicone oil, sodium benzoate solution or castor oil may also be used (Balasubramaniam et al., 2015). The specificities of the system may be dependent on the type of fermentation and the requirements of the microbial strain. For instance, aeration and agitation are highly beneficial for aerobic processes, while it can be dispensable for anaerobic fermentations. On the other hand, temperature control is usually an essential feature of the equipment, since even slight variation in temperature may be enough to compromise microbial cell growth and fermentation. The pressure range allowed by this type of pressure systems is usually low, since these fermentation applications do not require high pressure levels.

It is important to consider that it may be unpractical (and expensive) to acquire a HP equipment that is specific for cell growth and fermentation experiments. Therefore, some of the studies in literature were performed in different equipment layouts, with general features adapted for a wide range of HP experiments (food pasteurization and preservation, extractions, and many others). In order to perform fermentation studies in this type of systems, extensive process

optimization must be carried out, in attempt minimize the hurdles related to lack of agitation, aeration or temperature control.

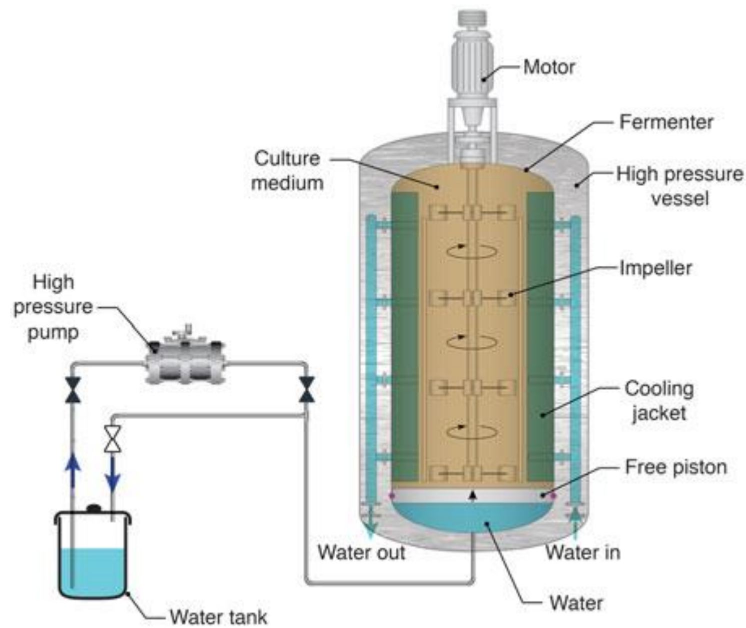

Figure 1.1. Schematic representation of microbial fermentation under high pressure used at laboratory scale. Reproduced from Mota et al. (2018) with permission of Taylor & Francis Group LLC in the format Thesis/Dissertation via Copyright Clearance Center.

High pressure systems have been highly studied and engineered in the last decades, resulting in considerable advances in the field. However, some challenges still remain, as the need to improve the strength of HP vessels and the capacity of the pumps, as well as the vessel's resistance to a high number of cycles. On the other hand, the main issue related to HP equipment for cell growth/fermentation is the adaptation to the particular and unique requirements of each microorganism and process. Therefore, these HP systems need to be highly adjustable, through the utilization of different modules and functions that should be adapted for each fermentation process.

1.3. Comparison between high pressure and other non-conventional technologies

Although many differences were observed between the technologies tested for this purpose, all of them seem to be suitable for application (under specific conditions) during the fermentative processes. Electric fields are able to promote beneficial effects to fermentation, but a careful optimization of process parameters is necessary to avoid loss of cell viability, since the application of electric fields (particularly in the case of high intensity PEF) are usually very aggressive to the

cells (Barba et al., 2015a; Varghese et al., 2014). In addition, the scaling up of PEF systems, their high initial cost, and the availability of commercial units are relevant drawbacks of this technology (Morales-de La Peña et al., 2011). In contrast, US are a well-studied technology, with several applications in different fields, and with lower equipment costs. A further benefit is that US is usually less aggressive to the cells, making this technology more feasible for application on fermentation processes (Chisti, 2003; Gogate and Kabadi, 2009). However, the application of electric fields or ultrasonication during fermentation implies an increase of temperature that affects the viability of the microbial strains and compromises the entire fermentative process. As a result, a refrigeration system is needed to control the temperature during the process, leading to higher energy requirements and thus higher energetic costs.

Therefore, HP seems to be particularly suitable for application to fermentation processes, since it is possible to use it continuously without inactivation problems or heat generation, in contrast to the other technologies. The application of sub-lethal HP has higher versatility, as it can be applied as stresses or continuously, without heating effects, leading to a higher variety of possibilities. Additionally, HP only needs energy to generate the pressure and no more energy is needed to maintain the pressure, and so application of HP stress during the whole fermentation process has also the advantage of not involving energetic costs.

Moreover, HP has interesting effects on living systems, offering great biotechnological potential. Such an example is the thriving of living organisms under deep-sea, since pressure increases with depth in the oceans, at an approximate rate of 10 MPa per km in the water column. The microorganisms living under these environments can be a valuable source of compounds with interesting biological activity and useful enzymes with novel properties. Consequently, these extremophiles are attracting the attention of several pharmaceutical companies, wishing to obtain novel compounds with biological activity. In fact, marine natural products used to drug development cover a very wide range of pharmacological effects (Newman and Cragg, 2004). From this perspective, fermentation under HP can be used to mimic the conditions present in deep-sea environments, challenging the microorganisms to respond and adapt, with possible production of several added-value products. Despite of the strong interest of pressure application on biological processes, the information in literature is very limited, possibly due to the low availability of HP systems and the high cost of the equipment, which correspond to the main challenges to a more widespread utilization of this approach.

Overall, the performance of fermentation under non-conventional conditions is still poorly explored, but the studies published so far suggest potential development within the next years, due to the wide biotechnological applications. At this point it is difficult to assess the economical feasibility of these methods, since there are many factors affecting the processes that still need better understanding and optimization. In addition, since most studies were performed at

laboratory-scale, the scale-up of these processes will be an important requirement to their implementation. Therefore, we expect that further work conducted in this field will focus on addressing these issues, as well as on the evaluation of these technologies on other fermentative processes with industrial relevance, mainly for non-food applications, which are considerably scarcer within this topic.

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CHAPTER II

Fermentation of glycerol for production of valuable bio-chemicals: A literature revision

2.1. The biodiesel industry and the glycerol surplus

Global energy demand is met by petrochemical sources, coal and natural gases. These sources are finite and the current high consumption rate may lead to depletion of fossil fuels. In addition to that, it is necessary to make great efforts to reduce emissions of sulphur, nitrogen and carbon oxides, which are released from the fossil fuel combustion and correspond to a fundamental source of global warming (Vivek et al., 2017). For these ecological and economic reasons, there is a growing awareness to look for fuels derived from renewable sources, such as biomass. In that context, biodiesel is one of the most promising alternatives to fossil fuels, since it is renewable, biodegradable, environmentally-friendly, and can be used directly in diesel engines without major modifications (da Silva et al., 2009; McNutt et al., 2017). It is manufactured from vegetable, plant derived oils or animal fat as feedstock, consisting of long chain alkyl esters. Biodiesel is chemically synthesized by transesterification of lipids with an alcohol in the presence of catalyst resulting in a mono alkyl ester (Vivek et al., 2017).

In United States and Europe, biodiesel is a fast growing product as their governments policies seek to spur the development of renewable transportation fuels (Lee et al., 2015). Global biodiesel production has significantly increased annually, achieving 22.7 million tons in 2012, and it is forecasted to reach 36.9 million tons by 2020 (Ciriminna et al., 2014; Katryniok et al., 2013; OECD, 2011; Sun et al., 2017). The fast growth of the biodiesel industry has created environmental and sustainability issues, such as the formation of glycerol as the major reaction by-product: in the transesterification process, 3 mol of fatty acid methyl esters and 1 mol of glycerol are synthesized. The glycerol by-product yields approximately 10 wt% of the total product (Lee et al., 2015). Due to the current increasing biofuel demand, worldwide production of glycerol climbed from 1 million tons in 2000 to 3 million tons in 2011 and is expected to reach 6 million tons by 2025 (Ciriminna et al., 2014; Katryniok et al., 2013; OECD, 2011; Sun et al., 2017). The increased availability of glycerol has caused a substantial reduction in its cost. According to a recent report, the price of crude glycerol is \$0.24/kg and that of pure grade glycerol is \$0.90/kg (Kumar and Park, 2018; San Kong et al., 2016). Effective and economic utilization of the enormous amounts of glycerol produced by the biodiesel industry is still very challenging (Khanna et al., 2012). Glycerol is traditionally used for many applications in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries (da Silva et al., 2009). However, it is essential to find alternative and sustainable applications for glycerol.

Extensive research has been conducted to investigate ways to utilize this surplus crude glycerol. The annual number of research articles addressing the use of glycerol has increased to >7000, doubling in number from the year 2000 to 2007 (Kumar and Park, 2018). One of the most popular approaches is the conversion of glycerol into high-value products by either biological or chemical transformations (Mattam et al., 2013). However, biological conversions are generally

preferred to chemical processes, due to the higher specificity of the reaction, lower temperature and pressure involved in the reaction process, and lower levels of chemical contaminants (Johnson and Taconi, 2007; Mattam et al., 2013; Vivek et al., 2017). The use of glycerol as substrate for microbial fermentation processes will be addressed in detail in the following sections.

2.2. Glycerol bio-conversion into valuable products

In the last decade, significant research efforts have been focusing on the use of glycerol as substrate for the production of a wide variety of valuable bio-chemicals, such as organic acids, alcohols, lipids and polymers (Mattam et al., 2013; Vivek et al., 2017). Some of the main products obtained from glycerol fermentation are indicated in Table 2.1, as well as the microbial strains usually involved in those processes.

Table 2.1. Some of the bioproducts obtained from glycerol in fermentation by different microorganisms.

Bioproduct	Main producing-microorganisms	References
Succinate	*Actinobacillus succinogenes; Anaerobiospirillum succiniciproducens; Basfia succiniciproducens*	Carvalho et al. (2014); Lee et al. (2001); Scholten et al. (2009)
Citrate	*Yarrowia lipolytica*	Rywińska and Rymowicz (2010)
Propionate	*Propionibacterium acidipropionici; Propionibacterium acnes; Clostridium propionicum; Propionibacterium freudenreichii* subsp. *Shermanii*	Barbirato et al. (1997); Wang et al. (2015); Zhu et al. (2010)
Lactate	*Escherichia coli; Lactobacillus rhamnosus; Enterobacter faecalis*	Hong et al. (2009); Murakami et al. (2016); Prada-Palomo et al. (2012)
1,3-Propanediol	*Clostridium butyricum; Klebsiella pneumonia; Citrobacter freundii; Lactobacillus reuteri; Lactobacillus brevis; Lactobacillus diolivorans; Lactobacillus panis*	Boenigk et al. (1993); Grahame et al. (2013); Lüthi-Peng et al. (2002); Menzel et al. (1997); Petitdemange et al. (1995); Pflügl et al. (2012); Vivek et al. (2016)
2,3-Butanediol	*Klebsiella pneumonia; Klebsiella oxytoca; Bacillus amyloliquefaciens*	Biebl et al. (1998); Metsoviti et al. (2012); Yang et al. (2013)
Butanol	*Clostridium pasteurianum*	Biebl (2001)
[a]PHA	*Cupriavidus necator; Burkholderia sacchari; Pseudomonas oleovorans; Pseudomonas putida; Zobellella denitrificans; Paracoccus denitrificans*	Ashby et al. (2004); Bormann and Roth (1999); Huijberts et al. (1992); Ibrahim and Steinbüchel (2010); Mothes et al. (2007); Rodríguez-Contreras et al. (2015)

[a]PHA: Polyhydroxyalkanoates

Because of its reduced nature, glycerol generates twice the number of reducing equivalents per carbon than do traditional carbohydrates (glucose, xylose, and sucrose) when converted into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate (Dharmadi et al., 2006). As a consequence, glycerol gives higher yields of reduced metabolites (e.g. succinate, ethanol, 1,3-propanediol, and butanol) than does glucose (Kumar and Park, 2018; Yazdani and Gonzalez, 2007) (Table 2.2).

Table 2.2. Comparison of maximum theoretical yield for production of different compounds from glycerol and glucose. Adapted from Kumar and Park (2018).

Bioproduct	[a]Maximum theoretical yield	
	Glucose (mol/0.5 mol)	**Glycerol (mol/mol)**
1,3-Propanediol	0.75	0.88
2,3-Butanediol	0.55	0.64
Ethanol	1.00	1.17
Succinate	0.86	1.00
Lactate	1.00	1.17
Acetate	1.50	1.75
Butanol	0.50	0.58

[a]The theoretical yields of metabolites were calculated on the basis of degree of reduction (Dugar and Stephanopoulos, 2011)

Although glycerol conversion is highly variable according to the microorganism, it is possible to define generalized metabolic pathways that are usually present in most glycerol-consumer strains. The initial step corresponds to the uptake of glycerol into the cells, which can happen passively since glycerol is a small and uncharged molecule, and thus permeable across the membranes (Johnson et al., 2016). However, cells that are limited to passive uptake have a growth disadvantage at low glycerol concentrations (da Silva et al., 2009). Facilitated diffusion can be achieved by an integral membrane protein, the glycerol facilitator GlpF (Darbon et al., 1999; Heller et al., 1980; Voegele et al., 1993), which is able to selectively diffuse glycerol across the cellular membranes (Fu et al., 2000).

The generalized metabolism of glycerol in microorganisms was reviewed by Khanna et al. (2012). It mainly occurs by two distinct and parallel pathways, oxidative and reductive (Yazdani and Gonzalez, 2007), both shown in Figure 2.1. Some microorganisms, such as the members of Enterobacteriaceae family, such as *Klebsiella*, *Citrobacter* and *Clostridium* exhibit both oxidative and reductive pathways.

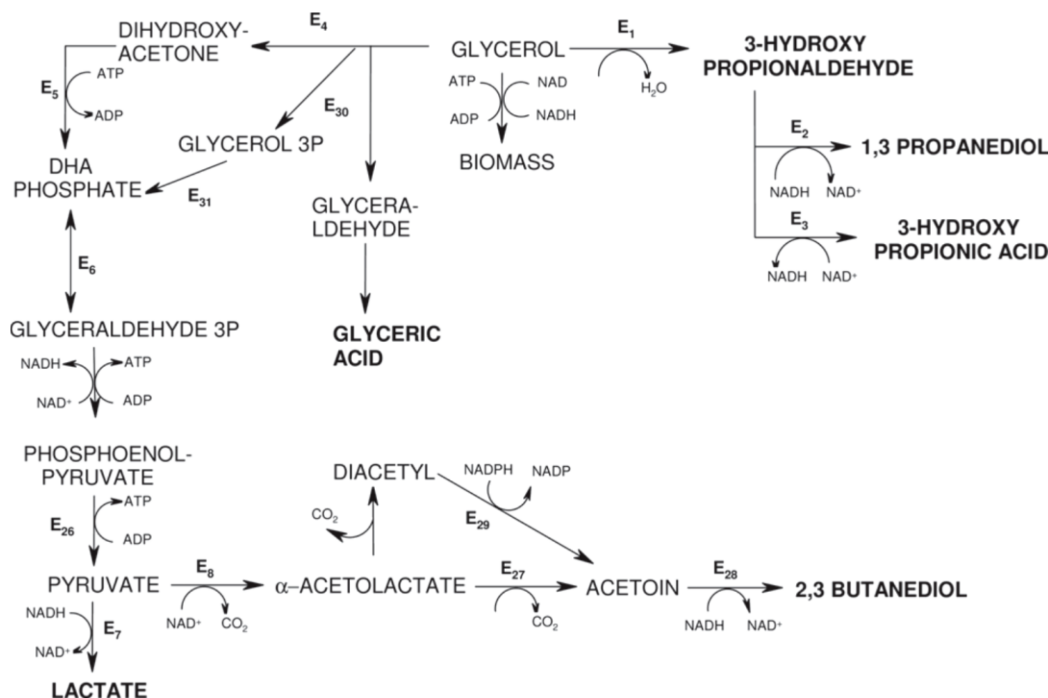

Figure 2.1. Metabolic pathway of glycerol bioconversion: oxidative and reductive wings of glycerol assimilation (E1–Glycerol dehydratase; E2–1,3 PDO dehydrogenase; E3–Aldehyde dehydrogenase; E4–Glycerol dehydrogenase type 1; E5–Dihydroxy acetone kinase; E6–Triose phosphate isomerase; E7–Lactate dehydrogenase; E8–Pyruvate decarboxylase; E26–Pyruvate kinase; E27–α-Acetolactate decarboxylase; E28–Acetoin reductase; E29–Diacetyl reductase; E31–Glycerol 3-phosphate dehydrogenase). Reproduced from Khanna et al. (2012) with permission of Taylor & Francis Group LLC in the format Thesis/Dissertation via Copyright Clearance Center.

The reductive pathway proceeds by the dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA), also called as reuterin, in presence of glycerol dehydratase. The 3-HPA is then reduced to 1,3-propanediol by a NADH-dependant 1,3-propanediol dehydrogenase, regenerating NAD^+ for the oxidative branch. 3-Hydroxypropionaldehyde can be further oxidized to 3-hydroxypropionic acid by NAD-linked aldehyde dehydrogenase (Khanna et al., 2012).

A parallel oxidative branch produces dihydroxyacetone and ultimately channels glycerol to Embden–Meyerhof–Parnas (EMP) pathway for the production of a number of important metabolites, such as ethanol, citrate, succinate, lactate, 2,3-butanediol, and others. The oxidative wing progresses by the oxidation of glycerol to dihydroxyacetone (DHA) by a NAD-linked type 1 glycerol dehydrogenase, that is further phosphorylated by an ATP-dependant DHA kinase to a glycolytic intermediate, DHA phosphate, which enters EMP pathway. The production of DHA

from glycerol oxidation is primarily observed in acetic acid bacteria, *Acetobacter* and *Gluconobacter.* These microorganisms are anaerobes, and hence, they lack glycolytic and Kreb's cycle enzymes and, as a consequence, further metabolism of DHA phosphate is not carried out by them (Khanna et al., 2012).

In aerobe microorganisms, DHA phosphate isomerizes to glyceralehyde-3-phosphate by triose phosphate isomerase. Glyceraldehyde-3-phosphate converts to phosphoenolpyruvate (PEP) through a series of steps and finally undergoes substrate level phosphorylation to form pyruvate by pyruvate kinase, generating ATP in the process. The pyruvate formed in the earlier step can follow different metabolic pathways (Khanna et al., 2012):

i) It can be reduced to lactate by NADH-dependant lactate dehydrogenase;

ii) It may undergo decarboxylation by pyruvate decarboxylase to form α-acetolactate, which is further decarboxylated to acetoin. α-Acetolactate may also decarboxylate to diacetyl, which in turn is reduced to acetoin by NADPH-dependant diacetyl reductase. The acetoin so formed, through any of the two routes, is reversibly reduced to 2,3-butanediol by NADH-dependant acetoin reductase (Syu, 2001);

iii) It may convert into formate in presence of pyruvate formate lyase, which is further cleaved to gaseous products H_2 and CO_2 by formate hydrogen lyase;

iv) It can be carboxylated by pyruvate carboxylase to oxaloacetate, which is further reduced by NADH-dependant malate dehydrogenase to malate. Malate then gradually forms fumarate by fumarase, and fumarate converts into succinate by fumarate reductase;

v) It can form acetyl CoA in presence of pyruvate dehydrogenase complex. Acetyl CoA is the precursor for the formation of acetate, butyrate, butanol and ethanol. Acetyl CoA can also enter in lipid biosynthesis pathway to produce triacylglycerols, also known as single cell oil.

Although the complete assimilation pathway of glycerol is rather complex, every step yields a particular product. The range of metabolites varies according to the microorganism and, particularly, the presence of an enzyme or a group of enzymes (Khanna et al., 2012).

2.3. Glycerol bio-conversion into 1,3-propanediol

1,3-Propanediol (1,3-PDO) is a diol monomer well known for numerous applications in cosmetics, solvents, adhesives, detergents, and resins (Vivek et al., 2017). Recently, this monomer has gained much attention in production of biodegradable polyester polytrimethylene terephthalate (PTT), having significant application in carpet and textile industry (Kaur et al.,

2012; Traub et al., 1995). It is also a GRAS (Generally Recognized as Safe) food and beverage ingredient, commercialized by DuPont Tate & Lyle Bio Products. It can be used as a flavor carrier, to reduce bitterness and enhance sweetness, or as a humectant, or processing aid. It is approved for use in different food products, such as seasonings and flavorings, confections and frostings, nuts and nut products, alcoholic beverages, and frozen dairy products (DuPont Tate & Lyle Bio Products, 2018). The global market of 1,3-PDO has increased considerably in recent years, due to the increasing market demand of its derivatives. While the global demand for 1,3-PDO was 60.2 kilotons in 2012, it is expected to reach approximately 150 kilotons in 2019 (Lee et al., 2015; Research TM, 2012).

The chemical synthesis of 1,3-PDO can be conducted by two main processes. The first one is "Degussa" (hold by DuPont Company) and implies catalytically oxidation of propylene to acrolein, which is hydrated next to 3-hydroxypropionaldehyde at medium pressure and temperature, followed by the hydrogenation to 1,3-PDO using a rubidium catalyst at high pressure. The second process carried out by Shell is based on oxidation of ethylene to ethylene oxide, followed by production of 3-HPA through the reaction called "hydroformylation" (also named "oxo synthesis") at high pressures (around 150 bar). The aldehyde extraction from the organic phase is performed using water, and the 3-HPA hydrogenation is conducted by using nickel as a catalyst under high pressure (Mitrea et al., 2017). However, these processes have numerous drawbacks, such as toxic intermediate products, the need to apply high pressures and temperatures, expensive non-renewable petroleum based catalysts, as well as moderate process efficiency and environmental noxiousness (Lin et al., 2005; Przystałowska et al., 2015; Raynaud et al., 2003). Therefore, biotechnology offers the potential to develop and/or to improve the production of these valuable compounds.

Currently, bio-based 1,3-PDO is produced by DuPont and Genencor International, Inc., by using a recombinant *Escherichia coli* strain, from glucose derived from corn as the sole carbon source (Jolly et al., 2014; Maervoet et al., 2010). The biological conversion of glycerol into 1,3-PDO is mainly attained by anaerobic bacteria fermentation process or micro-aerobic fermentation (Chen et al., 2003; Liu et al., 2007; Yang et al., 2007). The bacterial fermentation of glycerol to 1,3-PDO was first reported in 1881 by Zeng and Biebl (2002). Several members of the genus *Klebsiella* (Ji et al., 2009), *Citrobacter* (Boenigk et al., 1993), *Clostridium* (Petitdemange et al., 1995), *Lactobacillus* (Ricci et al., 2015), *Enterobacter* and *Shimwellia blattae* (Rodriguez et al., 2016) are reported in literature as wild type producers of 1,3-PDO from glycerol. Among all these microorganisms, *Clostridium butyricum* and *Klebsiella pneumoniae* are usually considered the best natural producers. For instance, in anaerobic fermentation with glycerol as the sole carbon source, *Clostridium butyricum* strains were observed to produce 1,3-PDO titers higher than 60 g L^{-1} (Wilkens et al., 2012), and *K. pneumoniae* strains were reported

to produce titers between 60 and 90 g L^{-1} (Durgapal et al., 2014; Zhao et al., 2009). However, *Klebsiella* family strains are classified as opportunistic pathogens and *Clostridium* family members are obligate anaerobes. In consequence, strict process control is required in fermentations with these microorganisms, thus making the process unattractive. Taking this into account, the members of the *Lactobacillus* genus offer great potential for production of 1,3-PDO from glycerol (Jolly et al., 2014). Some examples are indicated in Table 2.1, and include the utilization of *Lactobacillus reuteri*, *Lactobacillus panis*, *Lactobacillus diolivorans*, and *Lactobacillus brevis* for this purpose, achieving 1,3-PDO titers as high as 92 g L^{-1} (Lindlbauer et al., 2017).

2.3.1. Production of 1,3-propanediol by *Lactobacillus reuteri*

Lactic acid bacteria (LAB) are a well-known group of microorganisms that have been associated with humans throughout history. One important landmark was the first use of LAB as starter cultures for dairy production in 1890, which marked the beginning of industrial microbiology and established the industrial relevance of these microbial strains (Sauer et al., 2017). There are numerous advantages in using fermentation by lactic acid bacteria (LAB) for production of interest bio-based compounds. These include high carbon uptake rates, low biomass formation, high stress tolerance, as well as strictly regulated simple metabolic pathways that lead to a limited number of metabolites (Sauer et al., 2017). In addition, most members of LAB are non-pathogenic and generally regarded as safe (GRAS) and, in consequence, these microorganisms are not usually subjected to special legislative restrictions (Ricci et al., 2015).

Nevertheless, the utilization of LAB has remained mostly restricted to food and health, while their potential for the bio-based chemical industry is still unexploited. Several studies report that LAB have great potential for production of industrially valuable compounds, such as lactate, mannitol, or 1,3-PDO (Sauer et al., 2017). In this context, *Lactobacillus reuteri* presents itself as a LAB with high potential for bio-chemical production. It is a Gram-positive rod, facultative anaerobe and obligate heterofermentative microbe (Lüthi-Peng et al., 2002). It is able to produce 1,3-PDO from glycerol, using the 5 enzymes encoded in its propanediol-utilization operon (pdu), namely glycerol/diol dehydratase, propionaldehyde dehydrogenase, phosphotransacylase, propionate kinase and 1,3-PDO oxidoreductase (Sriramulu et al., 2008).

Lactobacillus reuteri uses glycerol only as an electron acceptor and not as a carbon source for growth. Even though *L. reuteri* is unable to grow on glycerol as the sole carbon source, the strain can be used in a co-fermentation of glucose and glycerol (Ricci et al., 2015). The general metabolic pathways of *L. reuteri* in a glycerol/glucose co-fermentation are represented in Figure 2.2.

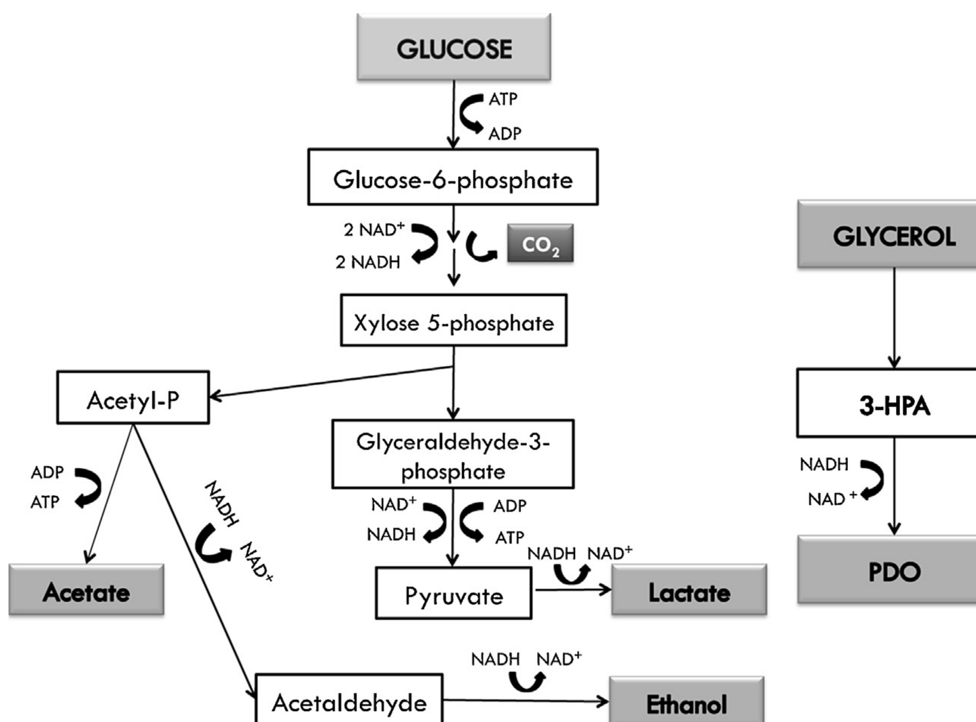

Figure 2.2. *Lactobacillus reuteri* metabolic pathways from glucose and glycerol. Reprinted from Vieira et al. (2015), with permission from Elsevier.

Glycerol enters the *L. reuteri* cell by the glycerol facilitator GlpF (Vivek et al., 2016) and fermentation occurs in two steps. In the first step, glycerol is dehydrated into 3-HPA by a coenzyme B12-dependent glycerol dehydratase (Burgé et al., 2015; Sardari et al., 2013a, 2013b; Sriramulu et al., 2008; Talarico and Dobrogosz, 1990). The second step involves the reduction of 3-HPA to 1,3-PDO by a NADH-oxidoreductase, requiring NADH (El-Ziney et al., 1998; Schütz and Radler, 1984; Talarico and Dobrogosz, 1989). Then, 3-HPA can also be oxidized into 3-hydroxypropinonic acid (3-HP) through a three-step reaction (Dishisha et al., 2014; Luo et al., 2011; Sabet-Azad et al., 2013). Sobolov and Smiley (1960) reported that 3-HPA was converted to equimolar quantities of 3-HP and 1,3-PDO by an aldehydic dismutation of 3-HPA, particularly when glycerol fermentation proceeded in the absence of sugar (glucose). On the other hand, 1,3-PDO production was elevated and 3-HP production was reduced in the presence of glucose concentrations (Kumar et al., 2013).

Therefore, glycerol/sugar co-fermentations are applied to stimulate cell growth and to avoid the formation of 3-HP instead of 1,3-PDO. During glycerol/glucose co-fermentation, glucose enters the glycolysis pathway, leading to the formation of various by-products, such as ethanol, lactate, acetate, and others (Doleyres et al., 2005). Although both substrates are metabolized by alternative pathways, the production of 1,3-PDO from glycerol is dependent on

glucose availability: as long as NADH is generated from glucose oxidative pathway, glycerol is used as the preferred electron acceptor leading to its enhanced consumption (Jolly et al., 2014). In addition, pyruvate obtained from the glycolysis pathway will compete with 3-HPA for NADH-oxidoreductase to form other by-products such as lactate (El-Ziney et al., 1998).

Some of the most relevant studies on 1,3-PDO production by *L. reuteri* co-fermentation (glycerol/sugar) are summarized in Table 2.3. In an attempt to increase 1,3-PDO production by *L. reuteri*, Ragout et al. (1996) first tested a co-fermentation process with a sugar (maltose) and glycerol. The results showed that *L. reuteri* changed its fermentation pattern to produce 4.4 g L^{-1} of 1,3-PDO as the major end product. Similarly, El-Ziney et al. (1998) observed that glucose/glycerol co-fermentation resulted in the production of 3-HPA and 1,3-PDO, at the expense of ethanol and lactate. More recently, several authors have been studying the co-fermentation of glucose/glycerol by *L. reuteri*. Baeza-Jiménez et al. (2011) obtained 1,3-PDO concentrations of 16.81 g L^{-1} in shake-flasks (100 mM glucose/200 mM glycerol), and 28.69 g L^{-1} in bioreactor studies (200 mM glucose/400 mM glycerol). Jolly et al. (2014) reached a 1,3-PDO concentration of 37.4 g L^{-1} after a batch fermentation with initial glucose and glycerol concentrations of 20 and 63.5 g L^{-1}, respectively. The authors also found that 1,3-PDO final concentrations and yields under unaerated conditions were close to those obtained under anaerobic conditions. Therefore, unaerated fermentation can be used to effectively produce 1,3-PDO in bioreactors, which is highly beneficial for large scale fermentation processes, since maintaining anaerobic conditions involves considerable costs.

Table 2.3. Main results of 1,3-PDO production of by *L. reuteri*, using glycerol as co-substrate.

Titer (g L^{-1})	Yield (g g^{-1})	Productivity (g L^{-1} h^{-1})	References
4.4	-	-	Ragout et al. (1996)
4.4	-	-	El-Ziney et al. (1998)
28.7	0.91	1.05	Baeza-Jiménez et al. (2011)
65.3	0.67	1.2	Jolly et al. (2014)
[a]9.8	[a]0.70	[a]4.92	Vieira et al. (2015)
46.0	0.74	0.66	Ricci et al. (2015)
2.5	0.36	0.07	Zaushitsyna et al. (2017)

[a]For bioreactor operated at continuous mode

Another important factor for this fermentation is the concentration of acetate during the process. Acetate is normally regarded as a product of anaerobic fermentation and it has been recognized as a microbial growth inhibitor. However, Iino et al. (2002) have stated that certain

acetate concentrations may have a positive influence on growth and fermentation of LAB. Jolly et al. (2014) observed that an initial acetate concentration of 5 g L^{-1} increased 1,3-PDO productivity, while concentrations above this value were found to be inhibitory. A possible explanation is related to the ability of acetate to act as an indirect proton and electron acceptor, leading to the activation of oxidative pathway at early stages of fermentation. However, at higher concentrations, acetate decreases the cells cytoplasmic pH. Since the cells expend energy to restore cytoplasmic pH, less energy is available for growth and bacterial metabolism (Heyndrickx et al., 1991; Jolly et al., 2014). Vieira et al. (2015) tested different bioreactor operation modes (batch, repeated batch and continuous) and each one was found to affect *L. reuteri* glycerol fermentation. The highest 1,3-PDO productivity (4.92 g L^{-1}) was achieved at continuous mode.

Ricci et al. (2015) tested the ability of different *L. reuteri* strains for the production of 1,3-PDO in a glucose/glycerol co-fermentation, and concluded that only the strain DSM 20016 produced a considerable 1,3-PDO concentration (10 g L^{-1}). In addition, this strain has some specific features, such as the production of 1,3-PDO closely linked to biomass accumulation. After optimization of substrate concentrations (0.4 M glucose, 0.8 M glycerol, in modified MRS without sodium acetate) and process variables (37 ºC, 200 rpm, and pH 5.5), the authors reached a 1,3-PDO concentration of 41 g L^{-1}, which is the highest reported so far for a batch fermentation with this microbial strain. Zaushitsyna et al. (2017) studied the use of *L. reuteri* as whole-cell biocatalyst for immobilization and conversion of glycerol into 3-HPA, 3-HP and 1,3-PDO. With that strategy, a 1,3-PDO concentration of 2.5 g L^{-1} was achieved.

Overall, the production of 1,3-PDO by *L. reuteri* from glycerol corresponds to a sustainable and strategic biotechnological approach, mainly due to three different aspects:

i) The use of glycerol as one of the substrates of the process;

ii) The use of a non-pathogenic and safe fermentative microorganism;

iii) The production of 1,3-PDO, a bio-chemical intermediate with broad applications, including in the production of biodegradable polyester polytrimethylene terephthalate (PTT).

On the contrary, the current main limitations of the process correspond to:

i) The use of glucose as a co-substrate;

ii) The need to carefully adjust several process variables to promote 1,3-PDO production.

Although there is no apparent straightforward strategy to overcome the former limitation, the latter can be more easily addressed by analyzing all variables and parameters that may affect the process, as well as their interaction. This would be the first step for further optimization of this promising biotechnological process.

2.4. Glycerol bio-conversion into polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) represent a complex class of naturally occurring bacterial polyesters that are synthesized intracellularly as carbon and energy reserve materials (Ashby et al., 2004). These reserve materials are synthesized when the microorganisms are grown under nutrient-limited conditions (Ashby et al., 2005), since they play a pivotal role in the long-term survival of bacteria under stressful conditions (Tan et al., 2014). Polyhydroxyalkanoates possess properties similar to various synthetic thermoplastics, such as polypropylene (Lee, 1996). However, PHA shows some unique properties, including biodegradability, biocompatibility, water resistance, and oxygen impermeability, and thus they can be used in a variety of disposable packaging goods (Tanadchangsaeng and Yu, 2012). They are also great candidates for high-valued applications in biomedical devices, e.g. surgical implants, sutures, meshes, or tissue engineering scaffolds (Brigham and Sinskey, 2012; Chen and Wu, 2005; Williams et al., 1999; Zinn et al., 2001). The intense research and commercial interest in PHA is evident from the rapid increment in PHA-related publications. For instance, Web of Science citation report (Thomson Reuters, New York, NY, USA) revealed that in the last 20 years, PHA-related documents have increased by almost 10-fold, while citations have increased by more than 500-fold, corresponding to an average citation count of about 1100 citations per year (Tan et al., 2014).

Industrial PHA production still has a high cost and cannot be economically competitive with conventional plastics (Brämer et al., 2001). The main factors that increase PHA production costs were reviewed by Choi and Lee (1999) and included PHA productivity, content and yield, the cost of the carbon substrate, and the recovery method applied. Therefore, the use of superior bacterial strains, inexpensive carbon sources, low-cost media, as well as efficient fermentation conditions and easier downstream processing could render PHA a commercially viable product (da Cruz Pradella et al., 2010). One of the most well studied strategies is the substitution of pure sugars (such as glucose or sucrose) by cheaper carbon sources as basis feedstock (Rodríguez-Contreras et al., 2015). Therefore, using glycerol as substrate for PHA production would be a good way to recycle a biodiesel by-product, and simultaneously reduce the high production cost of PHA (Tanadchangsaeng and Yu, 2012).

Depending upon the number of carbon atoms in the monomers, PHA are classified into two distinct groups: scl-PHA (short chain length PHA), consisting of 3-5 carbon atoms; and mcl-PHA (medium chain length PHA), consisting of 6-14 carbon atoms (Możejko-Ciesielska and Kiewisz, 2016). In both cases, the PHA biosynthetic pathways are intricately linked with the bacterium's central metabolic pathways including glycolysis, Krebs Cycle, β-oxidation, de novo fatty acids synthesis, amino acid catabolism, Calvin Cycle, and serine pathway (Lu et al., 2009; Madison and Huisman, 1999; Peplinski et al., 2010; Rothermich et al., 2000; Shimizu et al., 2013; Yamane, 1993). Anabolism of scl-PHA involves two enzymes that commonly condensate two

acetyl-CoA followed by the reduction of acetoacetyl-CoA to provide the ultimate substrate (R)-3-hydroxybutyryl-CoA for the PHA synthase (PhaC) (Suriyamongkol et al., 2007). In contrast, mcl-PHA production is closely linked to fatty acids metabolic routes: de novo fatty acid biosynthesis with non-related carbon sources, and β-oxidation which is the main metabolic pathway for related substrates like fatty acids (Shahid et al., 2013). In the former, the PHA composition depends directly on the carbon source, while in the latter there is no relationship between the carbon source and the resulting PHA composition.

The structural composition of PHA polymers depends not only on the carbon compound supplied as the growth substrate, but also on the microbial strain used for that purpose (Możejko-Ciesielska and Kiewisz, 2016). The PHA bioaccumulation trait is widespread among the bacterial and archaeal domains with PHA-producing microbes occurring in more than 70 bacterial and archaeal genera (Lu et al., 2009; Poli et al., 2011). It is predominantly investigated in *Cupriavidus necator* (formerly *Ralstonia eutropha*), which can store PHA up to 96 % of its cell dry mass (CDM) under conditions of nitrogen or phosphate limitation and excess of carbon source (Verlinden et al., 2007). Therefore, this microorganism is the most popular to produce PHA from glycerol. Other species commonly used for this process include bacteria from the genus *Pseudomonas* and *Burkholderia* (Koller and Marsalek, 2015). In the last years, other PHA-producers have been identified and evaluated. Some examples include *Zobellella denitrificans* (Ibrahim and Steinbüchel, 2010) and *Paracoccus denitrificans* (Kalaiyezhini and Ramachandran, 2015; Mothes et al., 2007), both able to achieve high PHA accumulation from glycerol (up to 87 % and 72 %, respectively, at optimized conditions). *Paracoccus denitrificans* is a microbial strain with interesting metabolic features, and it seems particularly suitable for growth under stressful conditions. For instance, *P. denitrificans* showed not only ability to survive, but also robust cellular growth, under extreme gravity accelerations - corresponding to 403,627 times *g* (the normal acceleration resulting from gravity at the Earth's surface) (Deguchi et al., 2011). The main findings regarding the production of PHA by *P. denitrificans* are briefly described in the following section.

2.4.1. Polyhydroxyalkanoate production by *Paracoccus denitrificans*

Formerly known as *Micrococcus denitrificans*, this bacterium was first isolated from soil more than one century ago by Beijerinck and Minkman (1910). It was then renamed to *Paracoccus denitrificans* in 1969. This is a Gram-negative facultative methylotrophic bacterium, able to synthesize PHA from many carbon sources, such as methanol, ethanol, 1-butanol, and 1-pentanol (Ueda et al., 1992; Yamane et al., 1996a, 1996b). It exhibits metabolic versatility, and it was shown to grow aerobically and anaerobically, performing complete or partial denitrification. Bacterial denitrification play an important role in determining the fate of reactive nitrogen in both

terrestrial and aquatic environments, especially when oxygen is limiting (Thomson et al., 2012). Denitrifying bacteria can switch between respiring oxygen and nitrate, using a process in which nitrate is reduced (via nitrite, nitric oxide and nitrous oxide) to di-nitrogen in a series of reactions (Giannopoulos et al., 2017).

Paracoccus denitrificans was found to accumulate a PHA copolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), during growth on n-pentanol (Yamane et al., 1996b). This strain was also reported to accumulate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) when methanol and n-amyl alcohol were added together to a nitrogen-limited medium (Ueda et al., 1992). There are only few studies regarding the production of PHA from glycerol by *P. denitrificans* that are summarized in Table 2.4.

Table 2.4. Main results of PHA production by *P. denitrificans*, using glycerol as substrate.

Titer ($g\ L^{-1}$)	**Yield ($g\ g^{-1}$)**	**Productivity ($g\ L^{-1}h^{-1}$)**	**Content (% of cell dry weight)**	**References**
-	0.5	-	70	Mothes et al. (2007)
10.7	0.3	0.15	72	Kalaiyezhini and Ramachandran (2015)
24.2	-	-	39.3	[a]Kumar et al. (2018)

[a]Study performed using *Paracoccus* sp. LL1, and not *P. denitrificans*

Mothes et al. (2007) published the first study on this subject, and obtained a maximum specific growth rate of 0.25 h^{-1}, which is higher than the observed in the same study for *C. necator* (0.13 h^{-1}). The molecular weight of poly(3-hydroxybutyrate) produced with *P. denitrificans* or *C. necator* from crude glycerol varied between 620,000 and 750,000 Da. These values are lower than the observed for the polymer produced from acetate or fructose (970,000 and 1,160,000 Da, respectively). However, these values are sufficiently high for processing by common techniques used in the polymer industry. The impact of common crude glycerol contaminants (NaCl or K_2SO_4) on *P. denitrificans* growth and poly(3-hydroxybutyrate) production was evaluated. These contaminants accumulate in the course of fermentation and could have an effect on growth and poly(3-hydroxybutyrate) synthesis. When using crude glycerol containing 5.5 % NaCl, a reduction in the poly(3-hydroxybutyrate) content (48 %) was observed. The poly(3-hydroxybutyrate) yield coefficient was also reduced, obviously due to osmoregulation. Interestingly, contamination with K_2SO_4 showed a less pronounced effect, and thus it seems to be more tolerable than contamination with NaCl.

Kalaiyezhini and Ramachandran (2015) have also studied poly(3-hydroxybutyrate) production from glycerol by *P. denitrificans*. In that case, the kinetics of poly(3-hydroxybutyrate) biosynthesis was evaluated in a batch bioreactor, testing different operational parameters, such as nitrogen source, carbon to nitrogen ratio, pH, aeration, and initial glycerol concentration. From all the nitrogen sources tested in the study (yeast extract, urea, ammonium sulfate, ammonium chloride, and ammonium nitrate), ammonium sulfate and yeast extracts were the most suitable ones in shake-flask experiments. However, yeast extracts have showed superior results (compared to ammonium sulfate) in bioreactor studies. In the case of oxygen transfer rate and initial glycerol concentration, the most suitable conditions for bacterial growth were not the same as for polymer production. While higher oxygen transfer rates promoted microbial growth, moderate oxygen transfer rates promoted poly(3-hydroxybutyrate) accumulation in the cells. Similarly, optimal glycerol concentration for cell growth was 40 g L^{-1}, while it was 20 g L^{-1} for poly(3-hydroxybutyrate) accumulation. The authors also reported that high initial carbon:nitrogen (C:N) ratio favored polymer accumulation and its productivity. At a C:N ratio of 21.4 (mol mol^{-1}), a poly(3-hydroxybutyrate) concentration of 10.7 g L^{-1} was achieved, corresponding to 72 % of CDM. Overall, the more suitable conditions for *P. denitrificans* for PHA production may be carefully selected, since cell growth and polymer accumulation may not be stimulated at the same culture conditions.

Recently, Kumar et al. (2018) reported the conversion of glycerol by a culture of *Paracoccus* sp. LL1, isolated from the Lonar Lake in India, with production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). The formation of this co-polymer by glycerol unveils interesting features of *Paracoccus* metabolism. However, it is relevant to note that the specific metabolic pathways behind glycerol conversion (into PHA and other products) by *Paracoccus* strains are still not extensively studied and understood. Likewise, the metabolic responses of *P. denitrificans* to stress are poorly documented, despite the evidences of its ability to survive and grow under extreme conditions. In fact, the use of stress to modulate the diverse and complex metabolism of *P. denitrificans* would potentially result in interesting effects, for the different purposes that *P. denitrificans* is usually applied (i.e. denitrification and PHA production).

2.5. Current challenges and possible improvements

Nowadays, it is widely accepted that several products usually obtained from petroleum can be produced biotechnologically from glycerol using specific microbial strains (da Silva et al., 2009). As a consequence, significant research efforts are focusing on this subject, with a wide range of microorganisms being tested and manipulated for glycerol consumption and conversion. Despite of the countless advantages of these glycerol-based bioprocesses, their application at

industrial scale still faces many challenges. According to Khanna et al. (2012), there are different aspects that need to be taken into account regarding the feasibility of glycerol bioconversion: the fluctuations in the demand and supply of each glycerol product in the international market; the cost of glycerol transportation (the integration of glycerol fermentation processes into biodiesel plants would be highly beneficial); the strict maintenance of sterility in the fermentation process, and the risks involved in the downstream processing.

There are other limitations related to biotechnological conversions *per se*, such as the usually slow kinetics of glycerol conversion (Khanna et al., 2012). Other drawbacks are related to the low product concentration in the fermentation broth, the co-production of low-value by-products (Cheng et al., 2012). To overcome these and other disadvantages, significant research has been conducted to optimize the conditions and configurations of these processes. The most commonly reported strategies are related to the isolation of novel glycerol-consuming strains and the development of genetically-engineered strains (Khanna et al., 2012; Mattam et al., 2013). However, breakthrough approaches related to the fermentation conditions applied throughout the process have rarely been tested. For instance, these glycerol-based fermentations were still not performed under non-conventional conditions, which could introduce significant improvements in these processes, potentially making them more attractive for industrial production.

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CHAPTER III

Scope and outline of the work

The microbial conversion of glycerol to value-added products constitutes a promising approach to dispose the excess glycerol generated by the biodiesel industry. However, there are still some limitations related to these biotechnological processes, such as the usually slow kinetics of glycerol conversion, the low product concentration, and the co-production of low-value by-products. Different strategies have been tested to overcome these issues. One possibility is the application of non-conventional conditions (such as HP, electric fields, or ultrasounds) to glycerol-based fermentations, possibly introducing significant improvements in these processes. Therefore, the main goal of the present work was to evaluate the application of sub-lethal HP on fermentative processes that use glycerol as substrate to the production of value-added products. The work intended to i) understand the possible effects of pressure on glycerol fermentations, providing fundamental knowledge about the microbial responses to pressure as a variable of life; and ii) if possible, contribute to the process improvement, by increasing fermentation rates, increasing the yields of desired products, and/or promoting the formation of different products.

In order to perform this study, two specific microbial processes were selected: one of them with *Lactobacillus reuteri*, which converts glycerol into 1,3-PDO; and the other with *Paracoccus denitrificans*, which is able to convert glycerol into PHA. These processes have different oxygen requirements, since the former corresponds to an anaerobic process, and the latter corresponds to an aerobic one. The combination of both was intentionally selected to allow a more comprehensive perspective regarding the potential of HP for application on glycerol-based processes. In both cases, only sub-lethal HP conditions (in the range of 10 to 50 MPa) were used, at controlled temperature, with proper fermentative media, and using glycerol as substrate (or co-substrate, in the case of *L. reuteri*).

The following chapters describe the results obtained for each of these glycerol-based processes. The first part of the study (Chapters IV – VI) is focused on the glycerol/glucose co-fermentation by *L. reuteri*. Chapter IV approaches the general effects of HP on this process, using different initial acetate concentrations in the culture medium, as a possible microbial growth inhibitor. Different HP levels (10, 25 and 35 MPa) were applied to *L. reuteri* fermentation, and the effects on cell growth, substrate consumption, and product formation were evaluated over time. In Chapter V, adaptation of *L. reuteri* to pressure was assessed during four consecutive fermentation cycles under sub-lethal HP levels (10 and 25 MPa). Similarly to the previous chapter, different initial acetate concentrations in the culture medium were tested. To evaluate the impact of pressure throughout the fermentation cycles, cell growth, substrate consumption and product formation were analyzed after each cycle. In addition, the effects of pressure were evaluated on cell membrane permeability, by determining the leakage of nucleic acids and extracellular proteins to the extracellular medium. To better understand the effects of these HP-cycles on the *L. reuteri* metabolome, the metabolic profiles of these samples were characterized

by ^{1}H NMR spectroscopy coupled with multivariate analysis, to evaluate the changes in the abundances of several metabolites relatively to atmospheric pressure (Chapter VI).

The work regarding glycerol fermentation by *P. denitrificans* is approached in Chapters VII and VIII. In this particular case, it was necessary to give special focus on air/oxygen availability, since this is a critical parameter that affects *P. denitrificans* growth and metabolic profile. It is important to highlight that the HP system used in the present work involves some process constrains, related to limited air volumes and the absence of agitation. Accordingly, the study presented in Chapter VII was divided in two main goals: *i)* to study the effects of air availability on *P. denitrificans*; *ii)* to assess if the strain was able to grow and maintain metabolic activity under HP (10, 25 and 35 MPa). Those aspects were evaluated in terms of biomass concentration, substrate consumption and formation of extracellular products. A more detailed analysis of the HP effects (10, 25, 35 and 50 MPa) on *P. denitrificans* is presented in Chapter VIII, which includes the evaluation of cell growth and substrate formation and PHA formation, as well as characterization of the PHA monomeric composition at different pressure conditions.

CHAPTER IV

Lactobacillus reuteri growth and fermentation under high pressure towards the production of 1,3-propanediol

Adapted from:

Mota, M.J., Lopes, R.P., Sousa, S., Gomes, A.M., Delgadillo, I., Saraiva, J.A., 2018. *Lactobacillus reuteri* growth and fermentation under high pressure towards the production of 1,3-propanediol. Food Res. Int. 113, 424-432.

4.1. Introduction

Biodiesel production has been raising some sustainability issues, and one of them corresponds to the formation of crude glycerol as a reaction by-product (da Silva et al., 2009; Kolesárová et al., 2011). Glycerol may be used as substrate in microbial fermentation processes, for production of high-value products, such as organic acids, alcohols, or biopolymers (Mattam et al., 2013). In this context, *Lactobacillus reuteri* presents itself as a lactic acid bacteria (LAB) with high potential for the production of different relevant compounds from glycerol, including reuterin, 3-hydroxypropionic acid and 1,3-propanediol (1,3-PDO) (Burgé et al., 2015; Dishisha et al., 2015, 2014; Jolly et al., 2014; Lee et al., 2015; Ricci et al., 2015; Vollenweider and Lacroix, 2004). 1,3-Propanediol is an important chemical intermediate used in food, cosmetic and pharmaceutical industries (Biebl et al., 1999; Katrlík et al., 2007; Menzel et al., 1997; Saxena et al., 2009). It is also a GRAS (Generally Recognized As Safe) food and beverage ingredient, commercialized by DuPont Tate & Lyle Bio Products. It can be used as a flavor carrier, to reduce bitterness and enhance sweetness, or as a humectant, or processing aid. It is approved for use in different food products, such as seasonings and flavorings, confections and frostings, nuts and nut products, alcoholic beverages, and frozen dairy products (DuPont Tate & Lyle Bio Products, 2018). The global market of 1,3-PDO has considerably increased in recent years, due to the increasing market demand of its derivatives – e.g. 60,000 tons in 2012 *versus* 150,000 tons expected to 2019 (Lee et al., 2015; Research TM, 2012).

The production of 1,3-PDO by *L. reuteri* from glycerol seems a promising biotechnological approach. For that, a co-fermentation with glycerol and a sugar is usually performed, since this LAB uses glycerol only as an electron acceptor and not as a carbon source for growth (Ricci et al., 2015). For instance, Ragout et al. (1996) showed that the production of 1,3-PDO by *L. reuteri* only occurred during the maltose/glycerol co-fermentation. An important factor for this fermentation is the presence of acetate, which has been recognized as a microbial growth inhibitor. However, Iino et al. (2002) have stated that certain acetate concentrations may have a positive influence on growth and fermentation of LAB. A possible explanation is the ability of acetate to act as an indirect proton and electron acceptor, leading to the activation of oxidative pathways at early stages of fermentation (Heyndrickx et al., 1991; Jolly et al., 2014). In fact, Jolly et al. (2014) reported that an initial acetate concentration of 5 g L^{-1} increased 1,3-PDO productivity, while concentrations above this value were found to be inhibitory.

The performance of fermentation under non-conventional conditions (such as high pressure, electric fields or ultrasounds) is a strategy that has been recently tested for different fermentation processes, aiming the stimulation of microbial growth and/or improvement of fermentation rates and yields (Mota et al., 2018). This approach involves the use of these technologies, at sub-lethal levels, prior and/or during the fermentation process, in order to affect

cell growth and metabolism, but without compromising cell viability. In some cases, considerable improvements were achieved, such as increased yields, productivities and fermentation rates, lower accumulation of by-products and/or production of different compounds.

High pressure (HP) is particularly advantageous in the context of microbial fermentations, since it can be maintained continuously during the entire fermentation process, without heat generation and no need of energy to maintain the pressure (Mota et al., 2018). The beneficial effects of HP on bioprocesses have been reported for several bacteria and yeasts (Mota et al., 2013). Picard et al. (2007) reported acceleration of alcoholic fermentation by *Saccharomyces cerevisiae* at 5 and 10 MPa, with the production of ethanol proceeding 3-fold faster at 10 MPa compared to atmospheric pressure. High pressure can also be used to change the metabolic selectivity of the fermentative strains. For instance, application of pressures of 7 and 17 MPa during fermentation by *Clostridium thermocellum* redirected the metabolism from the production of by-products (such as acetate) to ethanol, leading to an increase in the ratio ethanol:acetate up to 60-fold compared to atmospheric pressure (Bothun et al., 2004). Another example is the utilization of HP to modify the properties of biopolymers produced during fermentation. Production of bacterial cellulose by *Gluconacetobacter xylinus* under HP (30, 60 and 100 MPa) showed profound differences in morphological properties of the polymer, having a significantly higher density compared with the cellulose produced at atmospheric pressure (Kato et al., 2007). The effects of HP were already tested during fermentation of a food product: pressures up to 100 MPa were applied during lactic acid fermentation for probiotic yogurt production. High pressure reduced the fermentation rate, but it was still possible to produce yogurt at 5 MPa, showing that lactic acid fermentation can be performed at this pressure without compromising the fermentation activity of the bacterial strains (including the probiotic one) (Mota et al., 2015).

Based on the results published so far, application of HP seems to be a promising approach that could introduce significant improvements in glycerol-based fermentation processes. Therefore, the present work intended to study the effects of HP (10 - 35 MPa) on growth and fermentation of *L. reuteri* DSM 20016, and using different initial acetate concentrations in the culture medium, to test its possible microbial growth inhibitor effect under pressure.

4.2. Material and methods

4.2.1. Microorganism and culture media

A lyophilized culture of *Lactobacillus reuteri* DSM 20016 obtained from DSMZ, Germany, was used in this study. The strain was reconstituted on commercial MRS medium,

according to the manufacturer's instructions. Cells were maintained at -80 ºC in a cryoprotectant solution.

Commercial MRS medium was used for seed culture preparation and growth. For the fermentation experiments, two different media were used: *i)* with an initial acetate concentration of 5 g L^{-1}, correspondent to commercial MRS broth, composed by peptone (10 g L^{-1}), meat extract (10 g L^{-1}), yeast extract (5 g L^{-1}), dipotassium phosphate (2 g L^{-1}), ammonium citrate (2 g L^{-1}), magnesium sulphate (0.1 g L^{-1}), manganese sulphate (0.05 g L^{-1}), Tween 80 (1.0 g L^{-1}), glucose (20 g L^{-1}), sodium acetate (5 g L^{-1}), and glycerol (20 g L^{-1}); and *ii)* without initial acetate, prepared with the same components except the sodium acetate.

4.2.2. Seed culture preparation

A single cell colony was seeded into 10 ml MRS broth and incubated for 10-12 h at 37 ºC, in static conditions. The culture was then transferred to a 250 ml modified MRS medium in a 300 ml Erlenmeyer flask, incubated overnight at 37 ºC, in static conditions.

4.2.3. Fermentation experiments

The medium was inoculated with 10 % (v/v) of seed culture. The mixture was homogenized and then transferred to polyethylene bags, which were carefully heat-sealed with no air. All these steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination.

Fermentation was carried at 37 ºC under different HP conditions (10, 25, and 35), for 32 h. All fermentations were performed under unaerated conditions. These experiments were conducted in a Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom), with a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature, using a mixture of propylene glycol and water as pressurizing fluid. In parallel, a control sample carried out fermentation at atmospheric pressure (0.1 MPa), while maintaining constant the remaining process conditions. At all pressure conditions (including atmospheric pressure), two different types of samples were studied: *i)* with acetate, corresponding to samples with an initial acetate concentration of 5 g L^{-1}; *ii)* and without acetate, corresponding to samples with no acetate added in the culture medium. In all cases, fermentation experiments were performed in duplicate and the analyses were also performed in duplicate.

4.2.4. Determination of biomass concentration

Biomass concentration of the samples was determined by optical density measurement at 600 nm, with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.,

USA). Cell dry weight (CDW) was routinely determined using a standard curve relating *L. reuteri* optical density and CDW.

4.2.5. Determination of viable cell counts

For determination of viable cells, serial dilutions (using Ringer solution) of the culture samples were prepared and aliquots of 1.0 mL of proper dilutions were plated in MRS agar plates, incubated at 37 °C for 24 h.

4.2.6. Quantification of glycerol, glucose, and fermentation products

Culture samples were centrifuged at 10,000 rpm and 4 ºC for 10 min and the collected supernatants were filtered through a 0.22 µm filter membrane. Analysis by HPLC was performed using a HPLC Knauer system equipped with Knauer K-2301 RI and K-2501 UV detectors, and an Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase was 13 mM H_2SO_4, delivered at a flow rate of 0.6 mL min^{-1} and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards. Because of co-elution of glycerol and lactate, and since glycerol is only detected by the RI detector, but lactate is detected by both detectors, lactate concentration was estimated through the UV detector. The RI area corresponding to this lactate concentration was estimated using the corresponding RI calibration curve. Glycerol quantification was determined by the difference between the total area of the RI peak and the calculated contribution of lactate in the area of the peak.

4.3. Results and Discussion

4.3.1. Effect of sub-lethal pressure on cell growth

In order to understand how sub-lethal pressures affect *L. reuteri* growth, biomass concentration and viable cell counts were both determined. Figure 4.1 shows the variation of both parameters during fermentation at different pressure conditions (0.1, 10, 25, and 35 MPa), for the samples with acetate in the culture medium (Figures 4.1.a and 4.1.b), and the ones without added acetate (Figures 4.1.c and 4.1.d).

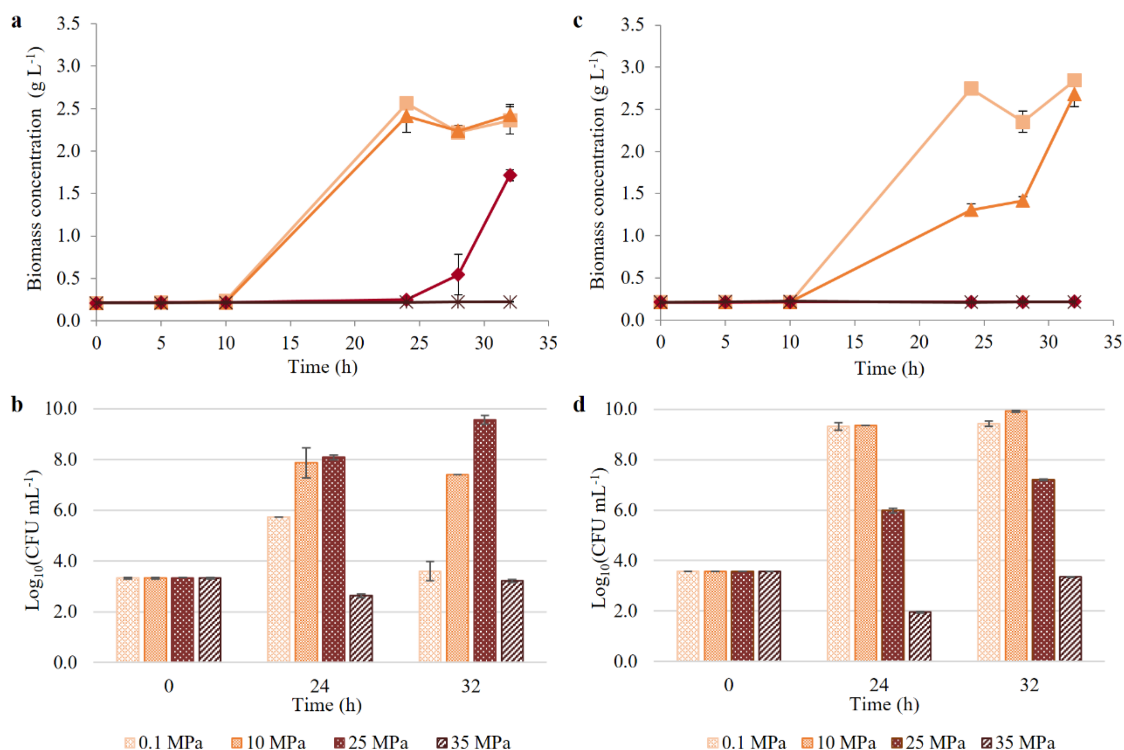

Figure 4.1. Biomass concentration and viable cell counts throughout fermentation, for samples with initial acetate (a and b) and without initial acetate (c and d), according to the pressure applied: 10 MPa (triangles, ▲), 25 MPa (diamonds ♦), or 35 MPa (stars *). Control samples (0.1 MPa) are represented as squares (■).

The presence of acetate in the culture medium was found to affect biomass production and cell viability, which confirms that this is an important aspect to consider in *L. reuteri* fermentation processes. At atmospheric pressure (0.1 MPa), samples without acetate showed higher cell counts and slightly higher biomass concentrations, compared to the ones with acetate, which may result from an inhibitory effect caused by accumulation of this metabolite: the acetate produced from glucose metabolism accumulates, in addition to the amount already present in the medium and, in consequence, the inhibitory concentration is more rapidly achieved. The effect of acetate on microbial growth is a highly debated subject on literature, since it is usually regarded as an inhibitor of microbial growth (Lasko et al., 2000), but some studies stated it may have a favorable influence on growth rates and energy yields of lactic acid bacteria (Iino et al., 2002; Jolly et al., 2014).

Application of HP was also shown to affect biomass concentration during *L. reuteri* fermentation. In general, HP decreased biomass concentration (Figures 4.1.a and 4.1.c), with a more pronounced effect at higher pressure levels. However, the presence of acetate in the culture media seems to increase the *L. reuteri* tolerance to HP. In the samples with acetate, biomass concentrations showed similar behavior at 0.1 and 10 MPa, reaching similar concentrations (2.36

and 2.43 g L^{-1}, respectively) after 32 h. When fermentation was carried out without acetate in the culture medium, the increase in biomass concentration was slower at 10 MPa than at 0.1 MPa, but it reached similar values after 32 h (2.84 at 0.1 MPa, and 2.68 g L^{-1} at 10 MPa). In samples with acetate fermenting at 25 MPa, variation in biomass concentration was minimal during the first 24 h, which suggests a longer lag phase for development of appropriate stress responses to ensure cell survival and growth at this pressure level. After that time, biomass concentration increased up to 1.72 g L^{-1} at 32 h of fermentation, which is still considerably lower than the concentrations achieved at 0.1 and 10 MPa. However, it is important to note that samples without acetate showed no variation in biomass concentration at 25 MPa, indicating higher sensitivity to this pressure in the absence of acetate. At 35 MPa, the pressure seems to severely affect *L. reuteri* cells, which were not able to withstand this level of pressure and, in consequence, biomass concentration remained constant over the fermentation time, regardless of the presence/absence of acetate in the medium.

Viable cell counts (Figures 4.1.b and 4.1.d) were also affected by HP, with the effect depending on the pressure level and the acetate in the medium. In the samples with acetate, cell counts were higher at 10 MPa than at 0.1 MPa, and even higher at 25 MPa. Similarly to the observed for biomass concentration, viable cell counts reflected higher sensitivity of *L. reuteri* to pressure in the absence of acetate. In this case, cell counts were still similar at 0.1 MPa and 10 MPa, but considerably lower at 25 MPa. The higher cell counts at 25 MPa in samples with acetate are not in accordance with the biomass concentration results, which were lower at this pressure compared to 10 and 0.1 MPa. It is important to note that biomass concentrations were estimated by measurement of optical density (at 600 nm), which may be affected by the presence of non-viable cells, or by the cell size and morphology. All of these aspects are pressure-sensitive and were possibly modified in the course of the present work. A possible explanation for the different results between both parameters relies on the longer lag phase at 25 MPa that lasted for the first 24 h of fermentation. After that time, cells at 10 and 0.1 MPa were already reaching the stationary phase, due to accumulation of acetate and other inhibitory metabolites, which resulted in a subsequent reduction of cell viability. In contrast, cells at 25 MPa were still growing at that time, promoting the increase of viable cell counts. In both cases, there was no cell growth at 35 MPa, suggesting microbial inhibition or even inactivation after 24 h, followed by a slight increase, possibly as a result of development of HP stress responses. This indicates a considerably longer lag phase when fermentation was carried out at 35 MPa.

The results in this section showed that fermentation under pressures between 10 and 35 MPa affected *L. reuteri* growth, with the effects being dependent on the initial acetate concentration. In fact, the presence of acetate seems to enhance the resistance of this strain to pressure. Although there is no explanation for this behavior on literature, this effect suggests that

acetate may have a role on the cell response mechanisms developed under HP-stress, possibly due to cross-protection mechanisms. It is documented in literature that LAB response to HP can be related to other factors, such as heat or cold, and their ability to react to pressure can be explained by a bacteria cross-protection system (Bucka-Kolendo and Sokołowska, 2017; Ljungh and Wadström, 2009; Scheyhing et al., 2004). However, these LAB response to HP is still not well documented and understood, and must be clarified by further transcriptome and proteome studies.

4.3.2. Effect of sub-lethal pressure on substrate consumption

The consumption of glycerol and glucose was monitored during fermentation at different pressure conditions, and the results are presented in Figure 4.2. In all cases, the uptake of glycerol (Figure 4.2.a and 4.2.c) and glucose (Figure 4.2.b and 4.2.d) seem to be correlated, with both showing similar behavior, independently of the pressure applied or the initial concentration of acetate. Although glycerol and glucose are metabolized by different pathways, these are, in fact, dependent of each other. The regulation of glycerol metabolizing pathway is dependent on the availability of glucose (Lüthi-Peng et al., 2002), since the NADH generated from glucose by the glycolysis pathway is recycled by the presence of glycerol and its transformation into 1,3-PDO (Doleyres et al., 2005). Therefore, high glucose consumption usually results in high glycerol uptake rate (Jolly et al., 2014).

The presence/absence of acetate was found to affect substrate consumption under different pressure conditions. With acetate (Figures 4.2.a and 4.2.b), fermentation at 0.1 and 10 MPa showed similar substrate consumption profiles over time. After 32 h, samples at 10 MPa reached glycerol and glucose concentrations of 6.37 and 2.02 g L^{-1}, respectively, which were similar to the obtained at 0.1 MPa (6.51 and 1.96 g L^{-1} for glycerol and glucose, respectively). These final concentrations correspond to consumption of ≈ 63 % of the glycerol and ≈ 83 % of the glucose present in the medium (Table 4.1). At 25 MPa, the lag phase was more extensive (as discussed in the previous section), with low substrate consumption during the first 24 h. After that time, the consumption of both substrates had a considerable increase, resulting in a glycerol consumption of ≈ 30 % and a glucose consumption of ≈ 42 % after 32 h (Table 4.1), which are still lower than the values for samples at 0.1 and 10 MPa. However, the extension of fermentation time at 25 MPa would possibly lead to substrate consumptions similar to the observed at 0.1 and MPa, since the *L. reuteri* cells seem to be able to grow and ferment at this pressure, suggesting the development of suitable response mechanisms that allowed the microorganism to withstand these stressful pressure conditions. Differently, application of 35 MPa promoted low substrate consumption during the entire fermentation time: glycerol concentration had no variation over time, while glucose concentration showed a slight decrease (correspondent to consumption of 6.28 % of the total glucose present in the medium).

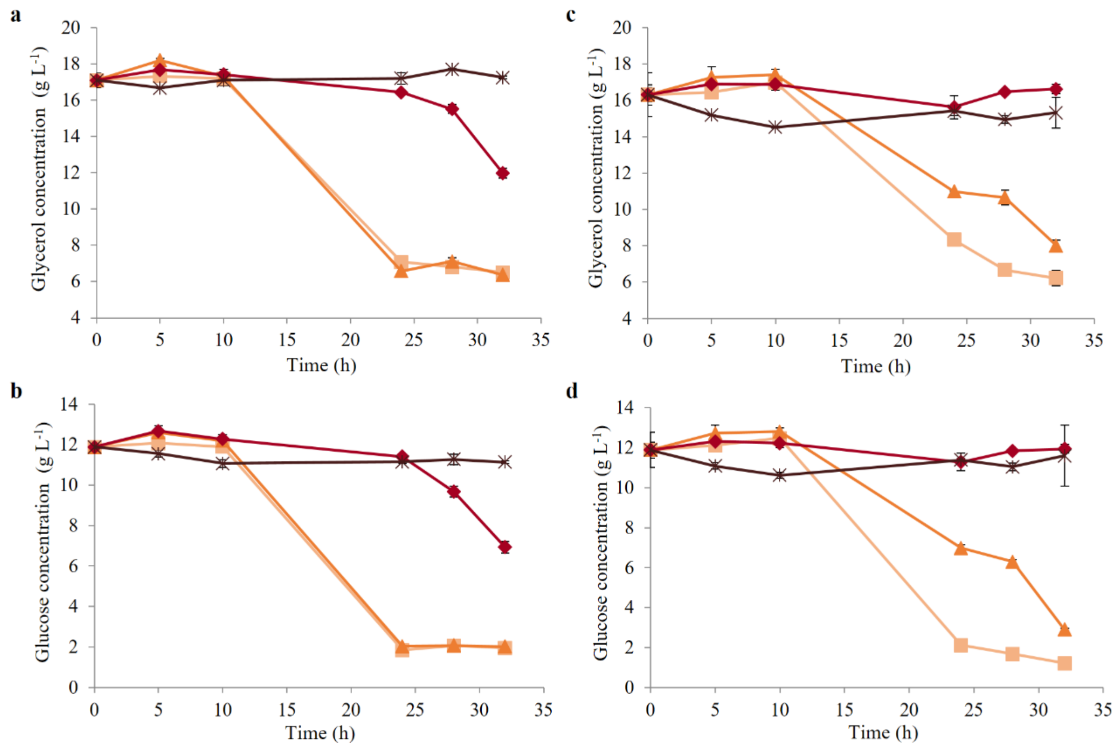

Figure 4.2. Glycerol and glucose concentrations throughout fermentation, for samples with initial acetate (a and b) and without initial acetate (c and d), according to the pressure applied: 10 MPa (triangles, ▲), 25 MPa (diamonds ♦), or 35 MPa (stars *). Control samples (0.1 MPa) are represented as squares (■).

For the samples without added acetate (Figures 4.2.c and 4.2.d), substrate consumption was more affected by the pressure increase. In this case, glycerol and glucose consumptions at 10 MPa (50.85 and 75.61 %, respectively, after 32 h) were significantly lower than at 0.1 MPa (61.85 and 89.72 %, respectively, after 32 h). At 25 MPa, there was no consumption of either glycerol or glucose during the 32 h, possibly due to the inhibition of *L. reuteri* metabolism. However, cell growth (i.e. increase of viable cell counts) was still observed in these samples. In contrast, *L. reuteri* was able to consume glycerol (≈ 2 % after 32 h) when fermentation was carried out at 35 MPa, but the viable cell counts after 24 h and 32 h were lower than in the beginning of the process. Considering these results, it is not clear if the HP effects at 25 and 35 MPa promote complete inhibition of cell growth and metabolism, but this aspect will be clarified in the following sections.

Table 4.1. Consumption (%) of glycerol and glucose after 32 h of fermentation at different pressure conditions, in samples with and without acetate.

Samples	Pressure	Glycerol consumed (%)	Glucose consumed (%)
With acetate	0.1 MPa	61.96	83.54
	10 MPa	62.76	83.05
	25 MPa	29.99	41.73
	35 MPa	-	6.28
Without acetate	0.1 MPa	61.85	89.72
	10 MPa	50.85	75.61
	25 MPa	-	-
	35 MPa	2.35	-

4.3.3. Effect of sub-lethal pressure on production of 1,3-propanediol

The production of 1,3-PDO during fermentation at different pressure conditions was also evaluated, with the results being presented in Figure 4.3. In addition, 1,3-PDO yields on glycerol ($Y_{1,3\text{-PDO/Gly}}$, g g^{-1}) and 1,3-PDO productivities ($Q_{1,3\text{-PDO}}$, g L^{-1} h^{-1}) are represented in Table 4.2.

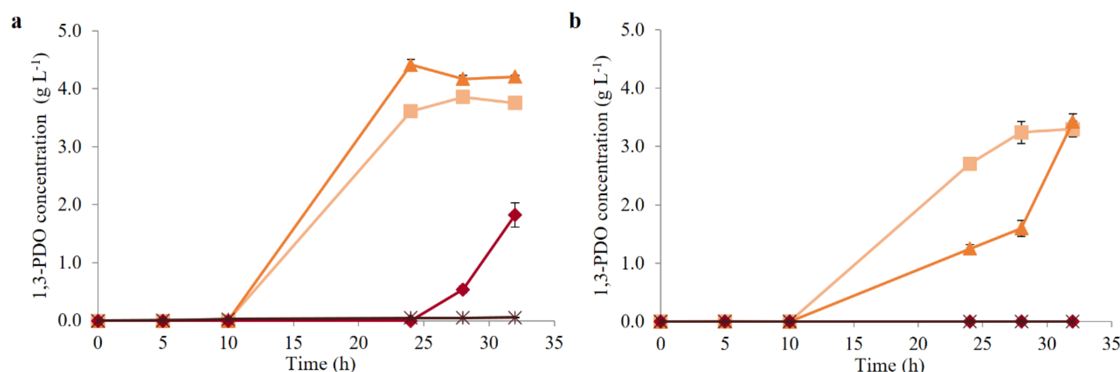

Figure 4.3. 1,3-Propanediol concentration throughout fermentation, for samples with initial acetate (a) and without initial acetate (b), according to the pressure applied: 10 MPa (triangles, ▲), 25 MPa (diamonds ♦), or 35 MPa (stars *). Control samples (0.1 MPa) are represented as squares (■).

At atmospheric pressure, *L. reuteri* reached 1,3-PDO titers of 3.76 and 3.30 g L^{-1} after 32 h, which are in the range of some of the values reported in literature for *L. reuteri* glycerol/sugar co-fermentation (≈ 4 g L^{-1}) (El-Ziney et al., 1998; Ragout et al., 1996). However, these are considerably lower than some of the values reported in literature for fermentation with LAB. For

instance, *Lactobacillus diolivorans* has been reported to produce 1,3-PDO concentrations as high as 74 g L^{-1} or 92 g L^{-1} in fed-batch experiments (Lindlbauer et al., 2017; Pflügl et al., 2012). For fermentation with *L. reuteri*, Jolly et al. (2014) reached 1,3-PDO concentrations of 65.3 g L^{-1} during fed-batch experiments, while Baeza-Jiménez et al. (2011) and Ricci et al. (2015) obtained concentrations of 29 and 41 g L^{-1}, respectively, for batch cultivations. Nevertheless, it is important to note that all of these studies were performed in bioreactors, with monitoring, adjustment and optimization of several important process parameters. In the present work, fermentation was carried out with a different and simpler experimental set-up, with lower volumes suitable for the HP experiments, as a proof of concept to evaluate if it was possible to carry out the process under these pressure conditions, and if it could promote any improvement.

Table 4.2. 1,3-PDO yields on glycerol ($Y_{1,3\text{-PDO/Gly}}$) and 1,3-PDO productivities ($Q_{1,3\text{-PDO}}$), for fermentation at different pressure conditions, in samples with and without acetate.

Samples	Pressure	$Y_{1,3\text{-PDO/Gly}}$ (g g^{-1})	$Q_{1,3\text{-PDO}}$ (g L^{-1} h^{-1})
With acetate	0.1 MPa	0.354	0.117
	10 MPa	0.392	0.131
	25 MPa	0.355	0.057
	35 MPa	-	0.002
Without acetate	0.1 MPa	0.328	0.103
	10 MPa	0.414	0.107
	25 MPa	-	-
	35 MPa	-	-

Yields and productivities were calculated from a single time-point corresponding to the end of the experiment (32 h). Values reported in the table represent the mean of two independent biological replicates.

As indicated in Figure 4.3, the presence/absence of acetate affected the production of 1,3-PDO under different pressure conditions. In general, higher rates and titers were achieved in the samples with acetate (Figure 4.3.a), compared to the ones without this compound in the culture medium (Figure 4.3.b). Interestingly, the opposite effect was noted for cell growth at atmospheric pressure, since the samples without acetate had higher cell counts and biomass concentrations, compared do the ones with acetate. This suggests that although acetate acts as an inhibitor of *L. reuteri* growth, it has a positive effect on the production of 1,3-PDO. Jolly et al. (2014) published similar findings, with an initial acetate concentration up to 5 g L^{-1} improving 1,3-PDO productivity. However, the authors also reported an improvement of *L. reuteri* cell growth promoted by acetate, which was not observed in the present work.

The presence of acetate in the culture media seems to increase the *L. reuteri* tolerance to HP, resulting in an enhancement of 1,3-PDO production at these conditions. The production of this compound was faster at 10 MPa and resulted in a higher final titer (4.21 g L^{-1}), when compared to 0.1 MPa (3.76 g L^{-1}). In consequence, fermentation at 10 MPa showed yield and productivity improvements of 11 and 12 %, respectively, relatively to 0.1 MPa. By increasing the pressure to higher levels, 1,3-PDO production tends to decrease. At 25 MPa, this compound was only detected after 24 h of fermentation, which is in accordance to the observed for cell growth and substrate consumption. Therefore, the final titer at this pressure (1.83 g L^{-1}) is significantly lower than at 10 and 0.1 MPa, which results in a decreased productivity of 1,3-PDO. In contrast, the yield reached at 25 MPa is similar to the obtained at 0.1 MPa (0.354 and 0.355 g g^{-1}, respectively), due to an also lower glycerol consumption at 25 MPa. When fermentation was carried at 35 MPa, the production of 1,3-PDO was minimal, indicating that this pressure might be enough to inhibit *L. reuteri* metabolism from glycerol, at least up to the fermentation time studied (32 h).

In samples without initial acetate, 1,3-PDO production was only observed at 0.1 and 10 MPa. At 10 MPa, 1,3-PDO was slower than at 0.1 MPa, resulting in lower concentrations after 24 and 28 h. However, the final titers after 32 h of fermentation were similar for both conditions (3.30 and 3.42 g L^{-1}, at 0.1 and 10 MPa, respectively), and even slightly higher at 10 MPa. The 1,3-PDO productivity was also similar at 0.1 and 10 MPa (0.103 and 0.107 g L^{-1} h^{-1}, respectively), while the yield was ≈ 26 % higher at 10 MPa. This improved yield is a result of similar 1,3-PDO titers obtained with a lower glycerol consumption, which suggests that a higher proportion of the consumed glycerol was being directed to the production of 1,3-PDO.

It can be concluded that, under specific conditions, application of pressure (10 MPa) on fermentation may stimulate production of 1,3-PDO by *L. reuteri*, promoting enhanced final titers, yields and productivities. Picard et al. (2007) reported a similar effect on alcoholic fermentation, when applying pressure in the range of 5-10 MPa to *Saccharomyces cerevisiae*. In that case, the production of bioethanol at 10 MPa proceeded 3-fold faster than at 0.1 MPa, and the fermentation yield was increased by 6 % and 5 %, at 5 and 10 MPa, respectively. However, fermentation was slowed down at pressures above 20 MPa. Despite the use of a different microorganism in a different fermentation process, these results are in accordance to the obtained in the present work. The mechanisms behind the increase of fermentative activity by HP are still not completely understood. Several effects of HP on microbial cells are related to modification of cell membrane composition and fluidity, which affect the exchange of substances (both substrates and metabolites) throughout the cell, with consequent effect on cell metabolism (Mota et al., 2013). Other factors may also be involved, such as the effects of HP on enzyme activity (Eisenmenger and Reyes-De-Corcuera, 2009) and on regulation of gene expression (Bravim et al., 2012;

Fernandes et al., 2004). Picard et al. (2007) indicated some possible explanations for the improvement of bioethanol production by *S. cerevisiae*. These included the increased activity of one or more enzymes involved in the fermentation pathways; the improvement of ethanol secretion from the cell, leading to a decreased intracellular concentration and to the reduction of retro-inhibition; and the enhancement of substrate consumption. The last one, however, is not valid for the present work, since substrate consumption was evaluated and it was demonstrated that HP did not promote the increase of glycerol uptake. Therefore, the improvements in 1,3-PDO production may be related to changes in cell membrane, gene expression and enzymatic activity.

4.3.4. Effect of sub-lethal pressure on formation of fermentation by-products

During glycerol/glucose co-fermentation, glucose enters into the glycolysis pathway, leading to the formation of different products, such as ethanol, lactate, acetate, and others. The presence of glycerol and its transformation into 1,3-PDO enables the cells to recycle the NADH generated during glycolysis (Doleyres et al., 2005). However, some of these products obtained from the glycolysis pathway will compete with 3-HPA (the precursor of 1,3-PDO) for NADH-oxidoreductase and, as a result, its formation may decrease the 1,3-PDO yield (El-Ziney et al., 1998). The production of these by-products is addressed in this section. Figure 4.4 shows the production of lactate (Figures 4.4.a and 4.4.d), acetate (Figures 4.4.b and 4.4.e) and ethanol (Figures 4.4.c and 4.4.f) during fermentation at different pressure conditions, while the respective yields on glucose (Y, g g^{-1}) are represented in Table 4.3.

Lactate was the major by-product of *L. reuteri* fermentation process, reaching final titers as high as 7.56 g L^{-1} at 0.1 MPa, in samples without initial acetate. Lactate is an interesting chemical building block and hence its formation could represent a valuable feature of this process (Ricci et al., 2015), even if it is not the intended in the present work. However, it is also important to consider that accumulation of lactate has an inhibitory effect on fermentation, due to its high toxicity levels (Ricci et al., 2015; Vieira et al., 2015).

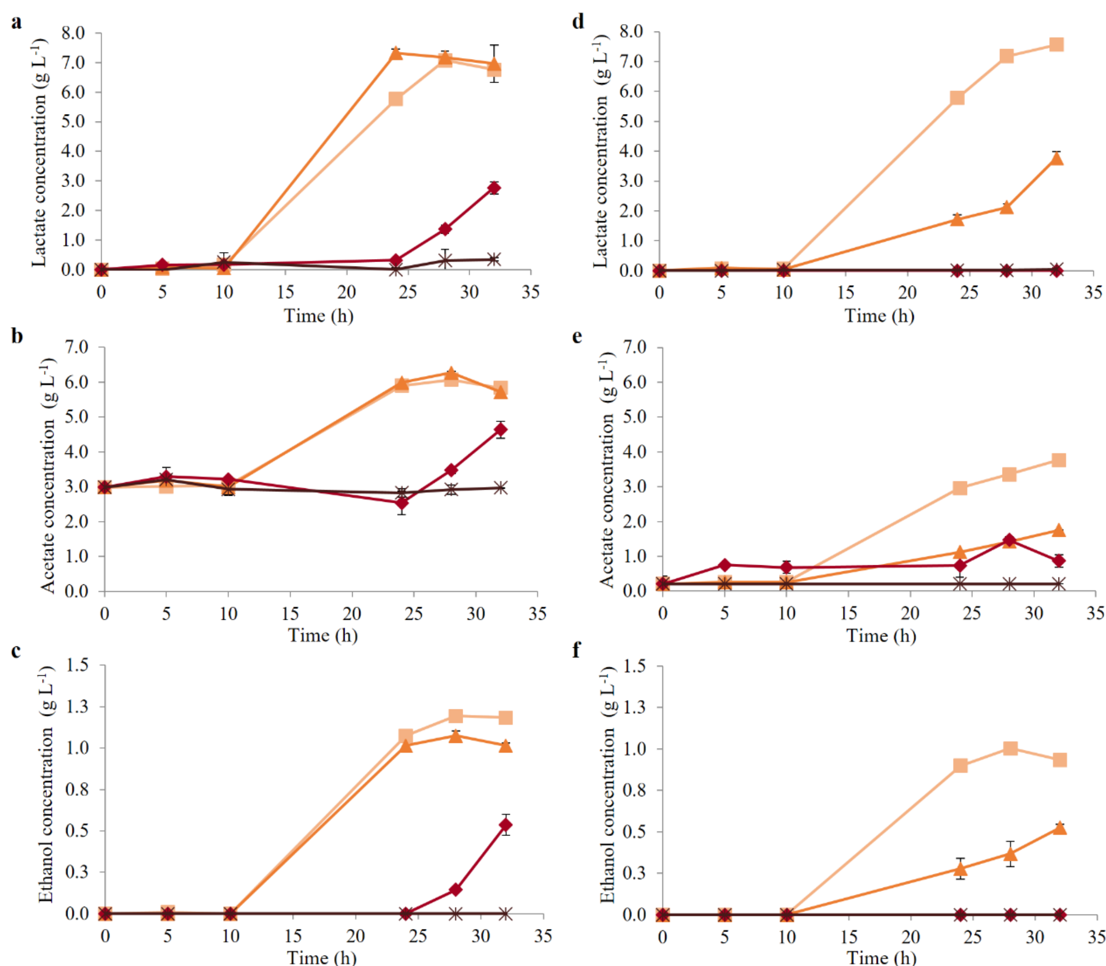

Figure 4.4. Lactate, acetate and ethanol concentrations throughout fermentation, for samples with initial acetate (a, b and c) and without initial acetate (c, d and e), according to the pressure applied: 10 MPa (triangles, ▲), 25 MPa (diamonds ♦), or 35 MPa (stars *). Control samples (0.1 MPa) are represented as squares (■).

At atmospheric pressure, the absence of acetate in the initial culture medium stimulated the production of lactate, leading to a higher final titer compared to the samples with initial acetate (7.56 and 6.77 g L^{-1}, respectively). In general, application of HP seems to inhibit lactate production, with this effect being more pronounced in the samples without acetate. However, in the presence of acetate (Figure 4.4.a), the production of lactate was faster at 10 MPa than at 0.1 MPa. By increasing the pressure to 25 and 35 MPa, lactate production over time was reduced, but still detected at both pressures (2.77 and 0.35 g L^{-1}, respectively).In the absence of acetate (Figure 4.4.d), application of HP at 10 MPa considerably decreased lactate production, leading to a final titer of 3.77 g L^{-1}, while no lactate production was detected at 25 MPa. At 35 MPa, a negligible concentration lactate (0.04 g L^{-1}) was obtained. Therefore, application of HP on *L. reuteri* fermentation had a general inhibitory effect on lactate production, except at 10 MPa when acetate

was added in the culture medium. A similar inhibitory effect was reported in a previous work using three different LAB (*Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis*) for probiotic yogurt production. In that study, application of HP in the range of 5 and 100 MPa negatively affected cell growth and lactic acid fermentation (Mota et al., 2015). However, in the particular case of the present work, lactate is not the desired metabolic product, and its production can even (indirectly) reduce the yields of 1,3-PDO. In addition, lactate is the fermentation product with the highest toxicity for *L. reuteri* (Ricci et al., 2015), with a concentration of 4 g L^{-1} being enough to inhibit cell growth (Vieira et al., 2015). Considering all those aspects, the reduction of lactate production under some HP conditions may represent a potential process improvement.

Table 4.3. Yields of lactate ($Y_{Lact/Glu}$), acetate ($Y_{Acet/Glu}$), and ethanol ($Y_{EtOH/Glu}$) on glucose, for fermentation at different pressure conditions, in samples with and without acetate.

Samples	Pressure	$Y_{Lact/Glu}$ (g g^{-1})	$Y_{Acet/Glu}$ (g g^{-1})	$Y_{EtOH/Glu}$ (g g^{-1})
With acetate	0.1 MPa	0.682	0.287	0.119
	10 MPa	0.706	0.277	0.103
	25 MPa	0.557	0.332	0.108
	35 MPa	0.469	-	-
Without acetate	0.1 MPa	0.709	0.333	0.088
	10 MPa	0.419	0.172	0.059
	25 MPa	-	-	-
	35 MPa	-	-	-

Yields were calculated from a single time-point corresponding to the end of the experiment (32 h). Values reported in the table represent the mean of two independent biological replicates.

Acetate is another by-product of *L. reuteri* fermentation, which has a relevant role on the regulation of this process. The presence/absence of initial acetate in the culture medium was found to affect its production during fermentation. As expected, the final titer was higher in samples with acetate added in the medium, but higher acetate production was observed in the samples without initial acetate (≈ 3.6 g L^{-1} of acetate produced, compared to ≈ 2.9 g L^{-1} in samples without acetate). The increase of pressure promoted a general decrease in acetate production during fermentation. With initial acetate (Figure 4.4.b), fermentation at 0.1 and 10 MPa showed close acetate concentration profiles over time, with similar final titers of 5.84 and 5.72 g L^{-1}, respectively. At 25 MPa, acetate concentration only started to increase after 24 h, achieving a final titer of 4.64 g L^{-1}. In this case, acetate yield was higher than at 0.1 and 10 MPa (with an

increase of 12 % and 20 %, respectively), suggesting a stimulation of acetate formation at 25 MPa. In samples without acetate (Figure 4.4.e), acetate production was considerably reduced when fermentation was performed at 10 and 25 MPa, leading to lower final titers (1.75 and 0.87 g L^{-1}) compared to 0.1 MPa (3.76 g L^{-1}). As a consequence, the acetate yield at 10 MPa was nearly 2-fold lower than the yield at 0.1 MPa, which demonstrates a clear inhibitory effect of HP on acetate production in these samples. At 35 MPa, no acetate production was detected during fermentation, regardless of the initial acetate concentration.

Another product that can be obtained from *L. reuteri* glucose metabolism is ethanol, but usually at low concentrations in glycerol/glucose co-fermentation processes (Jolly et al., 2014; Ricci et al., 2015). In fact, the highest ethanol concentration in this study corresponded to 1.19 g L^{-1}, which is considerably lower than the concentrations of other fermentation by-products. Ethanol production was even lower under HP, regardless of the presence or absence of acetate in the initial culture medium. In samples with acetate (Figure 4.4.c), ethanol production was detected not only at 0.1 MPa, but also at 10 and 25 MPa. In the case of samples without acetate (Figure 4.4.f), ethanol was only produced at 0.1 and 10 MPa, with considerably lower concentrations at 10 MPa.

Overall, the results in this section suggest an inhibitory effect of HP on the formation of lactate, acetate and ethanol. In order to obtain an optimum yield of 1,3-PDO, these compounds should be maintained at the lowest level or even completely stopped (Lee et al., 2015). It is important to note that these by-products and 1,3-PDO are produced from different substrates using different metabolic pathways, but both are necessarily related, due to NADH/NAD^{+} recycling and competition. Because of that, regulation of glycerol bioconversion is highly dependent on glucose metabolism. In addition, acetate, ethanol, and mostly lactate may act as inhibitors of *L. reuteri* growth and fermentation (Ricci et al., 2015; Vieira et al., 2015). Because of all that, 1,3-PDO biosynthetic ability and yield may be improved by minimizing by-product formation (Jolly et al., 2014; Lüthi-Peng et al., 2002).

To better understand the effects of HP on *L. reuteri* metabolic selectivity, molar ratios between 1,3-PDO and the by-products were estimated for all pressure conditions, in the presence and absence of initial acetate (Table 4.4). Regardless of the presence/absence of acetate, the highest 1,3-PDO:by-products ratios were achieved at 10 MPa, indicating that *L. reuteri* product selectivity has shifted towards production of 1,3-PDO at this pressure. This effect was considerably more pronounced when fermentation was carried out without initial acetate: in this case, the ratio at 10 MPa has more than doubled relatively to 0.1 MPa (0.546 and 0.260, respectively). Since the highest 1,3-PDO yield (0.414 g g^{-1}) was also reached at these fermentation conditions, these seem to be the most suitable to perform *L. reuteri* fermentation for 1,3-PDO

production. By increasing pressure to higher pressure levels (25 and 35 MPa), the 1,3-PDO/by-products ratios decreased, or were even impossible to estimate.

Table 4.4. Molar ratios between 1,3-PDO and by-products (lactate, acetate, and ethanol) produced after 32 h of fermentation at different pressure conditions, in samples with and without acetate.

Samples	Pressure	Molar ratio 1,3-PDO:by-products
With acetate	0.1 MPa	0.249
	10 MPa	0.284
	25 MPa	0.200
	35 MPa	0.014
Without acetate	0.1 MPa	0.260
	10 MPa	0.546
	25 MPa	-
	35 MPa	-

Modification of product selectivity by application of HP at 10 MPa would represent a valuable improvement of the process, not only due to enhancement of 1,3-PDO yields, but also reduction of formation of undesired by-products that may limit the scale-up of fermentation and its widespread application in the industry. The mechanisms promoting these HP effects are still not clearly explained in literature. Bothun et al. (2004) observed shifts in product selectivity towards ethanol production by *Clostridium thermocellum* under HP (7 and 17 MPa), and explained it with possible mass-action effects and changes in membrane fluidity. Both of these aspects may also be related to the modifications in product selectivity observed in the present work, even if concerning a different microorganism and a different fermentation process. Although the impact of HP on microbial cells vary according to the microbial strain in question, pressure exerts some general effects on living organisms, predominantly affecting some key pressure sensitive structures and processes (Mota et al., 2013).

4.4. Conclusions

This corresponds to the first study regarding the effects of HP on *L. reuteri*, or on any glycerol fermentation process. High pressure (in the range of 10 and 35 MPa) was found to affect *L. reuteri* growth and co-fermentation, with the effects varying not only according to the applied pressure level, but also to the initial acetate content in the samples. In general, fermentation was

less inhibited by HP when acetate was present in the initial culture medium, indicating that acetate enhances the resistance of *L. reuteri* to pressure. This suggests that acetate may have a role on the cell response mechanisms developed under HP stress.

Under specific conditions, application of HP on *L. reuteri* co-fermentation stimulated 1,3-PDO production. At 10 MPa, higher titers, yields and productivities were observed, compared to the same process at 0.1 MPa. The enhancement effect in 1,3-PDO titer was more pronounced in the presence of acetate in the culture medium: in this case, 1,3-PDO production was faster at 10 MPa, while yield and productivity were increased by 11 and 12 %, respectively, relatively to 0.1 MPa. In samples without initial acetate, 1,3-PDO production was slower at 10 MPa than at 0.1 MPa, but similar titers were obtained after 32 h, and the yield was ≈ 26 % higher at 10 MPa. In any case, the 1,3-PDO concentrations achieved in this work were lower compared to some studies in literature. However, similarly to other studies of fermentation under pressure, we intended to perform a study in a small scale, as a proof of concept to assess possible improvements in microbial growth and fermentation. Further process optimization and scale-up could lead to enhanced production of 1,3-PDO, with concentrations in the range of those reported for fermentation in bioreactors.

Overall, HP can be a useful tool to modify and improve *L. reuteri* fermentation, by using specific conditions adapted to that purpose. Moreover, this opens the way for utilization of this technology to stimulate other fermentations processes, resulting in a real impact of HP technology in the food and biotechnological fields.

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CHAPTER V

Utilization of glycerol during consecutive cycles of *Lactobacillus reuteri* fermentation under pressure: The impact on cell growth and fermentation profile

Adapted from:

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5.1. Introduction

Lactobacillus reuteri is a lactic acid bacterium (LAB) with high biotechnological value, since it is able to produce relevant biochemical compounds from glycerol, including 1,3-propanediol (1,3-PDO) (Burgé et al., 2015; Dishisha et al., 2015, 2014; Jolly et al., 2014; Lee et al., 2015; Ricci et al., 2015; Vollenweider and Lacroix, 2004). This compound is a chemical intermediate, widely used in different industries (Biebl et al., 1999; Katrlík et al., 2007; Menzel et al., 1997; Saxena et al., 2009). It is also a GRAS (Generally Recognized As Safe) food and beverage ingredient, commercialized by DuPont Tate & Lyle Bio Products (DuPont Tate & Lyle Bio Products, 2018). The global market of 1,3-PDO has considerably increased in recent years, with the global demand achieving 60.2 ktons in 2012, and being expect to reach ≈ 150.0 ktons in 2019 (Lee et al., 2015; Research TM, 2012).

For the production of 1,3-PDO by *L. reuteri*, a co-fermentation with glycerol and glucose is usually performed, since glycerol is used only as an electron acceptor, and not as a carbon source for growth (Ricci et al., 2015). Glycerol metabolism in *L. reuteri* occurs in two steps: in the first one, glycerol is dehydrated into 3-hydroxypropionaldehyde (3-HPA); and in the second step, 3-HPA is reduced into 1,3-PDO (El-Ziney et al., 1998; Schütz and Radler, 1984; Talarico and Dobrogosz, 1989). In contrast, glucose is metabolized by the glycolysis pathway, leading to the formation of various by-products, such as ethanol, lactate, acetate, and others (El-Ziney et al., 1998; Talarico and Dobrogosz, 1989). Although both substrates are metabolized by alternative pathways, the production of 1,3-PDO from glycerol is dependent on glucose availability (Lüthi-Peng et al., 2002), as well as other factors, such as aeration and acetate concentration (Jolly et al., 2014). Acetate has been recognized as a microbial growth inhibitor, but some studies report a positive influence of certain acetate concentrations on LAB growth and fermentation (Iino et al., 2002; Jolly et al., 2014). A possible explanation is the ability of acetate to act as an indirect proton and electron acceptor, leading to the activation of oxidative pathway at early stages of fermentation (Heyndrickx et al., 1991; Jolly et al., 2014).

The concept of sub-lethal high pressure (HP) is gaining relevance in the last decade, mainly due to piezophilic microorganisms, which are showing to have considerable interest for use in biotechnology (Mota et al., 2013). For instance, piezophilic microorganisms living under deep-sea environments are attracting the attention of several pharmaceutical companies wishing to obtain sources of compounds with relevant biological activity and novel properties (Mota et al., 2018). Piezophiles are not the only types of microorganisms with capacity to withstand high pressure, since some mesophilic microorganisms develop mechanisms to improve pressure resistance, which allow them to grow under these stress conditions (Mota et al., 2013; Oger and Jebbar, 2010). The exposure of bacterial cells to stressful conditions, such as HP, during growth and fermentation, involves a complex network of response mechanisms, with several metabolic

activities that will reflect upon the metabolome of the fermentative microorganisms, and thus on the bioproducts and on the bioprocess itself (Serrazanetti et al., 2009). Some of the changes promoted by these stress responses may have a positive outcome, such as improved fermentation rates, yields and productivities (Bravim et al., 2012; Picard et al., 2007), changes in metabolic selectivity (Bothun et al., 2004), or even production of compounds with different properties (Kato et al., 2007). In this context, application of sub-lethal HP is particularly suitable and has high versatility, as it can be applied as intermittent stresses or continuously during the whole fermentation time, without serious cell loss and no heating effect. In addition, it is only necessary to provide energy to generate the pressure (and not to maintain it) and so, application of HP stress during the whole fermentation process has minimal energetic costs.

Since stress is one of the major driving forces of microbial evolution and adaptation (Serrazanetti et al., 2009), application of sub-lethal HP may promote the development of microorganisms with new features, some of them with potential technological interest. Considering that, the present work intended to assess adaptation of *L. reuteri* DSM 20016 to pressure, during four consecutive fermentation cycles under sub-lethal HP levels (10 and 25 MPa). Cell viability, growth and fermentation profiles were evaluated throughout the study, to understand how pressure affected each fermentation cycle, giving special focus to the production of 1,3-PDO.

5.2. Material and methods

5.2.1. Microorganism and culture media

A lyophilized culture of *Lactobacillus reuteri* DSM 20016 obtained from DSMZ, Germany, was used in this study. The strain was reconstituted on commercial MRS medium, according to the manufacturer's instructions. Cells were maintained at -80 ºC in a cryoprotectant solution.

Commercial MRS medium was used for seed culture preparation and growth. For the fermentation experiments, two different media were used: *i)* with an initial acetate concentration of 5 g L^{-1}, correspondent to commercial MRS broth, composed by peptone (10 g L^{-1}), meat extract (10 g L^{-1}), yeast extract (5 g L^{-1}), dipotassium phosphate (2 g L^{-1}), ammonium citrate (2 g L^{-1}), magnesium sulphate (0.1 g L^{-1}), manganese sulphate (0.05 g L^{-1}), Tween 80 (1.0 g L^{-1}), glucose (20 g L^{-1}), sodium acetate (5 g L^{-1}), and glycerol (20 g L^{-1}); and *ii)* without initial acetate, prepared with the same components except the sodium acetate.

5.2.2. Seed culture preparation

A single cell colony was seeded into 10 mL MRS broth and incubated for 10 - 12 h at 37 ºC, in static conditions. The culture was then transferred to a 250 mL MRS medium without acetate in a 300 mL Erlenmeyer flask, and incubated overnight at 37 ºC, in static conditions.

5.2.3. Fermentation experiments

The medium was inoculated with 10 % (v/v) of seed culture. The mixture was homogenized and then transferred to polyethylene bags, which were carefully sealed with the minimal air possible. All these steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination.

Fermentation experiments were performed during four consecutive cycles of 24 h, at 37 ºC, under different pressure conditions (10 and 25 MPa). After each cycle, the fermentative medium was collected, analyzed, and used as inoculum for the subsequent fermentation cycle, carried out at the same pressure conditions (Figure 5.1). All fermentations were performed under unaerated conditions.

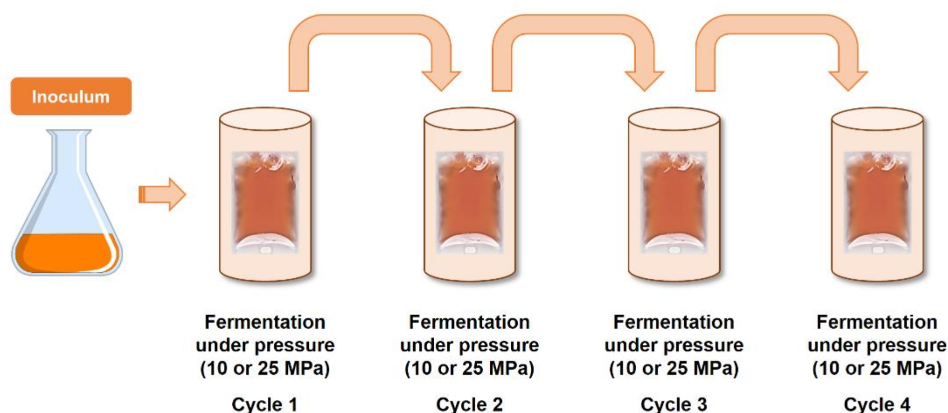

Figure 5.1. Schematic representation of the fermentation experiments, carried out in four consecutive cycles of 24 h, at 10 or 25 MPa. Fermentation cycles were also performed at 0.1 MPa, to use as control.

The experiments were conducted in a HP vessel SFP FPG7100 (Stansted Fluid Power Ltd, Essex, UK), using a mixture of propylene glycol and water (40:60) as pressurizing fluid. This equipment is a hydrostatic press with a pressure vessel of 100 mm inner diameter and 250 mm height, surrounded by an external jacket to control the temperature. In parallel, a control sample carried out fermentation at atmospheric pressure (0.1 MPa), while maintaining constant the remaining process conditions. To all pressure conditions (including atmospheric pressure), two different types of samples were studied: *i)* with acetate, corresponding to samples with an initial

acetate concentration of 5 g L^{-1}; *ii)* and without acetate, corresponding to samples with no acetate added in the culture medium. In all cases, fermentation experiments were performed in duplicate. Samples were collected after each cycle, and all the analyses were also performed in duplicate.

5.2.4. Determination of biomass concentration

Biomass concentration of the samples was determined by optical density measurement at 600 nm, with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA). Cell dry weight (CDW) was routinely determined using a standard curve relating *L. reuteri* optical density and CDW.

5.2.5. Determination of viable cell counts

For determination of viable cells, serial dilutions (using Ringer solution) of the culture samples were prepared and aliquots of 1.0 mL of proper dilutions were plated in MRS agar (using the pour plate technique), and incubated at 37 °C for 24 h.

5.2.6. Quantification of glycerol, glucose, and fermentation products

Culture samples were centrifuged at 8,000 *g* and 4 ºC for 10 min and the collected supernatants were filtered through a 0.22 μm filter membrane. Analysis by HPLC was performed using a HPLC Knauer system equipped with Knauer K-2301 RI and K-2501 UV detectors and an Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase was 13 mM H_2SO_4, delivered at a flow rate of 0.6 mL min^{-1} and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards. Because of co-elution of glycerol and lactate, and since glycerol is only detected by the RI detector, but lactate is detected by both detectors, lactate concentration was estimated through the UV detector. The RI area corresponding to the lactate concentration was estimated using the corresponding RI calibration curve. Glycerol quantification was determined by the difference between the total area of the RI peak and the calculated contribution of lactate in the area of the peak.

5.2.7. Analysis of the leakage of nucleic acids and proteins

Effect of HP on membrane permeability of *L. reuteri* was measured by determining the contents of nucleic acids and proteins on extracellular medium, according to Dai et al. (2017). After each fermentation cycle, changes of nucleic acid and extracellular protein contents were determined based on their maximum absorption wavelength at 260 nm and 280 nm, respectively. The absorbance (A) of the samples (fermented under HP conditions or at 0.1 MPa) was measured at 260 nm and 280 nm after centrifugation at 8,000 *g* for 10 min. Nucleic acid or extracellular

protein contents (%) were calculated by comparison between the values obtained at HP and the ones at 0.1 MPa, using the following equation:

$$Nucleic\ acid\ or\ extracellular\ protein\ (\%) = \frac{A_{high\ pressure} - A_{0.1\ MPa}}{A_{0.1\ MPa}} \times 100$$

where $A_{high\ pressure}$ corresponds to the absorbance of samples obtained at HP (10 or 25 MPa), and $A_{0.1\ MPa}$ corresponds to the absorbance of samples obtained at atmospheric pressure (0.1 MPa).

5.3. Results and discussion

5.3.1. Cell growth

To understand how different levels of sub-lethal HP affected *L. reuteri* cells throughout the fermentation cycles, biomass concentration and viable cell counts were determined. Figure 5.2 shows the variation of both parameters after each fermentation cycle, at different pressure conditions (10 and 25 MPa, as well as 0.1 MPa as control), for the samples with added acetate in the culture medium (Figures 5.2.a and 5.2.b), and the ones without added acetate (Figures 5.2.c and 5.2.d). In the course of these fermentation cycles, the initial acetate concentration and the pressure applied were both found to affect cell growth.

In samples with initial acetate, biomass concentration showed a general decreasing trend throughout the cycles at 0.1 and 10 MPa, indicating no improvement of cell growth. At 25 MPa, biomass concentrations were always lower than at 0.1 and 10 MPa, and varied differently over the cycles, with the highest concentration being reached at the second cycle (1.73 g L^{-1}), and gradually decreasing thereafter. Still, biomass concentration at the fourth cycle (0.97 g L^{-1}) was about 2-fold higher that the observed for first one (0.57 g L^{-1}), which contrasts with the trend at the other pressures, and suggests an improvement in cell ability to grow at 25 MPa. Regardless of the pressure conditions applied, viable cell counts showed only slightly variation throughout the cycles. In addition, cell counts were considerably higher at 10 and 25 MPa compared to 0.1 MPa, which may result from differences in cell growth curves under different pressure conditions.

In samples without added acetate, biomass concentrations at 0.1 MPa remained relatively stable over the cycles, with a slight trend to increase (from 2.52 g L^{-1} in the first cycle, to 2.79 g L^{-1} in the last one). Application of HP to the process, 10 or 25 MPa, led to lower biomass concentrations in the first fermentation cycle: 0.66 and 0.24 g L^{-1} were observed for 10 and 25 MPa, respectively. However, biomass concentration was found to considerably increase in the subsequent HP-cycles, indicating adaptation of *L. reuteri* cells to these conditions. At 10 MPa,

biomass concentration increased to 2.87 g L^{-1} in the second cycle, and remained in this range of values during the following ones. At 25 MPa, the highest biomass concentration (2.13 g L^{-1}) was reached at the third cycle, but decreased to 0.63 g L^{-1} in the fourth one, showing that the eventual resistance and adaptation mechanisms developed at 25 MPa were not stable throughout the fermentation cycles.

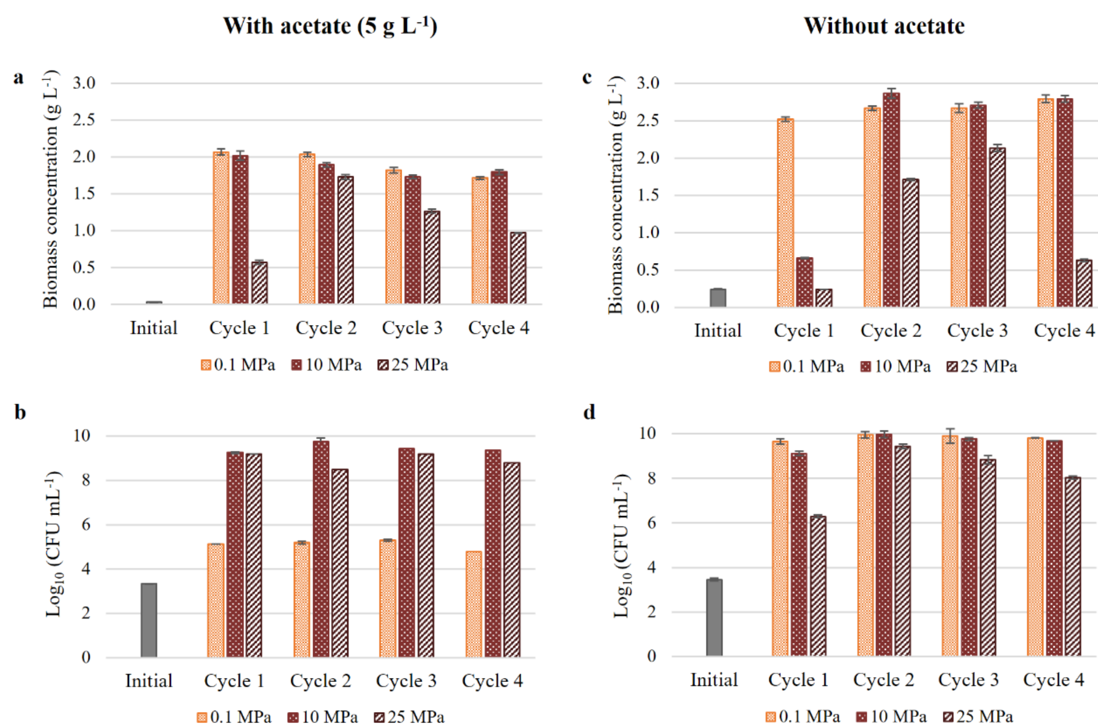

Figure 5.2. Biomass concentration and viable cell counts after each fermentation cycle (24 h), for samples with an initial acetate concentration of 5 g L^{-1} (a and b), and for samples without initial acetate (c and d), according to the pressure applied: 0.1 MPa (control samples), 10 MPa, and 25 MPa.

Similarly, the effects of HP on cell counts were more prominent in the first fermentation cycle, with lower values at 10 and 25 MPa compared to 0.1 MPa. However, such as observed for biomass concentration, cell growth was stimulated during the subsequent fermentation cycles, reaching cell counts of 9.69 and 8.04 log_{10}(colony forming units (CFU) mL^{-1}) after the fourth cycle at 10 and 25 MPa, respectively. These results suggest development of stress response mechanisms to improve HP tolerance. Interestingly, this effect seems to be dependent of the initial acetate concentration in the culture medium, since it was solely observed for samples without acetate.

Pavlovic et al. (2008) have also reported adaptation of a lactobacilli strain to HP, by isolating a piezotolerant *Lactobacillus sanfranciscensis* after incubation at 50 MPa during 25

cycles. Compared to the wild type, that strain showed enhanced ability to grow at 50 MPa. The authors proposed that piezotolerance was promoted by increased expression of ssrA, leading to higher amounts of tmRNA, a small RNA that provides a quality control function for translation by recognizing stalled ribosomes and targeting the incomplete proteins for degradation. Therefore, tmRNA might help to prevent accumulation of truncated, potentially harmful proteins at higher pressures and make proteolysis more efficient (Pavlovic et al., 2008). Several other genes were reported to be upregulated under sub-lethal HP conditions, in different microorganisms. These include lipid synthesis genes, to increase the proportion of unsaturated fatty acids in the cellular membrane and thus improve fluidity, and chaperone-encoding genes, to help in maintaining protein folding and thus protein function (Oger and Jebbar, 2010). Increased growth under HP may be also promoted by expression of HP-specific genes (Kato and Qureshi, 1999), and adaptation of the biomolecules structure to sustain pressure (Chilukuri and Bartlett, 1997). Most of these pressure-adaptation mechanisms would affect not only the cell ability to grow under pressure, but also the overall metabolic and biological profile of the microorganism. With this in mind, the main substrates and products involved in *L. reuteri* fermentation were evaluated, being the results reported in further sections.

5.3.2. Substrate consumption

During fermentation, *L. reuteri* simultaneously consumes two carbon sources: glucose, which is directed to the glycolysis pathway and results in the production of several products (e.g. lactate, acetate and ethanol); and glycerol, which is metabolized by a different pathway, using enzymes encoded in its propanediol-utilization (pdu) operon (Zaushitsyna et al., 2017). Therefore, the consumption of both substrates was monitored throughout the fermentation cycles, at different pressure conditions. The results are presented in Figure 5.3, for samples with added acetate in the culture medium (Figures 5.3.a and 5.3.b), and for the ones without added acetate (Figures 5.3.c and 5.3.d). In general, the uptake of glycerol and glucose showed similar profiles throughout the cycles, as already observed in previous studies (Doleyres et al., 2005; Jolly et al., 2014; Lüthi-Peng et al., 2002). This effect is explained by the dependency between them, since the NADH generated from glucose by the glycolysis pathway is recycled by the presence of glycerol and its transformation into 1,3-PDO (Doleyres et al., 2005).

At 0.1 MPa, the samples with acetate showed only slight variations in substrate uptake over the cycles, for both glycerol and glucose. At 10 MPa, substrate uptake was lower in the first cycle, but increased in the following ones, reaching residual concentrations similar to 0.1 MPa at the fourth cycle. This increase in substrate consumption at 10 MPa was not related to cell growth, since biomass concentration was reduced over the cycles at this pressure (Figure 5.2.a). This indicates enhanced production of specific metabolites at 10 MPa, which may be related to the *L.*

reuteri response to pressure. At 25 MPa, substrate uptake seems to be inhibited, being usually lower than at 0.1 and 10 MPa. The highest uptake was reached in the second cycle, but it decreased thereafter in the subsequent cycles, similarly to the observed for cell growth (Figure 5.2.a).

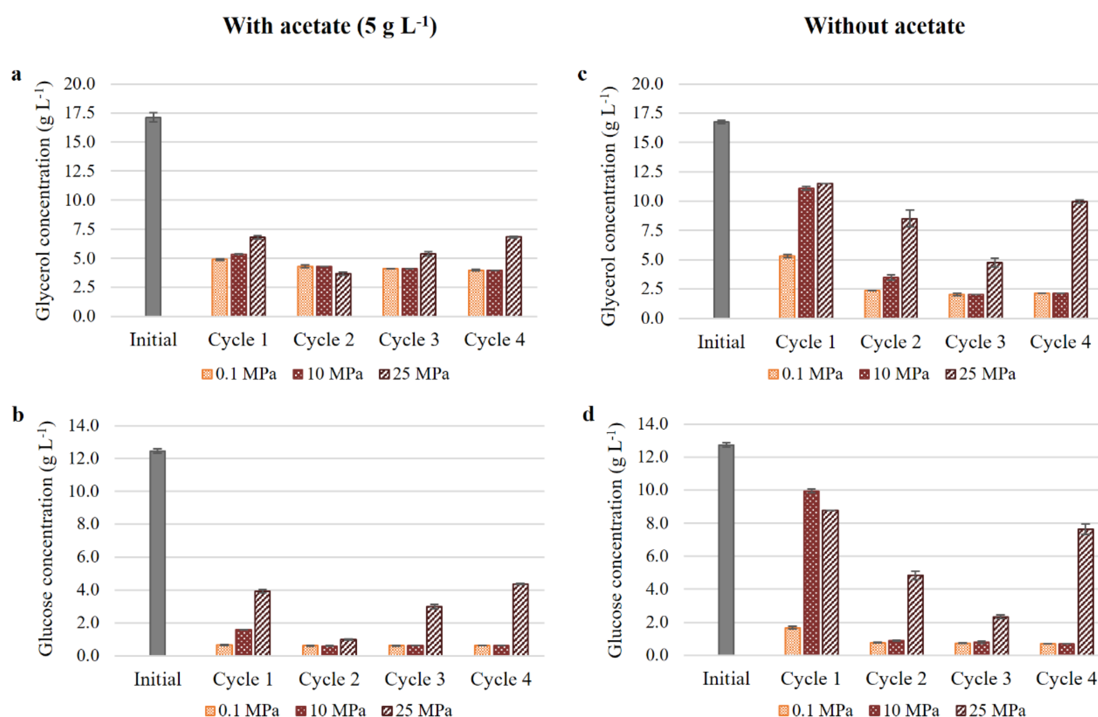

Figure 5.3. Glycerol and glucose concentrations after each fermentation cycle (24 h), for samples with an initial acetate concentration of 5 g L^{-1} (a and b), and for samples without initial acetate (c and d), according to the pressure applied: 0.1 MPa (control samples), 10 MPa, and 25 MPa.

Regarding the samples without initial acetate, substrate uptake increased over the fermentation cycles at 0.1 MPa. At 10 MPa, substrate consumption was low in the first cycle, but considerably increased in the subsequent ones. At the fourth cycle, the residual glycerol and glucose concentrations were identical to the ones observed at the correspondent cycle at 0.1 MPa. This pronounced increase in substrate consumption can be related to metabolic changes in response to pressure, and even development of adaptation mechanisms to improve the *L. reuteri* ability to grow and ferment under pressure. Fermentation cycles at 25 MPa showed similar behavior, with low substrate uptake in the first cycle, and a subsequent increase in the second and third cycles. However, glycerol and glucose consumption decreased in the fourth one, with the residual concentrations coming closer to the ones in the first cycle. This effect of “improvement and decline” was detected in both fermentation samples (with and without acetate) at 25 MPa, but at different timelines: in samples with acetate, the peak of substrate consumption was at the

second cycle, while in samples without acetate the peak was at the third one. A similar effect was also observed for *Saccharomyces cerevisiae* in another work of our research group (unpublished results).

Therefore, application of consecutive fermentation cycles was found to improve substrate consumption of both glycerol and glucose. This improvement was observed not only under HP conditions, but also at 0.1 MPa. However, the enhancement of substrate consumption was more pronounced under HP, particularly at 10 MPa. The samples without acetate were also more prone to enhance substrate consumption and, thus, they seem more able to adapt to HP. The increase in glucose consumption over the cycles might be associated to cell growth, but only when biomass concentration was also increased, e.g. in samples without acetate. The cases with no cell growth throughout the cycles (e.g. samples with acetate) imply that the surplus of glucose consumed has been used to other purposes, such as increased production of metabolites involved in cell maintenance, with functions that include osmoregulation, cell motility, turnover of macromolecular compounds and defense mechanisms (Van Bodegom, 2007). Since glycerol is not a carbon source for growth, the increase in glycerol uptake is certainly not related to cell growth, and might indicate the increased production of some glycerol-derived compounds, such as 1,3-PDO.

5.3.3. Production of 1,3-propanediol

In our previous study with *L. reuteri*, the production of 1,3-PDO was found to be stimulated during fermentation at 10 MPa (one 32 h-cycle), in samples with acetate (unpublished results). In the present work, we intended to assess if the application of consecutive cycles of fermentation under pressure could improve even further the production of this bio-compound. The results for these 24 h-fermentation cycles are presented in Figure 5.4, for samples with added acetate in the culture medium (Figure 5.4.a), and for those without added acetate (Figure 5.4.b). The 1,3-PDO yields ($Y_{1,3\text{-PDO/Gly}}$, g g^{-1}) and productivities ($Q_{1,3\text{-PDO}}$, g L^{-1} h^{-1}) were also estimated, as indicated in Table 5.1.

When fermentation was performed at 0.1 MPa, the production of 1,3-PDO increased between the first and the fourth cycles, regardless of the presence/absence of initial acetate. As consequence, higher 1,3-PDO titers, yields and productivities were achieved at the fourth cycle, compared to the first one. This positive effect at 0.1 MPa demonstrated that the use of consecutive fermentation cycles itself stimulates the production of 1,3-PDO, as well as substrate consumption and, in some cases, even cell growth. This can be explained by an adaptive process to the stresses naturally involved in fermentation (even at 0.1 MPa), such as osmotic pressure, oxidative stress, reduction of pH, and others (Serrazanetti et al., 2009).

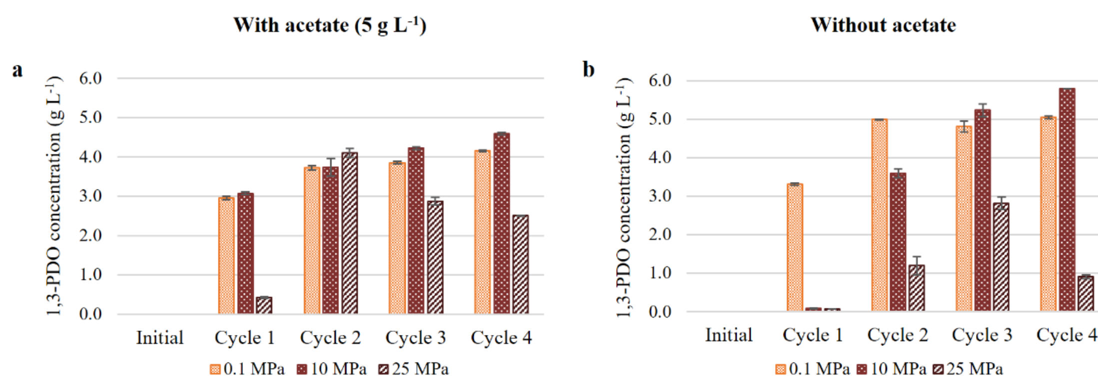

Figure 5.4. 1,3-Propanediol concentration after each fermentation cycle (24 h), for samples with an initial acetate concentration of 5 g L^{-1} (a), and for samples without initial acetate (b), according to the pressure applied: 0.1 MPa (control samples), 10 MPa, and 25 MPa.

In fermentation cycles under HP, the profiles of samples with and without added acetate were different from each other, and will be discussed separately. In the case of samples with acetate, the production of 1,3-PDO at 10 MPa was usually higher than at 0.1 MPa. Over the cycles, 1,3-PDO production gradually increased at both conditions, but more pronouncedly at 10 MPa. As consequence, the final titer at the fourth cycle was significantly higher at 10 MPa than at 0.1 MPa (4.59 and 4.16 g L^{-1}, respectively). Yields and productivities were also higher at 10 MPa, corresponding to improvements of ≈ 10 % relative to same cycle at 0.1 MPa. It is worthy to note that, in our previous study, similar yield and productivity improvements were already achieved at 10 MPa, for these type of samples (with acetate), after only one fermentation cycle but with longer duration (32 h) (unpublished results). Therefore, although these fermentation cycles under HP were favorable for the production of 1,3-PDO, it is arguable if this corresponds to a meaningful improvement. It may not be worth to use it in this particular case, since this approach involves more complex, expensive and time-consuming processes. However, adaptation to pressure might open new possibilities, with different metabolic features and different process applications.

At 25 MPa, the production of 1,3-PDO was considerably improved between the first and second cycles (from 0.43 to 4.11 g L^{-1}), but this effect was not stable and did not persist in the following cycles. At the fourth cycle, a titer of 2.51 g L^{-1} was achieved, which is higher than the obtained at the first cycle, but lower than the found at the second and third ones. At the moment, there is still no explanation for this "improvement and decline" effect, but it suggests that *L. reuteri* might not be able to adapt (at least not steadily) to a pressure of 25 MPa. In fact, continuous application of this pressure may cause extensive damaging effects to the bacterial cells. For instance, pressures between 10 – 26 MPa in *E. coli* are enough to impair relevant cellular processes, such as motility, substrate transport and cell division (Oger and Jebbar, 2010).

Table 5.1. 1,3-Propanediol yields on glycerol ($Y_{1,3\text{-PDO/Gly}}$) and 1,3-PDO productivities ($Q_{1,3\text{-PDO}}$), at the end of each fermentation cycle (24 h), at different pressure conditions, in samples with and without acetate.

Samples	Pressure	Cycle	$Y_{1,3\text{-PDO/Gly}}$ (g g^{-1})	$Q_{1,3\text{-PDO}}$ (g L^{-1} h^{-1})
With acetate	0.1 MPa	1	0.242	0.123
		2	0.291	0.155
		3	0.296	0.161
		4	0.317	0.173
	10 MPa	1	0.262	0.128
		2	0.292	0.156
		3	0.325	0.176
		4	0.350	0.191
	25 MPa	1	0.041	0.018
		2	0.306	0.171
		3	0.245	0.120
		4	0.244	0.104
Without acetate	0.1 MPa	1	0.290	0.138
		2	0.347	0.208
		3	0.327	0.200
		4	0.346	0.211
	10 MPa	1	0.016	0.004
		2	0.272	0.150
		3	0.356	0.218
		4	0.397	0.242
	25 MPa	1	0.016	0.003
		2	0.145	0.050
		3	0.236	0.118
		4	0.135	0.038

Yields and productivities were calculated from a single time-point corresponding to the end of each cycle (24 h). Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated.

If the use of consecutive fermentation cycles under HP did not seem to bring sufficient gain in the samples with acetate, the same is not the case in the samples without acetate. At the fourth cycle, fermentation at 10 MPa had significantly higher 1,3-PDO titer (5.80 g L^{-1}), as well as higher yield and productivity (0.397 g g^{-1}, and 0.242 g L^{-1} h^{-1} respectively), compared to 0.1 MPa. These correspond to yield and productivity improvements of $\approx$ 15 %. In our previous study with one 32 h-cycle (unpublished results), the samples without acetate at 10 MPa showed higher

1,3-PDO yield (0.414 g g^{-1}), but considerably lower titer (3.42 g L^{-1}) and productivity (0.107 g L^{-1} h^{-1}) after 32 h. Therefore, in samples without acetate, the consecutive cycles of fermentation at 10 MPa seem to have a positive effect on *L. reuteri* fermentation, in terms of 1,3-PDO production. In contrast, over the cycles at 25 MPa, the 1,3-PDO production was always lower than at 0.1 MPa. The highest titer (2.82 g L^{-1}) was achieved at the third cycle, and then decreased in the subsequent one, thus indicating that *L. reuteri* was not able to adapt to these pressure conditions, at least within the number of cycles studied.

The 1,3-PDO titers reached in this work are in the range of some of the values reported in literature for *L. reuteri* glycerol/sugar co-fermentation (≈ 4 g L^{-1}) (El-Ziney et al., 1998; Ragout et al., 1996). However, these are considerably lower compared to other fermentation studies with LAB. For instance, *Lactobacillus diolivorans* has been reported to produce 1,3-PDO concentrations as high as 74 g L^{-1} or 92 g L^{-1} in fed-batch experiments (Lindlbauer et al., 2017; Pflügl et al., 2012). For fermentation with *L. reuteri*, Jolly et al. (2014) reached 1,3-PDO concentrations of 65.3 g L^{-1} during fed-batch experiments, while Baeza-Jiménez et al. (2011) and Ricci et al. (2015) obtained concentrations of 29 and 41 g L^{-1}, respectively, for batch cultivations. Nevertheless, it is important to note that all of these studies were performed in bioreactors, with monitoring, adjustment and optimization of several important process parameters. In the present work, fermentation was carried out in batch, with different and simpler experimental set-up, as a proof of concept to evaluate if it was possible to carry out the process under these pressure conditions, and if it could promote any improvement.

In short, application of consecutive fermentation cycles at 10 MPa stimulated the production of 1,3-PDO, regardless of the presence/absence of acetate in the initial culture medium. However, the improvement of 1,3-PDO production was more pronounced in samples without acetate, with an increase of 1,3-PDO titer, yield and productivity by 15 % at the fourth fermentation cycle, relative to the same cycle at 0.1 MPa. By comparing the use of fermentation cycles at 10 MPa with "conventional fermentation", i.e. without the fermentation cycles and at 0.1 MPa, improvements of 52 %, 37 %, and 75 % were achieved for titer, yield and productivity, respectively. This represents a meaningful process enhancement and can have a real impact in the biotechnological production of 1,3-PDO. The reasons behind these improvements are not completely understood, but might be related to the development of general and specific stress responses, as well as adaptive mechanisms triggered by repeated exposure to sub-lethal HP.

5.3.4. Production of acetate, lactate and ethanol

Lactate, acetate, and ethanol are important reaction by-products obtained from glucose metabolism. The effects of consecutive fermentation cycles (at 0.1, 10 and 25 MPa) on these bio-chemicals are represented in Figure 5.5 – for lactate (Figures 5.5.a and 5.5.d), acetate (Figures

5.5.b and 5.5.e) and ethanol (Figures 5.5.c and 5.5.f). The yields (*Y*, g g^{-1}) of each of these compounds on glucose are indicated in Table 5.2.

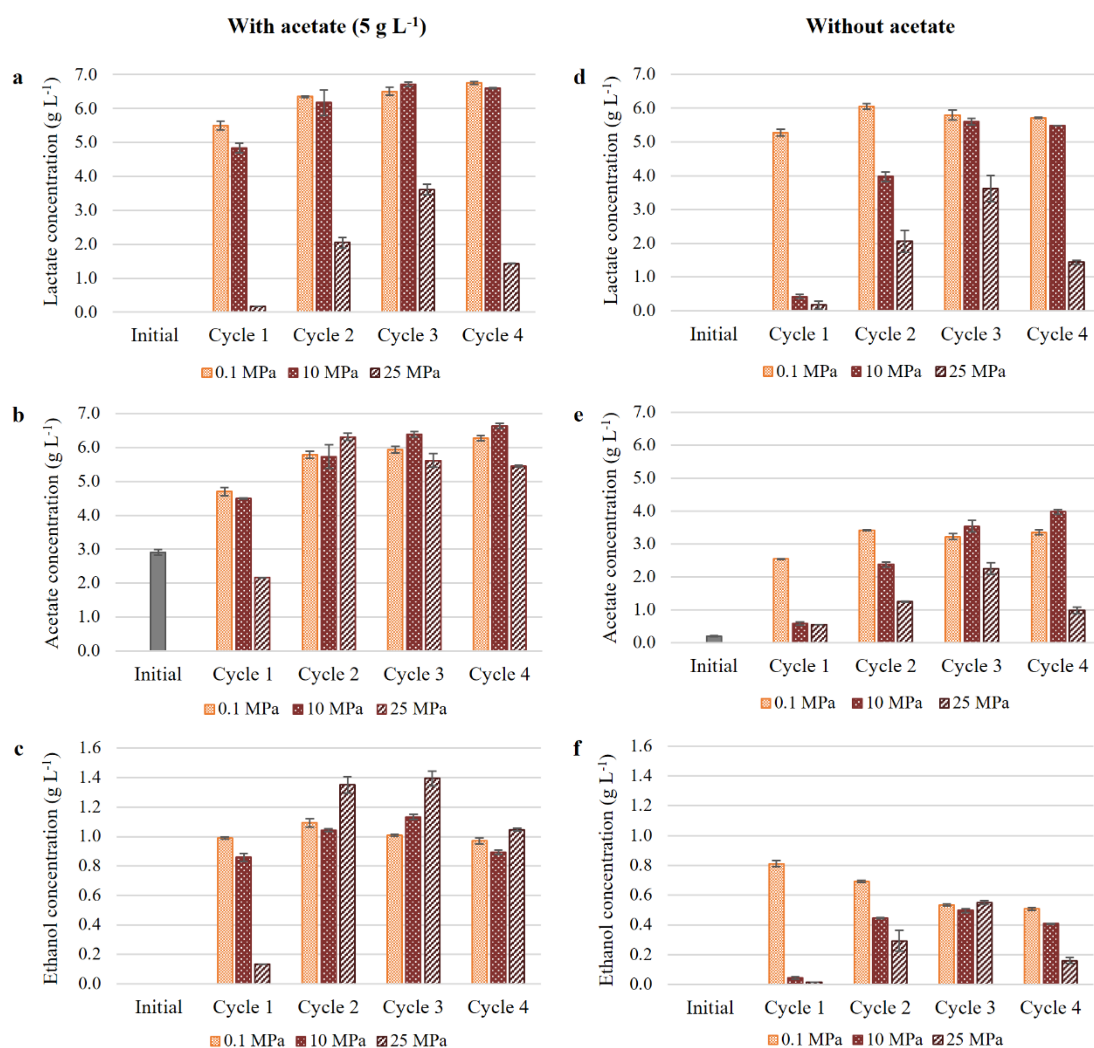

Figure 5.5. Lactate, acetate and ethanol concentrations after each fermentation cycle (24 h), for samples with an initial acetate concentration of 5 g L^{-1} (a, b and c), and for samples without initial acetate (d, e and f), according to the pressure applied: 0.1 MPa (control samples), 10 MPa, and 25 MPa.

Table 5.2. Yields of lactate ($Y_{Lact/Glu}$), acetate ($Y_{Acet/Glu}$), and ethanol ($Y_{EtOH/Glu}$) on glucose, at the end of each fermentation cycle (24 h), at different pressure conditions, in samples with and without acetate.

Samples	Pressure	Cycle	$Y_{Lact/Glu}$ (g g^{-1})	$Y_{Acet/Glu}$ (g g^{-1})	$Y_{EtOH/Glu}$ (g g^{-1})
With acetate	0.1 MPa	1	0.466	0.152	0.084
		2	0.536	0.242	0.093
		3	0.549	0.255	0.085
		4	0.571	0.284	0.081
	10 MPa	1	0.445	0.146	0.080
		2	0.521	0.238	0.088
		3	0.568	0.294	0.096
		4	0.558	0.315	0.075
	25 MPa	1	0.134	-	0.016
		2	0.468	0.297	0.116
		3	0.449	0.286	0.149
		4	0.463	0.314	0.129
Without acetate	0.1 MPa	1	0.477	0.212	0.073
		2	0.506	0.268	0.058
		3	0.483	0.251	0.045
		4	0.475	0.262	0.042
	10 MPa	1	0.153	0.135	0.016
		2	0.335	0.183	0.038
		3	0.469	0.279	0.042
		4	0.456	0.313	0.034
	25 MPa	1	0.043	0.087	0.004
		2	0.260	0.132	0.037
		3	0.348	0.197	0.053
		4	0.283	0.154	0.032

Yields were calculated from a single time-point corresponding to the end of each cycle (24 h). Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated.

In general, the production of all these by-products increased through the fermentation cycles, but with specific profiles that varied according to the by-product in question. In samples with acetate, by-product production at 10 MPa was generally similar to 0.1 MPa, with some slight differences throughout the cycles: for instance, at the fourth fermentation cycle, acetate concentration was higher at 10 MPa than at 0.1 MPa, while the opposite effect was observed for lactate and ethanol. The yields estimated for each by-product confirmed this effect, and indicate a metabolic shift promoted by the consecutive fermentation cycles at 10 MPa, favoring the production of acetate and impairing the production of lactate and ethanol. In samples without acetate, the by-product production at 10 MPa was low during the first two cycles (similarly to the production of 1,3-PDO), but reached values similar to 0.1 MPa in the subsequent cycles. In these last cycles, glucose metabolism have also shifted to the production of acetate, such as observed in the samples with initial acetate. These metabolic changes at 10 MPa may be related to the regulation of the NAD^+/NADH ratio, since the formation of lactate and ethanol competes with glycerol-derived 3-HPA for NADH. Consequently, if the cells are able to convert 3-HPA into 1,3-PDO, a lower amount of reduced fermentation products (lactate and ethanol) are formed, and a higher amount of oxidized products (acetate) can be obtained (Ricci et al., 2015). For that reason, the production of 1,3-PDO and acetate were positively correlated: the production of 1,3-PDO at 10 MPa was improved over the cycles (relative to 0.1 MPa), and promoted the formation of acetate, while reducing the formation of lactate and ethanol.

At 25 MPa the effect on by-product formation was highly variable according to the presence or absence of acetate in the medium. In samples with acetate, lactate production was significantly lower during all cycles, compared to 0.1 and 10 MPa. Acetate and ethanol production were also lower (at the first cycle, but increased in the following ones, and reached the range of concentrations obtained at 0.1 and 10 MPa. At the fourth cycle, acetate concentration at 25 MPa was lower than for other pressures, but ethanol concentration was significantly higher. In fact, the highest ethanol concentrations and yields were obtained for these samples at 25 MPa, at the third and fourth cycles. Once again, the explanation might be related to the regulation of the NAD^+/NADH ratio. At 25 MPa, the production of 1,3-PDO was generally low (Figure 5.4), possibly due to inhibition of one or more reactions involved in the glycerol-metabolizing pathway. If the conversion of 3-HPA to glycerol is inhibited, NADH generated by glycolysis has to be drained to other pathways, such as the acetylphosphate reduction to ethanol, which would be preferred over lactate, because it allows re-oxidation of twice the amount of NADH. However, this higher ethanol production at 25 MPa was not observed in the samples without acetate. In fact, these samples accumulated lower concentrations of all by-products, compared to 0.1 and 10 MPa (except ethanol, in the third cycle).

Overall, variations in by-product formation provided useful information about the *L. reuteri* metabolism under HP. Fermentation at 0.1 and 10 MPa showed an increasing by-product production over the cycles, which resulted in higher concentrations and yields. This stimulating effect throughout the cycles was also observed for 1,3-PDO production, and, in some cases, for cell growth. At 10 MPa, these improvements in product (and biomass) formation were also accompanied by an increase in substrate uptake. However, at 0.1 and 25 MPa, changes in substrate consumption were low, or even inexistent, which suggests that improved product formation in the later cycles resulted from a more efficient energy use by *L. reuteri* cells. As for the specific effects of HP, the use of consecutive cycles at these conditions promoted shifts in metabolic selectivity, which differed according to the applied pressure level: at 10 MPa, the production of acetate tended to increase over the cycles (such as previously observed for 1,3-PDO), while lactate and ethanol tended to decrease; at 25 MPa, the formation of all by-products tended to decrease, with exception of ethanol in the samples with acetate. To better understand how these fermentation cycles affected metabolic selectivity, molar ratios between 1,3-PDO and by-products were estimated for the four fermentation cycles performed at different pressure conditions (0.1, 10 and 25 MPa), in the presence and absence of initial acetate (Table 5.3).

The ratios showed a general upward trend over the cycles at 0.1 and 10 MPa, but not at 25 MPa. In addition, the ratios at 10 MPa were always higher than at the other conditions, possibly due to a shift in the metabolism towards the production of 1,3-PDO. High pressure may directly affect the conversion of glycerol into 1,3-PDO, or may affect it indirectly, by modifying glucose metabolism, which is closely related to glycerol metabolism. In fact, there are some reports in literature about HP affecting the energy metabolism, particularly in the case of glycolysis (Bucka-Kolendo and Sokołowska, 2017; Picard et al., 2007). Therefore, it is possible that some of the HP effects observed in the present work result from changes in glycolysis and glucose metabolism. The information regarding glycerol metabolism under HP is still scarce, and thus it is not certain how pressure impacts this metabolic pathway. In order to understand it, further studies are of interest, using, for instance, proteomic and metabolomics tools.

Table 5.3. Molar ratios between 1,3-PDO and by-products (lactate, acetate, and ethanol) produced after each fermentation cycle (24 h), at different pressure conditions, in samples with and without acetate.

Samples	Pressure	Cycle	Molar ratio 1,3-PDO:by-products
With acetate	0.1 MPa	1	0.242
		2	0.257
		3	0.263
		4	0.273
	10 MPa	1	0.274
		2	0.263
		3	0.270
		4	0.297
	25 MPa	1	0.109
		2	0.279
		3	0.221
		4	0.213
Without acetate	0.1 MPa	1	0.367
		2	0.472
		3	0.488
		4	0.510
	10 MPa	1	0.078
		2	0.507
		3	0.522
		4	0.560
	25 MPa	1	0.097
		2	0.313
		3	0.414
		4	0.335

Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated.

5.3.5. Leakage of proteins and nucleic acids

Several effects of HP on microbial cells are related to modification of cell membrane composition and fluidity (Mota et al., 2013). Lipid membranes are particularly pressure sensitive, due to its high compressible potential. Membrane permeability can be evaluated by the changes in the content of nucleic acids and proteins in the extracellular medium. The increase of these compounds in the extracellular medium suggests its leakage, indicating higher permeability of the cell membrane. Figure 5.6 shows the leakage of nucleic acids and proteins contents (%) in the

extracellular medium after each fermentation cycle at 10 and 25 MPa, relative to control samples (0.1 MPa). Other studies used this method on microorganisms submitted to ultrasound or pressurized CO_2 and obtained positive values for protein and nucleic acid leakage, relative to the control samples (without treatment) (Dai et al., 2017; Hashemi et al., 2018; Yao et al., 2014). However, in the present work, almost all samples at 10 and 25 MPa showed negative leakage values, which showed that HP did not increase leakage of cell constituents compared to 0.1 MPa - on the contrary, HP was found to decrease it. The implications of these results are still not completely understood, but suggest modification of cell membrane permeability under HP conditions. With increasing pressure, lipid bilayers lose fluidity and became impermeable to water and other molecules, with modification of protein-lipid interactions (Mota et al., 2013). Another possibility, only applicable to protein leakage, could be related to denaturation and aggregation of proteins caused by HP, but this phenomenon is rather unlikely at these low pressure levels, since partial protein denaturation usually occurs at pressures of 100 MPa or above (Huang et al., 2014).

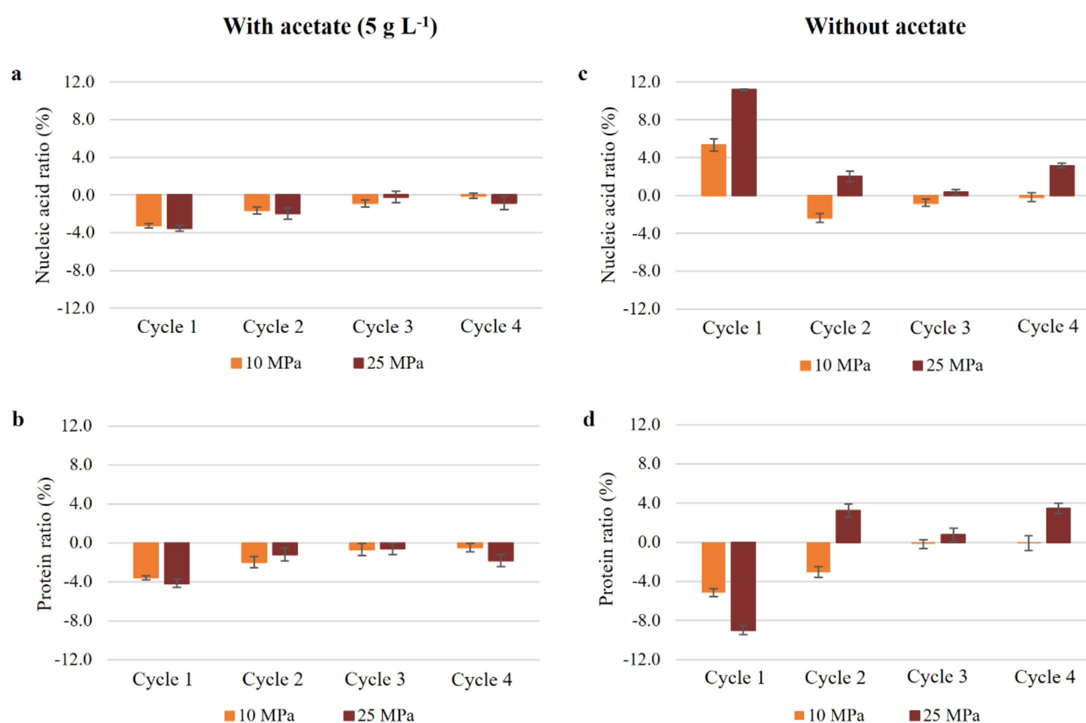

Figure 5.6. Ratio of extracellular nucleic acid and protein contents (%) after each fermentation cycle (24 h) at 10 and 25 MPa, relative to 0.1 MPa, for samples with an initial acetate concentration of 5 g L^{-1} (a and b), and for samples without initial acetate (c and d).

Considerable differences were observed between samples with and without acetate in the initial medium. In samples with acetate, negative values were obtained for all cycles at 10 and 25 MPa, indicating that these pressures decreased leakage of nucleic acids and proteins relative to 0.1 MPa, possibly due to loss of membrane fluidity and permeability. However, throughout the cycles, the relative leakage values (%) tended to come closer to zero, indicating that the HP effect on cell membrane was attenuated, as a possible adaptation to pressure. Under HP environments, several cell structures and components may be modified, due to fine tuning of gene expression, that is expected to play an important role in low pressure environments (below 40 MPa) (Oger and Jebbar, 2010). For instance, there is evidence of some microorganisms upregulating the expression of lipid synthesis genes (Allen et al., 1999; Fernandes et al., 2004; Iwahashi et al., 2005; Vezzi et al., 2005). As a result, cells may change the composition of lipid membrane under HP, through the increase of unsaturated fatty acid content, in order to increase membrane fluidity (Winter and Jeworrek, 2009). Thus, it is possible that *L. reuteri* begins to adapt to HP through the cycles, by modifying cell membrane and making it more fluid and permeable.

In samples without acetate, the first cycle at 10 MPa had a positive value for nucleic acid leakage (5.33 %), indicating higher leakage compared to 0.1 MPa. The release of nucleic acids may be a result of membrane damage and, in some cases, it might correlate with microbial inactivation (Lu et al., 2014; Pillet et al., 2016; Yao et al., 2014). Therefore, the use of HP at 10 MPa in these samples affected membrane integrity, and it may have caused bacterial inactivation. In fact, low biomass concentration was observed at these conditions, and cell counts were slightly lower compared to 0.1 MPa (Figure 5.2). However, in the following cycles at 10 MPa, the relative leakage of nucleic acids was negative (i.e. lower at 10 MPa than at 0.1 MPa), with tendency to come closer to zero. Regarding protein leakage at 10 MPa, the values were negative for all cycles (including the first one, which contrasts with the nucleic acid leakage at this cycle).

In samples without acetate fermenting at 25 MPa, the nucleic acid leakage was positive for all cycles, and more pronounced at the first cycle (11.18 %). In fact, biomass concentration and cell counts (Figure 5.2) were lower at this pressure, and the fermentative activity was reduced. Therefore, in these samples, fermentation at 25 MPa seems to cause membrane damage and, to some extent, cell inactivation. In the case of protein leakage, a negative value was observed for the first cycle, while the following ones all showed positive values. The results for these latter cycles are in accordance with the leakage of nucleic acids, and support the possibility of *L. reuteri* inactivation and cell destruction. However, the lower protein leakage in the first cycle remains to be explained.

It is also interesting to note that HP only increased leakage of cell content (relatively to atmospheric pressure) in samples without acetate, which suggests higher sensitivity of *L. reuteri* to pressure in these samples, compared to the ones with acetate. This effect was even clearer at

25 MPa, with little or no adaptive effect being visible over the cycles. This effect was observed along the study, and suggests that acetate may have a role on cell protection against HP, or may be involved in the stress responses triggered by HP.

All in all, the results obtained for nucleic acid and protein leakage provided a first insight into the HP effects on *L. reteuri* cell membrane, and how adaptation to these conditions may affect membrane permeability. However, this was evaluated by an indirect measurement and thus, to confirm these results, a more accurate and in-depth analysis should be performed to *L. reteuri* cell membrane, in order to evaluate the effect of HP not only in terms of permeability, but also regarding its composition.

5.4. Conclusions

Application of HP (10 and 25 MPa) during consecutive fermentation cycles was found to affect *L. reuteri* cell growth and fermentative profile. In general, 1,3-PDO production tended to increase over the fermentation cycles, especially at 10 MPa, which showed the highest 1,3-PDO concentrations. The most suitable conditions for 1,3-PDO production were 10 MPa, in culture medium without acetate. In that case, the highest titers, yields and productivities were achieved at the fourth cycle: titers increased 15 % relative to the respective sample at 0.1 MPa, and even more (52 %) relative to the "conventional approach", i.e. fermentation without cycles, at 0.1 MPa. The reasons behind these improvements are not completely understood, but might be related to development general and specific stress responses, as well as adaptive mechanisms triggered by repeated exposure to sub-lethal HP. In future work, it will be important to assess what is behind these HP effects using proteomic and metabolic tools, and also to discern how HP-cycles impact the composition and permeability of cell membrane. Our preliminary study regarding the effect of HP-cycles on protein and nucleic acid leakage suggests a general decrease of cell membrane permeability at 10 and 25 MPa. However, in some cases *L. reuteri* seems to gradually increase membrane permeability over the cycles, as a possible adaptation to HP. All these modifications in cell membrane will affect the exchange of substances (both substrates and products) throughout the cell, with certain effect on cell metabolism.

Overall, this work provided relevant information regarding *L. reuteri* fermentation and its adaptation to sub-lethal HP conditions (10 and 25 MPa). It also confirmed that HP may be useful to develop new strategies for production of 1,3-PDO, a relevant food ingredient and bio-chemical intermediate, with interest for several industries. One of the main advantages of HP in this context is related to the possibility of applying it continuously without inactivation problems or heat generation. Additionally, it is only necessary to provide energy to generate the pressure (and not to maintain it), and so application of HP stress during the whole fermentation process has minimal

energetic costs. Therefore, this approach can be easily applied to different fermentation processes, resulting in a real impact of HP technology in the food and biotechnological fields.

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CHAPTER VI

Comparative metabolomic profiling of *Lactobacillus reuteri* under high pressure fermentation cycles

6.1. Introduction

Stress is one of the major driving forces of microbial evolution and adaptation (Serrazanetti et al., 2009). When microorganisms are exposed to sub-lethal stress conditions, such as high pressure (HP), general and specific stress response mechanisms are activated to allow adaptation to the new conditions (Huang et al., 2014; Lado and Yousef, 2002). These stress responses involve a complex and coordinated expression of genes, which will reflect upon the microbial activity and, thus, on its metabolome (Serrazanetti et al., 2009). The metabolome is formally defined as the collection of all small molecule metabolites or chemicals that can be found in a cell, organ or organism. These small molecules can include a range of endogenous and exogenous compounds, such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, minerals and just about any other chemical that can be used, ingested or synthesized by a given cell or organism (Wishart, 2008). The effects of stress on microbial metabolome can be particularly relevant in the case of fermentative microorganisms (i.e. microbial strains involved in fermentation processes), since these metabolic changes will certainly affect the bioproducts and the bioprocess itself.

Metabolomics is an emerging field of “omics” research concerned with the high-throughput identification and quantification of small molecule (< 1500 Da) metabolites in the metabolome (German et al., 2005). The techniques most commonly used in metabolomics are: mass spectrometry (MS) as an analytical technique, together with gas or liquid chromatography (GC or LC, respectively) as additive methods; or nuclear magnetic resonance (NMR) spectroscopy (Kruk et al., 2017). Each technique has their own advantages and disadvantages, as indicated in Table 6.1. Whereas MS measures the ratio of mass to charge of ionized particles, nuclear magnetic spectroscopy takes advantage of the magnetic properties of certain nuclei, such as ^{1}H, ^{13}C, ^{31}P and others (Brennan, 2014; Serkova and Niemann, 2006; Zhang et al., 2012). NMR consists of exciting nuclear spins in an external magnetic field and then recording the electromagnetic radiation emitted. When a given frequency of the electromagnetic wave is used (dependent on the strength of the external magnetic field) only the nuclei with such resonance frequency absorb it. As the local chemical environment of a nucleus affects its resonance frequency it is possible to distinguish nuclei in different chemical groups (the chemical shift) in a given compound by NMR. In addition, under certain conditions, peak intensities can be used to quantify the compounds present in the samples.

Table 6.1. Comparison between different metabolomics technologies (NMR spectroscopy, GC-mass spectrometry, and LC-mass spectrometry). Adapted from Wishart (2008).

Technology	Advantages	Disadvantages
NMR spectroscopy	• Quantitative; • Non-destructive; • Fast (2-3 min/sample); • Requires no derivitization; • Requires no separation; • Detects all organic classes; • Allows identification of novel chemicals; • Robust, mature technology; • Can be used for metabolite imaging; • Large body of software and databases for metabolite identification; • Compatible with liquids and solids.	• Not very sensitive; • Expensive instrumentation; • Large instrument footprint; • Cannot detect or identify salts and inorganic ions; • Cannot detect non-protonated compounds; • Requires larger (0.5 mL) samples.
GC-mass spectrometry	• Robust, mature technology; • Relatively inexpensive; • Quantitative (with calibration); • Modest sample size need; • Good sensitivity; • Large body of software and databases for metabolite identification; • Detects most organic and some inorganic molecules; • Excellent separation reproducibility.	• Sample not recoverable; • Requires sample derivitization; • Requires separation; • Slow (20-30 min/sample); • Cannot be used in imaging; • Novel compound identification is difficult.
LC-mass spectrometry	• Superb sensitivity; • Very flexible technology; • Detects most organic and some inorganic molecules; • Minimal sample size requirement; • Can be done without separation (direct injection); • Has potential for detecting largest portion of metabolome.	• Sample not recoverable; • Not very quantitative; • Expensive instrumentation; • Slow (20-30 min/sample); • Poor separation resolution and reproducibility (vs. GC); • Less robust instrumentation than NMR or GC-MS; • Limited body of software and databases for metabolite identification; • Novel compound identification is difficult.

Over the past few years, two distinct approaches have emerged for processing and interpreting metabolomics data. In one version (called the chemometric or non-targeted approach), chemical compounds are not generally identified, and only their spectral patterns and intensities are recorded, statistically compared and used to identify the relevant spectral features that distinguish sample Classes (Nicholson et al., 1999; Trygg et al., 2007). These statistical comparisons and feature identification techniques usually involve unsupervised clustering (Principal Component Analysis or PCA) or supervised classification (Partial Least Squares Discriminant Analysis or PLS-DA). While chemometric approaches like PCA and PLS-DA, on their own, do not permit direct identification or quantification of compounds, they still allow an unbiased (or untargeted), chemically comprehensive comparison to be made among different samples (Wishart, 2008). In the other approach to metabolomics (called quantitative metabolomics or targeted profiling), the focus is on attempting to identify and/or quantify as many compounds in the sample as possible. Once the constituent compounds are identified and quantified, the data are then statistically processed (using PCA or PLS-DA) to identify the most important biomarkers or informative metabolic pathways (Weljie et al., 2006).

In the particular case of this work, we intended to analyze the general extracellular metabolic changes in *Lactobacillus reuteri* DSM 20016 exposed to fermentation cycles under HP (10 MPa). This may help to understand the effects of these HP-cycles on the *L. reuteri* metabolome, and possibly disclose some mechanisms of pressure adaptation. Therefore, we profiled the changes in the abundances of several metabolites (i.e. non-targeted strategy) using ^{1}H NMR spectroscopy. This technique was selected to perform this preliminary metabolomics study, because it is fast, with no need for derivatization or preceding separation methods. In addition, it detects all organic classes and allows identification of novel species, which could be important in the course of the work.

6.2. Material and methods

6.2.1. Fermentation experiments

Seed culture preparation and fermentation experiments were performed according to Chapter V (section 5.2, Materials and methods). From these samples, specific case studies were selected to perform metabolomics profiling, due to its pertinence to the work. These corresponded to samples without acetate in the initial culture medium, collected after the first and fourth cycles at 0.1 MPa (atmospheric pressure) and at 10 MPa. The samples without acetate were selected due to the considerable adaptive effect observed throughout the cycles at 10 MPa, and to the higher concentrations of 1,3-propanediol achieved.

6.2.2. ^{1}H NMR experiments

Extracts were prepared by centrifugation (at 10,000 *g* for 15 minutes) followed by filtration (0.45 μm pore diameter) of the supernatants. Extracts were then dried in a vacuum centrifuge, followed by storage in a desiccator until NMR analysis. Before NMR spectral acquisition, samples were reconstituted using 600 μL of phosphate buffer (100 mM, pH 3.0) containing 0.01 % of 3-(trimethylsilyl)propionic-2,2,3,3-*d4* acid, sodium salt (TSP-*d4*) as a chemical shift and intensity reference. The mixture was then centrifuged (4500 *g*, 25 °C, 5 min) and transferred into 5 mm NMR tubes to be analyzed.

^{1}H NMR spectra were recorded at 300 K on a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Germany), operating at a proton frequency of 500.13 MHz, equipped with an actively shielded gradient unit with a maximum gradient strength output of 53.5 G cm^{-1} and a 5 mm inverse probe. For each sample, a 1D ^{1}H NMR spectrum was acquired using the *noesypr1d* pulse sequence (Bruker pulse program library) with water presaturation. For all spectra, 128 transients were collected into 32,768 (32 K) data points with a spectral width of 10000 Hz, an acquisition time of 3.3 s and relaxation delay of 5 s. Each free induction decay (FID) was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transformation. iNMR software was used to manually phase and baseline correct the spectra. The spectra were exported as a matrix using R-Studio in-house scripts and subsequently normalised to TSP-*d4*. The spectra were overlaid and checked in iNMR to see whether alignment was required. If required, the speaq package was used in R.

6.2.3. Multivariate data analysis

The multivariate analysis were applied to the aligned spectra, using the ropls package (Thévenot et al., 2015) in R software (for statistical computing). Differences among samples were visualized by Pareto-scaled for principal component analysis (PCA). The identification of relevant metabolites was carried out by comparing the spectra with those of standard compounds from the Biological Magnetic Resonance Data Bank and the Yeast Metabolome Database, and confirmed by the Chenomx NMR Suite software. The relative amounts of the NMR metabolites and the effect size were determined by integrating the area under the most well-separated metabolite peak using in-house R scripts. Pairwise t-tests were carried out using the False Discovery Rate (FDR) to adjust for multiple testing. Effect sizes were calculated and corrected for small sample sizes using the formula:

$$Effect\ size = \left(1 - \left(\frac{3}{(4n1 + n2 - 2) - 1}\right)\right)\left(\frac{x1 - x2}{pooled\ SD}\right)$$

where pooled SD is the pooled standard deviation, $x1$ and $x2$ are the mean levels of metabolite x and $n1$ and $n2$ are the number of replicates.

6.3. Results and discussion

To obtain a wide perspective on how the HP fermentation cycles affected *L. reuteri* metabolic profile, samples collected after the first and fourth cycles at 0.1 MPa (atmospheric pressure) and at 10 MPa were analyzed by 1D ^{1}H NMR and evaluated using different metabolomic tools. It was intended not only to investigate the differences between fermentation at 0.1 and 10 MPa, but also to analyze the evolution of each type of samples between the first and fourth fermentation cycles. The characteristic 1D ^{1}H NMR spectra of the samples obtained at atmospheric pressure (C1 and C4, for cycles one and four, respectively) and at 10 MPa (P1 and P4, for cycles one and four, respectively) are shown in Figure A.1 (Appendix A). Before peak identification, a code name was assigned to each peak (M1 and M73) ranging from the higher to lower ppm values. It is important to highlight that each metabolite can generate more than one signal, when the compound has two or more protons in different “environments”. Spectral comparisons with databases and an appropriate software were performed in an attempt to identify some of the metabolites present in the samples. The obtained results are indicated in Table 6.2.

Table 6.2. List of relevant compounds identified in the samples by comparison with databases and an appropriate software, with the respective chemical shifts and code names (attributed between M1 and M73, from higher to lower ppm values).

Compounds	Chemical shifts (ppm)	Code name
1,3-Propanediol	3.66 – 3.73, 1.75 – 1.83	M44, M60
2,3-Butanediol	1.11 – 1.16	M69
Acetate	2.00 – 2.07	M58
Citrate	2.76 – 2.87, 2.66 – 2.76	M52, M53
Ethanol	1.26 – 1.30, 1.19 – 1.24	M67, M68
Glucose/glycerol	5.22 – 5.25, 4.53 – 4.70, 3.87 – 3.93, 3.73 – 3.75, 3.62 – 3.66, 3.53 – 3.59	M32, M36, M41, M43, M45, M46
Isobutyrate	2.36 – 2.43, 1.02 – 1.06	M56, M71
Lactate	4.12 – 4.22, 2.07 – 2.15, 1.30 – 1.38	M39, M57, M66

Insets for aromatic (6.0 - 9.0 ppm) and aliphatic (0.6 - 3.0 ppm) regions of the ^{1}H NMR spectra are presented (Figures A.1.b and A.1.c, respectively). The aromatic region is characteristic of aromatic amino acids (such as phenylalanine, histidine, tyrosine and tryptophan), while the aliphatic region is typically assigned to organic acids, alcohols, and aliphatic amino acids. Therefore, peaks of *L. reuteri* specific fermentation products can be easily identified in the aliphatic region, as indicated in Table 6.2. The region between 3.5 - 5.3 ppm is characteristic of sugars (glucose, in this case) and glycerol, which are overlapped with other signals. For instance, in this region, signals of 1,3-PDO (3.66 - 3.73 ppm) and lactate (4.12 - 4.22 ppm) were also identified. By comparing the full spectra (Figure A.1.a) of all samples, slight differences can be observed according to the pressure and the fermentation cycle. The most evident differences were detected in the P1 samples, correspondent to the first fermentation cycle at 10 MPa. In fact, the results of our previous work (Chapter V) showed that *L. reuteri* fermentative activity was very low during the first fermentation cycle at 10 MPa.

In order to screen the differences of metabolites between fermentation samples, a Principal Component Analysis (PCA) was performed from the 1D ^{1}H NMR data. PCA is a dimensional reduction technique that allows to easily plot, visualize and cluster multiple metabolomic data sets based on linear combinations (known as principal axes) of their shared features. As a clustering technique, PCA is most commonly used to identify how one sample is different from another and which variables contribute most to this difference (Wishart, 2008). In the present work, the PCA model showed a good fit of R^2X (0.95). The score plot resulting from PCA achieved by combining PC1 (81 % explained variance) and PC2 (14 % explained variance) is shown in Figure 6.1, while the respective loadings are presented in Figure A.2 (Appendix A).

The PCA score plot revealed a clear separation between the first and the fourth cycles, by primarily PC1. The loading plot (Figure A.2.a) revealed that glycerol and glucose seem to be the major metabolites that positively contributed to PC1, while organic acids and alcohols (in particular 1,3-PDO) negatively contributed to PC1. Therefore, the samples obtained after only one fermentation cycle showed higher PC1, due to higher content of substrates (glycerol and glucose) and lower content of typical fermentation products. In contrast, the samples obtained after four fermentation cycles showed lower PC1, indicating higher content of fermentation products and lower content of substrates, a profile characteristic of higher fermentative activity. This suggests that the application of fermentation cycles (under HP or not) stimulated *L. reuteri* fermentation, but with a considerably more pronounced effect at 10 MPa. The variables associated to PC2 had less contribution (14 %) to discriminate the samples, but clearly separated the one-cycle fermentation at 0.1 MPa from the remaining ones. The loading plot (Figure A.2.b) showed that the main metabolites that contributed to PC2 were in the sugars and glycerol region (4.70 – 4.90 ppm; 3.50 – 4.00 ppm), as well as in the aliphatic region (1.00 – 2.00 ppm). However, these

metabolites were not clearly identified, and thus it was not possible to confirm the differences in the metabolic profile of these samples.

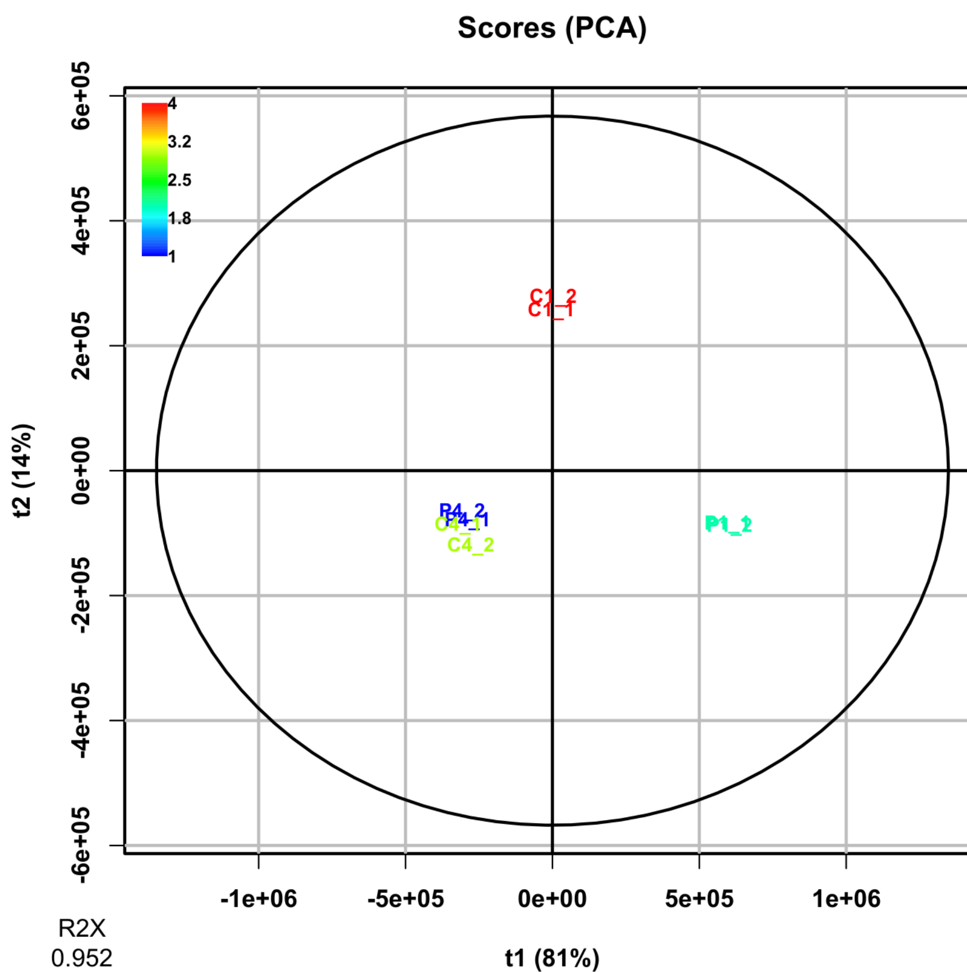

Figure 6.1. PCA scores plot of *L. reuteri* fermentation metabolites, obtained by 1D ^{1}H NMR. C1 and C4 samples correspond to the first and fourth fermentation cycles at atmospheric pressure, respectively; P1 and P4 samples correspond to the first and fourth fermentation cycles at 10 MPa, respectively. In all cases, the results are presented in duplicated (indicated as 1 and 2).

Changes in the metabolites levels in *L. reuteri* fermentation samples were visualized according to a heat map (Figure 6.2). As expected, the heat map showed considerable differences in the metabolic profile of the samples at 10 MPa after one fermentation cycle. In this case, higher abundance of glucose and glycerol (M32, M45, M46) was observed, as well as other unidentified metabolites (M24, M27, M47, M48). On the other hand, the metabolites with lower abundance in these samples were 1,3-PDO and lactate, as well as M1 and M2 (unidentified).

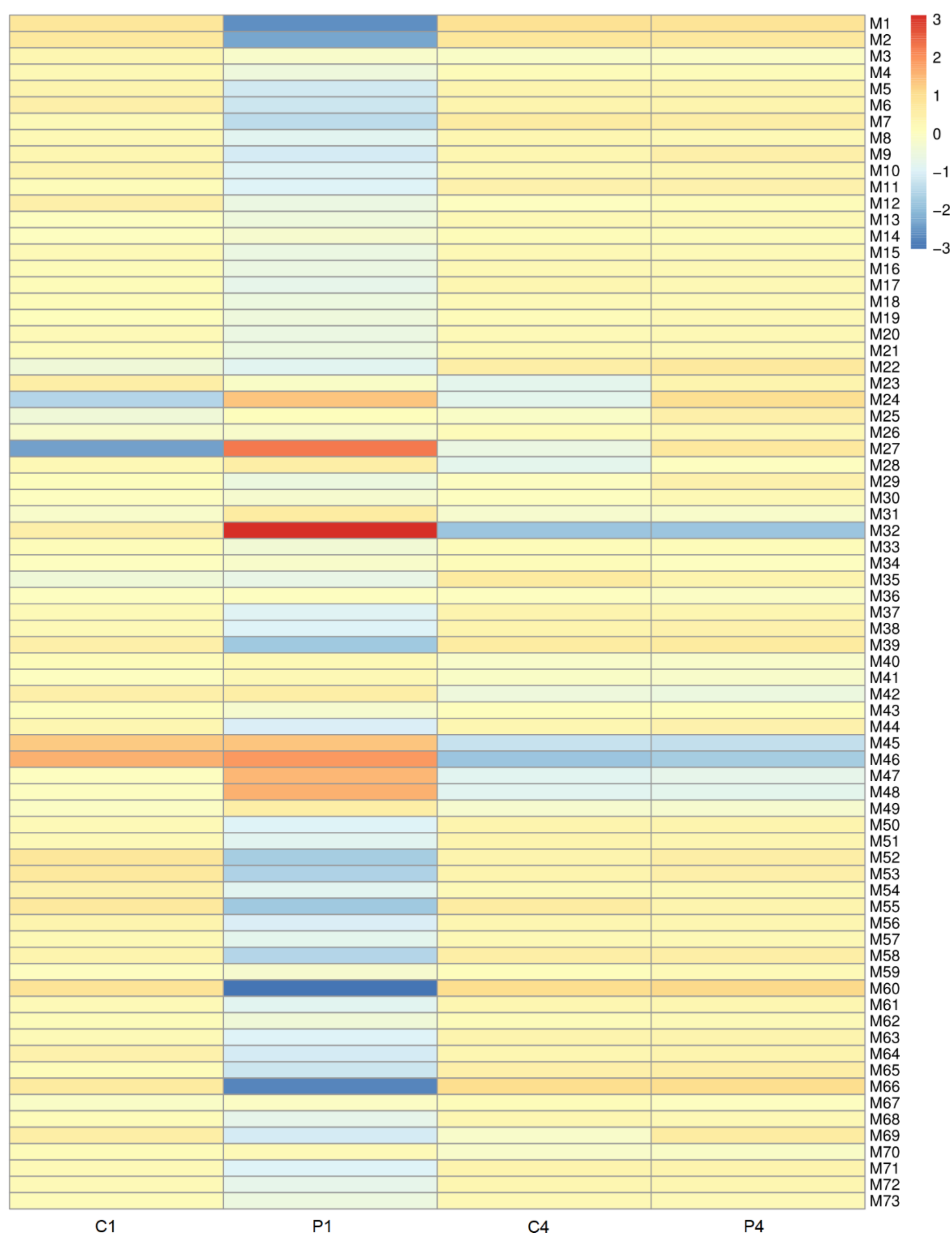

Figure 6.2. Heat map of *L. reuteri* metabolites at different fermentation cycles and pressure conditions. Scale is based on colors from red to blue representing an increase and a decrease in metabolite levels, respectively. The C1 and C4 samples correspond to the first and fourth fermentation cycles at atmospheric pressure, respectively; P1 and P4 samples correspond to the first and fourth fermentation cycles at 10 MPa, respectively. The signals are indicated by a code name, between M1 and M73, from higher to lower ppm values.

The profile of the one-cycle samples at 0.1 MPa showed less metabolite variation, with only higher abundance of substrates (M45 and M46) and lower abundance of M24 and M27 (unidentified). In samples collected after the fourth fermentation cycle, the metabolic profiles at 0.1 and 10 MPa were clearly similar, such as already suggested by the PCA score plot. The main differences may be attributed to M23, M24, M27 and M28, which are all in lower levels at 0.1 MPa. These metabolites were not identified, but seem to be part of the HP response and adaptation, since showed lower abundance in both samples at atmospheric pressure.

To better understand the effects of fermentation cycles on *L. reuteri* metabolism, the differential abundances between the first and the fourth cycles were analyzed using Volcano plots. These plots permitted the visualization of the relationship between effect size and *p*-values. Figure 6.3 shows the differential abundances between the cycles, for fermentation at 0.1 MPa (Figure 6.3.a) and at 10 MPa (Figure 6.3.b).

In both cases, the fermentation cycles affected the metabolite abundance in the samples, which suggested adaptation of *L. reuteri* to the fermentative conditions. At atmospheric pressure, this effect may result from an adaptive process to the stresses naturally involved in fermentation, such as osmotic pressure, oxidative stress, reduction of pH, and others (Serrazanetti et al., 2009). The changes in metabolite abundance between the cycles at 0.1 MPa were subtle, but were significantly more accentuated at 10 MPa. At this HP level, the metabolic profiles at the first and at the fourth fermentation cycles are unequivocally different, which suggest adaptation to the pressure conditions, possibly due to upregulation of general and specific sets of stress-response genes (Oger and Jebbar, 2010), leading to a different metabolic profile. In fact, some of the metabolites with higher relative abundance after the fourth cycle at 10 MPa (M1, M2, M16, M18, M21, M55, M56, M63 and M71) were not assigned to any of the typical *L. reuteri* fermentative substrates and products, and may be related to stress response mechanisms. From these, M16 and M18 were correspondent to aromatic amino acids, probably phenylalanine, tyrosine, tryptophan. In addition, M56 and M71 were assigned to isobutyrate, while the remaining signals were still not assigned. In further studies, it will be relevant to properly identify these metabolic products, by employing metabolomics tools suitable for that purpose.

In a different perspective, differential abundances between the samples at 10 and 0.1 MPa (at the fourth cycle) were plotted in a Volcano plot (Figure 6.4). Similarly to the PCA score plot (Figure 6.1) and to the heat map (Figure 6.2) presented above, the Volcano plot suggested similar metabolic profiles at both pressure conditions, after four fermentation cycles. Therefore, these fermentation cycles under pressure promoted adaptation of *L. reuteri*, resulting in a profile comparable to the obtained at atmospheric pressure. Despite the similarities between the samples, it was possible to identify two differentiating metabolites, each one specific for a pressure condition. At 0.1 MPa (orientation to the right in the Volcano plot), the metabolite with slightly

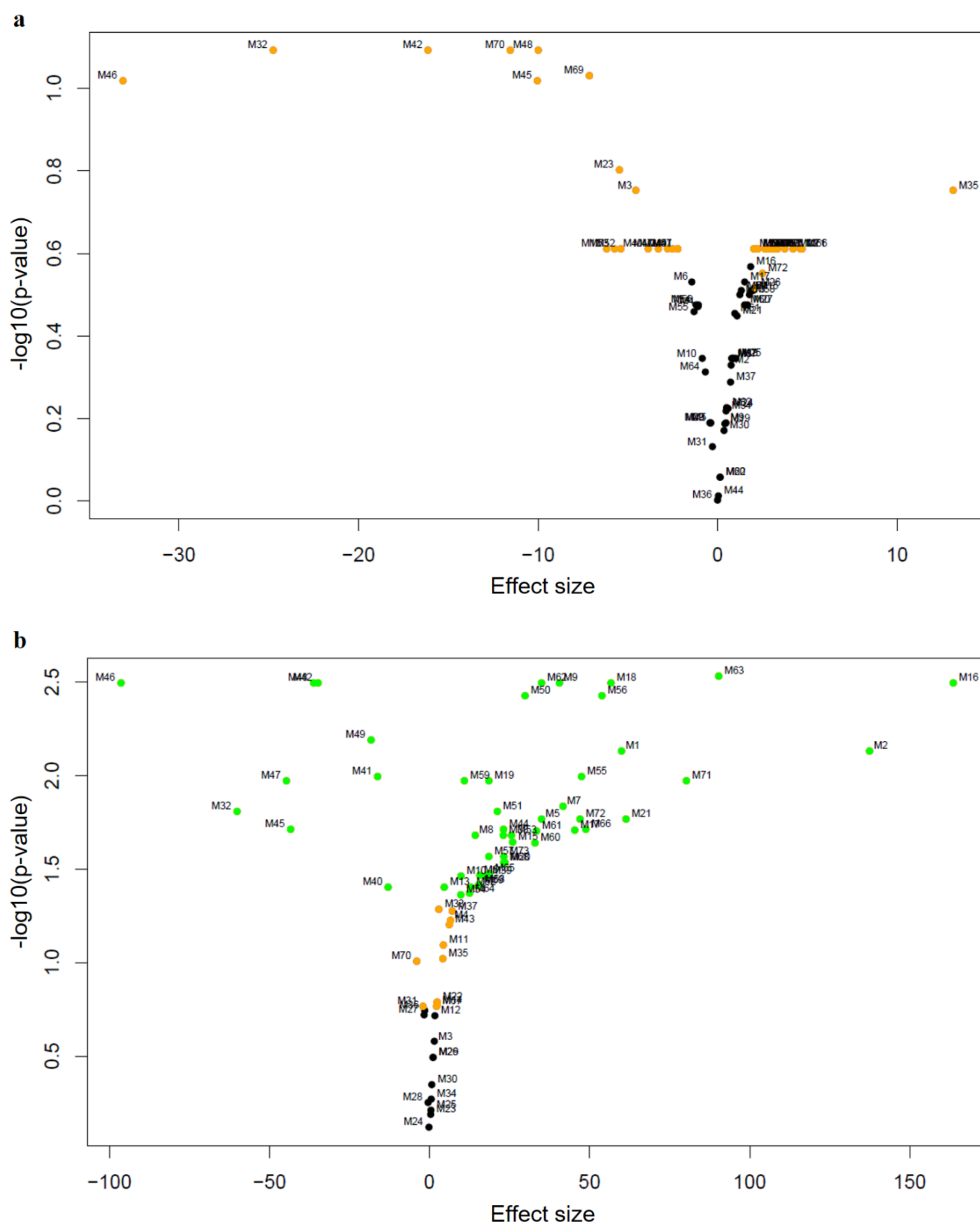

Figure 6.3. Volcano plots showing the differences in the metabolic profiles of *L. reuteri* between the first and the fourth fermentation cycles, at 0.1 MPa (a) and at 10 MPa (b). The signals/metabolites are indicated by a code name, between M1 and M73, as previously indicated. The x-axis represents the effect sizes (plotted on a log 2 scale) of the relative abundance of each metabolite between the samples after the first and the fourth cycles. The y-axis represents the statistical significance p-value of the ratio fold-change for each metabolite. Metabolites whose abundance is unchanged between the two samples will plot at the x-axis origin. Metabolites that hyper-accumulate in one of the two samples under analysis will plot either to the left (first cycle) or right (fourth cycle) of the x-axis origin.

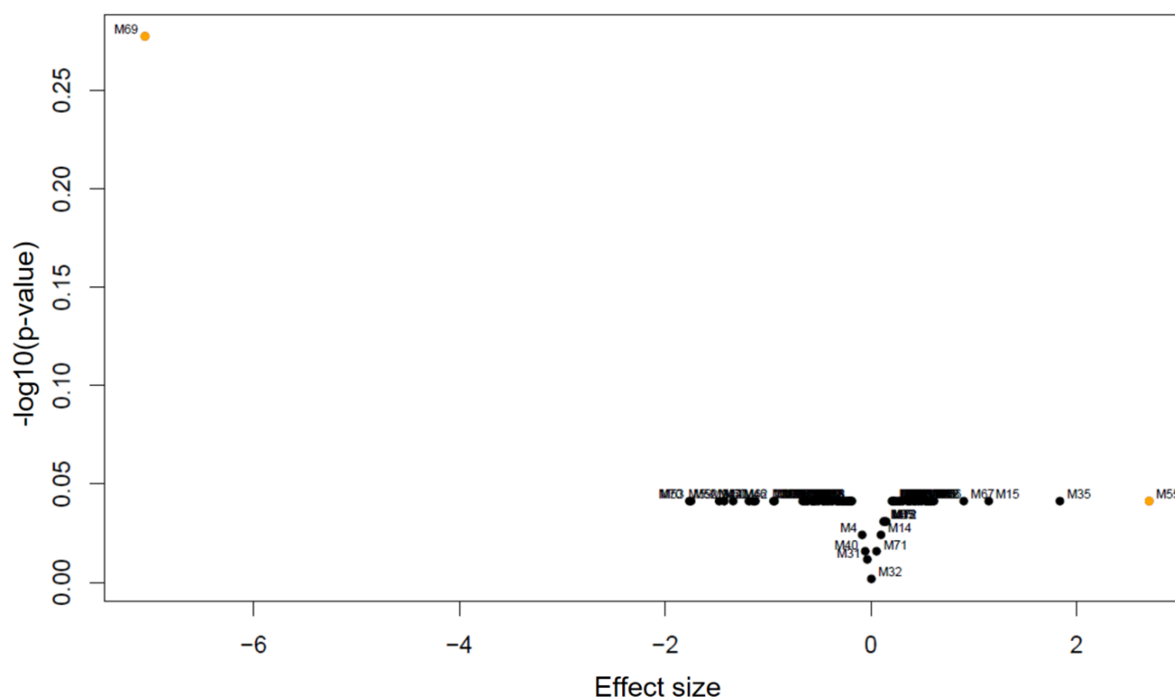

Figure 6.4. Volcano plots showing the differences between the metabolic profiles of *L. reuteri* at 10 and 0.1 MPa, after the fourth fermentation cycle. The signals/metabolites are indicated by a code name, between M1 and M73, as previously indicated.. The x-axis represents the effect sizes (plotted on a log 2 scale) of the relative abundance of each metabolite between the samples after the first and the fourth cycles. The y-axis represents the statistical significance p-value of the ratio fold-change for each metabolite. Metabolites whose abundance is unchanged between the two samples will plot at the x-axis origin. Metabolites that hyper-accumulate in one of the two samples under analysis will plot either to the left (10 MPa) or right (0.1 MPa) of the x-axis origin.

higher relative abundance is the M55 (unidentified), with chemical shift in the aliphatic region, and thus it may correspond to an organic acid, alcohol, or aliphatic amino acid. On the other hand, 2,3-butanediol (2,3-BDO), indicated as M69, showed evident higher relative abundance at 10 MPa (orientation to the left in the Volcano plot).

2,3-Butanediol is a bulk chemical with multiple practical applications, such as the production of synthetic rubber, plasticizers, fumigants, and also as an antifreeze agent, fuel additive, octane booster, and many others. The production of bio-based 2,3-BDO is mainly attained by *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Bacillus polymyxa*. It is produced from pyruvate via several intermediate compounds, including α-acetolactate and acetoin (Celińska and Grajek, 2009). A schematic representation of this metabolic pathway is shown in Figure 6.5.

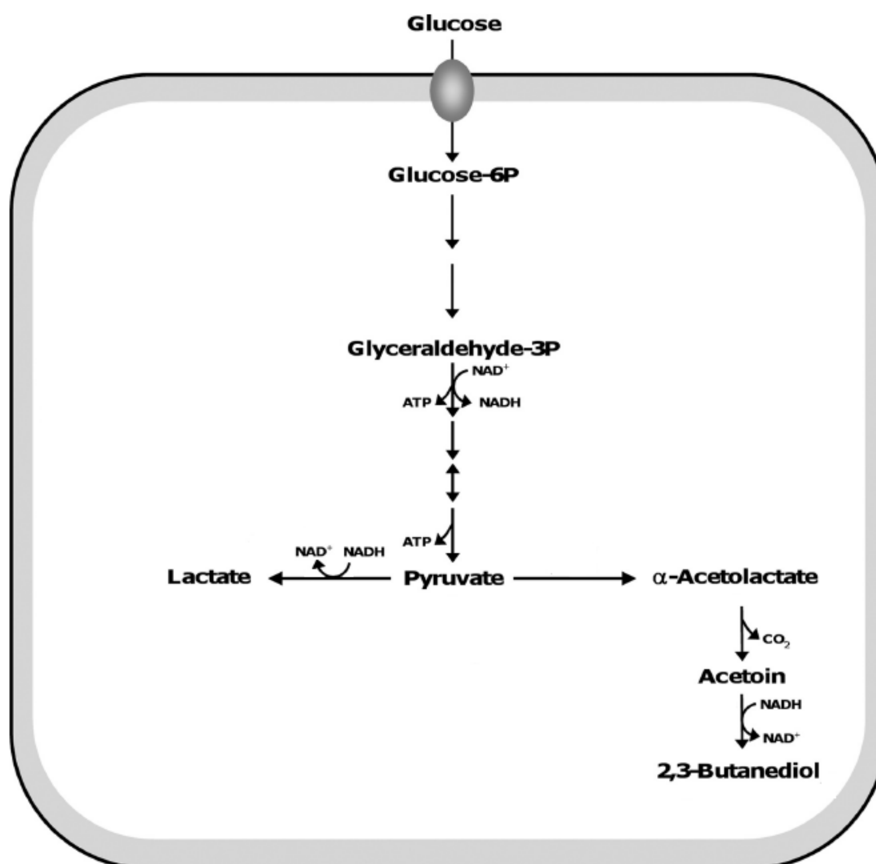

Figure 6.5. Schematic representation of general metabolic pathways for 2,3-butanediol (2,3-BDO) production by glucose metabolism, in lactic acid bacteria. Adapted from Gaspar et al. (2011) and Paul (2010).

First, pyruvate from glycolysis can be either converted either into lactate in a reaction that requires NADH (catalysed by lactate dehydrogenase, LDH) or into α-acetolactate (catalysed by α-acetolactate synthase). α-Acetolactate is mostly produced under low NADH availability. Further, α-acetolactate can be converted to acetoin by α-acetolactate decarboxylase, under anaerobic conditions. Finally, butanediol dehydrogenase reduces acetoin to 2,3-BDO.

A strategy to increase 2,3-BDO production is the use genetic engineering to reduce LDH activity (Gaspar et al., 2011; Paul, 2010). The manipulation of this enzyme (as the major player in the regeneration of NAD^+) allows to re-route the carbon flux from lactate to the production of 2,3-BDO (Gaspar et al., 2011). In the case of *L. reuteri* fermentation under HP cycles, pressure may also affect this metabolic pathway, and consequently stimulate the production of 2,3-butanediol. Two main mechanisms are possibly involved:

i) The cycles at 10 MPa may directly affect LDH activity, since HP is reported to modify the activity of several enzymes (Oey, 2016). In fact, Kouassi et al. (2007) reported loss

of LDH activity with HP treatments in the range of 206 - 620 MPa, for 6 and 12 min. They observed that even the lowest HP treatment (206 MPa) induced a reduction in LDH activity, and complete inactivation was detected at 482, 515, and 620 MPa. However, it is important to point out that these pressure levels are considerably higher than the ones applied in the present work. The duration and application of the treatment are also different, with the present work applying more prolonged times, and with use of consecutive cycles. Therefore, it is not possible to foresee if the HP-cycles at 10 MPa are able to affect or not the LDH activity.

ii) HP may also affect the NAD^+/NADH equilibrium in *L. reuteri* metabolism, such as suggested by the results in our previous study (Chapter V). Changes in NAD^+/NADH ratio can be related to the stimulation of 2,3-BDO production under pressure, since that metabolic pathway (Figure 6.5) is highly dependent on NADH availability.

The production of 2,3-BDO by *L. reuteri* corresponds to an interesting metabolic feature, which is poorly documented in literature, since the production of this compound is usually attributed to other *Lactobacillus* strains - such as *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus plantarum*. Because of that, the presence of 2,3-BDO was not assessed in our previous studies with *L. reuteri* under HP (Chapters IV and V), but should certainly be addressed in subsequent studies with this microorganism.

Another relevant aspect of this work concerns the formation of 1,3-PDO under the different pressure conditions. In our previous study (Chapter V), 1,3-PDO production was stimulated throughout the fermentation cycles at 10 MPa and, in consequence, the final titer at the fourth cycle was significantly higher compared to 0.1 MPa. However, this effect was not confirmed by the ^{1}H NMR results. It is important to take into account that one of the main disadvantages of ^{1}H NMR corresponds to its low sensitivity, such as indicated in Table 6.1. In this particular case, ^{1}H NMR was used as a qualitative method, to perform general chemical characterization and to provide a wide perspective on the metabolic profile of the samples. The methodology applied was not the most suitable for quantitative analysis, in comparison to the chromatographic methods (HPLC-RI/UV) applied in Chapter V, which used appropriate standards and a suitable quantification methodology.

To further analyze this subject, metabolite plots showing the abundance of 1,3-PDO in all samples are represented in Figure A.3 (Appendix A). The plots showed higher mean of 1,3-PDO abundance at the fourth cycle at 10 MPa. However, due to the high standard deviation values, these differences did not represent significant improvements relatively to the respective cycle at 0.1 MPa. Therefore, it is highly probable that 1,3-PDO production was, in fact, stimulated by the cycles at 10 MPa, such as suggested in our previous work (Chapter V), even if this was not

certainly confirmed by the ^{1}H NMR results. The high standard deviation values can be explained by the use of only two biological replicates for each sample, which were not enough to provide more accurate results. Therefore, additional biological replicates should be included in future metabolic profiling studies, in order to increase the accuracy of the analysis. In addition to that, further studies should comprise 2D NMR experiments and comparison with appropriate databases, to perform all peak assignments. With that information, it will be possible to fully understand the impact of HP on *L. reuteri* metabolic profile, and to identify some of the pressure adaptation mechanisms developed throughout the fermentation cycles.

6.4. Conclusions

The comparative metabolomic study between *L. reuteri* fermentation samples showed distinct metabolic profiles according to the pressure applied (0.1 or 10 MPa) and the application of fermentation cycles (one or four cycles). The PCA score plot revealed a clear separation between the first and the fourth cycles, with the samples obtained after only one fermentation cycle having higher abundance of fermentation substrates (glycerol and glucose), and the samples obtained after four fermentation cycles having higher content of fermentation products (such as organic acids and alcohols). This suggests that application of fermentation cycles (under HP or not) stimulated *L. reuteri* fermentation, possibly due to development of adaptive mechanisms. Visualization by Volcano plots showed that this adaptive effect throughout the cycles was considerably more accentuated at 10 MPa. After four fermentation cycles, the metabolic profile at 10 MPa was similar to 0.1 MPa, indicating that *L. reuteri* was able to adapt to pressure, with a response comparable to the one observed at atmospheric pressure. Interestingly, one of the metabolites characteristic of fermentation cycles at 10 MPa was 2,3-BDO, which showed higher relative abundance at this pressure, relatively to the respective cycle at 0.1 MPa. Two mechanisms were proposed to explain the stimulation of 2,3-BDO under HP: modification of lactose dehydrogenase activity; and/or modification of the NAD^+/NADH equilibrium.

Overall, the results of the present study unveil relevant information regarding the adaptation of LAB to sub-lethal HP. These mechanisms are intrinsically related to the development of different metabolic features, which certainly have an impact on the fermentation process itself, and also on the resulting bioproducts. Therefore, HP can be applied to regulate microbial metabolism to different purposes, through the optimization of pressure conditions able to stimulate preferred metabolic pathways and/or to inhibit undesirable ones.

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CHAPTER VII

The use of different fermentative approaches on *Paracoccus denitrificans*: Effect of high pressure and air availability on growth and metabolism

Adapted from:

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7.1. Introduction

Biodiesel is a fuel originated from biomass, produced from vegetable oils and animal fats, and represents one of the most promising alternatives to fossil fuels (da Silva et al., 2009). The increasing biodiesel production is raising constrains that may compromise the sustainability of the process, and one of the major problems corresponds to the formation of crude glycerol as a reaction by-product (da Silva et al., 2009; Kolesárová et al., 2011). In order to maintain the viability of biofuel economy, it became necessary to develop new and sustainable applications for glycerol, such as the use as substrate for microbial fermentation processes, resulting in the production of different value-added products, such as organic acids, alcohols, polymers, among others (Mattam et al., 2013).

Paracoccus denitrificans is a Gram-negative microorganism able to grow in glycerol, as well as in many other carbon sources, including methanol, ethanol, 1-butanol, and 1-pentanol (Ueda et al., 1992; Yamane et al., 1996a, 1996b). The first strain of *P. denitrificans* was isolated from soil more than one century ago by Beijerinck and Minkman (1910). It exhibits metabolic versatility, and it was shown to grow both aerobically and anaerobically, performing complete or partial denitrification. Air availability is a critical parameter in *P. denitrificans* growth and fermentation. It affects not only cell growth, but also some other relevant metabolic features. Kalaiyezhini and Ramachandran (2015) observed that *P. denitrificans* specific growth rates increased with the increase in oxygen transfer rate, while moderate oxygen transfer rate promoted poly(3-hydroxybutyrate) production. In aerobic bioprocesses, oxygen is a key substrate, and must be continuously supplied (Garcia-Ochoa and Gomez, 2009). However, this dependence on oxygen availability may be a limitation to some fermentation processes, particularly for high-scale industrial processes. The requirement for high oxygen availability also presents a limitation for the performance of fermentation under high pressure conditions since, currently, many high pressure equipments are not adapted to allow continuous air supply. Therefore, in some specific cases, it might be necessary to perform aerobic microbial processes under limited-air conditions.

The interest in exposing microbial cells to high pressure (HP) is related to growth stimulation and/or improvement of fermentation (Mota et al., 2018). This approach involves the use of sub-lethal HP levels that affect cell growth and metabolism, but without compromising cell viability. In some cases, these modifications can represent considerable improvements, such as increased yields, productivities and fermentation rates, lower accumulation of by-products and/or production of different compounds. For instance, Picard et al. (2007) accelerated alcoholic fermentation and increased ethanol yields in *Saccharomyces cerevisiae* by performing fermentation at 5 and 10 MPa. High pressure was also tested to change the metabolic selectivity of fermentative strains: application of pressures of 7 and 17 MPa during fermentation by *Clostridium thermocellum* redirected the metabolism from the production of by-products (such as

acetate) to ethanol, compared to fermentation at atmospheric pressure (Bothun et al., 2004). Another example is the use of HP to modify the properties of biopolymers produced during fermentation. Production of bacterial cellulose by *Gluconacetobacter xylinus* under HP (30, 60 and 100 MPa) showed profound differences in morphological properties of the polymer depending on the applied pressure conditions. The cellulose produced under HP had a significantly higher density compared with the cellulose produced at atmospheric pressure (Kato et al., 2007). Regarding polymer production, Follonier et al. (2012) applied a low pressure level (0.7 MPa) to *Pseudomonas putida* KT2440 and enhanced productivity of medium-chain-length polyhydroxyalkanoate production, even with a significant decrease in specific growth rates. The effects of HP have also been evaluated in the context of food fermentation: on lactic acid fermentation, for production of probiotic yogurt (Mota et al., 2015); and in the beginning of malolactic fermentation by *Oenococcus oeni* (Neto et al., 2016). In the first case, HP was found to reduce the fermentation rate, but it was still possible to produce yogurt under pressure by extension of the fermentation time (Mota et al., 2015). The probiotic yogurt produced at 5 MPa showed different biochemical composition (unpublished results), and possibly different organoleptic properties. In the study with *O. oeni* (microorganism used by the wine industry to perform malolactic fermentation), the strain was able to perform fermentation during and after HP-stresses of 50 and 100 MPa, with some metabolic changes. For instance, the HP-stress of 100 MPa stimulated the production of the D-lactate isomer, relative to the L-isomer.

There is still a great potential to explore in this field, with the studies conducted so far showing promising results, not only regarding food fermentations, but also for biotechnological processes. Considering that HP can be a useful tool for improving the glycerol-based fermentation processes, the present work was divided in two main goals: *i)* perform a preliminary study regarding the effects of air availability on *P. denitrificans* growth and metabolism; *ii)* assess if *P. denitrificans* is able to grow and maintain metabolic activity under HP (10, 25 and 35 MPa), even with limitations in terms of volume and air supply.

7.2. Material and methods

7.2.1. Microorganism and culture media

A lyophilized culture of *Paracoccus denitrificans* DSM 413 (ATCC 17741), obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, *Braunschweig*, Germany), was used in this study. The strain was reconstituted on nutrient broth according to the manufacturer's instructions. The strain was sub-cultured on nutrient agar plates and incubated at 30 ºC for 24 h and then preserved at 4 ºC for a maximum period of 1 month.

Rich Medium reported by Hori et al. (1994) was used for inoculum preparation. It included polypeptone (10 g L^{-1}), yeast extract (10 g L^{-1}), meat extract (5 g L^{-1}) and $(NH_4)_2SO_4$ (5 g L^{-1}). Mineral medium reported by Mothes et al. (2007) was used for the fermentation experiments. It contained glycerol (20 g L^{-1}), yeast extract (4.5 g L^{-1}), K_2HPO_4 (5 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), $CaCl_2$•$2H_2O$ (20 mg L^{-1}), $MgSO_4$•$7H_2O$ (1 g L^{-1}), and trace elements solution (2 mL L^{-1}). The composition of trace elements solution is as follows: $FeSO_4$•$7H_2O$ (4.98 g L^{-1}), $ZnCl_2$ (0.44 g L^{-1}), $CuSO_4$•$5H_2O$ (0.78 g L^{-1}), Na_2MoO_4•$2H_2O$ (0.24 g L^{-1}), $MnSO_4$•$4H_2O$ (0.81 g L^{-1}), dissolved in 1 N HCl solution.

7.2.2. Inoculum preparation and inoculation

A single cell colony was seeded into 100 mL of rich medium and incubated overnight at 35 ºC for 16 – 20 h, in a rotary incubator (160 rpm). Mineral medium was inoculated with 5 % (v/v) of standard inoculum, in an aseptic environment, within a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain), to avoid contamination.

7.2.3. Fermentation experiments: Effect of air availability

For these experiments, three different types of samples were prepared: *i)* samples “with air”, which performed fermentation in shake-flasks, with medium:air volume ratio (V_{medium}:V_{air} ratio) of 1:5, and agitation speed of 135 rpm; *ii)* samples “without air”, which fermented in polyethylene bags, sealed with no air; *iii)* samples “24 h with air + 48 h without air”, which fermented in shake flasks (with air availability) during the first 24 h, and then were transferred to polyethylene bags (with no air), where they remained during the following 48 h of fermentation. To all samples, fermentation was then carried at 35 ºC, at atmospheric pressure (0.1 MPa), for 72 h. Fermentation samples were collected over time in duplicate and all the analyses were also performed in duplicate.

In a subsequent study, fermentation was performed in polyethylene bags with air, under two slightly different V_{medium}:V_{air} ratios (1.0:1.8 and 1.0:2.2), to test the most suitable conditions for fermentation under pressure, considering volume limitations of the high pressure vessel. Fermentation was carried at 35 ºC, at atmospheric pressure (0.1 MPa), for 24 h. Fermentation samples were collected over time in duplicate and all the analyses were also performed in duplicate.

7.2.4. Fermentation experiments: Effect of high pressure

Fermentation was carried out in polyethylene bags, with controlled V_{medium}:V_{air} ratio (1.0:2.2), at 35 ºC under different HP conditions (10, 25, and 35 MPa), for 72 h. The experiments were conducted in a Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United

Kingdom), with a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature, using a mixture of propylene glycol and water (40:60 v/v) as pressurizing fluid. In parallel, a control sample (at atmospheric pressure, 0.1 MPa) was also performed, maintaining the exact same conditions of the HP-samples. Fermentation samples were collected over time in duplicate and all the analyses were also performed in duplicate.

7.2.5. Analysis of biomass concentration

Biomass concentration of the samples was determined by optical density measurement at 600 nm, with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Cell dry weight (CDW) was routinely determined using a standard curve relating *P. denitrificans* optical density and cell dry weight (CDW).

7.2.6. Glycerol quantification

Glycerol measurement was performed in the samples supernatants using the Glycerol GK Assay Kit (Megazyme, Ireland), accordingly to the manufacturer's instructions for use in 96-well microplates. The absorbance was measured with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA). The results were further confirmed by analysis with high performance liquid chromatography (HPLC) coupled with refraction index detector (HPLC-RI), by the method described in the following section.

7.2.7. Characterization of the extracellular medium

Culture samples were centrifuged at 10,000 rpm and 4 ºC for 10 min and the collected supernatants were filtered through a 0.22 μm filter membrane. Analysis by HPLC was performed using a HPLC Knauer system equipped with Knauer K-2301 RI detector and a Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase was 13 mM H_2SO_4, delivered at a flow rate of 0.6 mL min^{-1} and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with the respective standards.

7.3. Results and discussion

7.3.1. Effect of air availability on *Paracoccus denitrificans* growth and fermentation at atmospheric pressure

In the first part of this work, *P. denitrificans* metabolism was evaluated under different conditions of air availability and agitation, in order to understand how they affected growth and

fermentation. For that purpose, three different approaches were tested: (1) in the absence of air (or, at least, minimizing the air availability) and without agitation, i.e. samples "without air"; (2): in the presence of air (V_{medium}:V_{air} ratio of 1:5) and with agitation, i.e. samples "with air"; and (3): in a mixed process, which corresponded to fermentation with air in the first 24 h, and without air in the remaining time, i.e. samples "24 h with air + 48 h without air" (without adding fresh culture medium). We intended to assess if *P. denitrificans* was able to maintain growth and activity under all these conditions, and to analyze potential metabolic differences between them. The results for variation of biomass and glycerol concentrations are indicated in Figure 7.1.

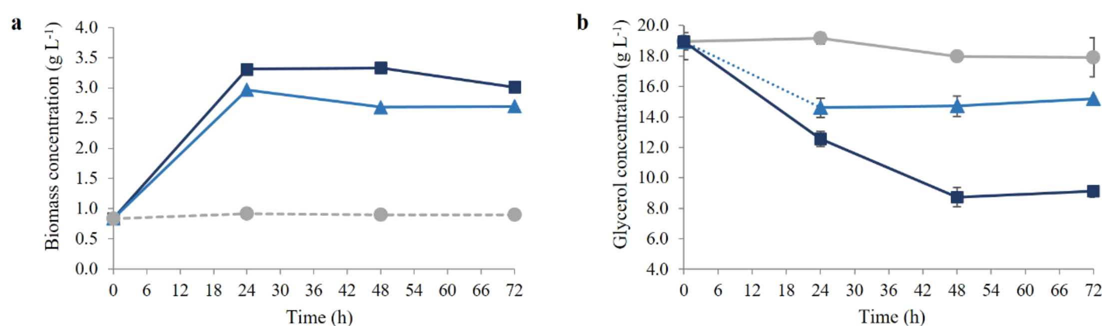

Figure 7.1. Concentrations of biomass (a) and glycerol (b) throughout fermentation, for different air availability conditions: without air during the entire process (●); with air during the first 24 h, and without air during the remaining 48 h (▲); or with air during the entire process (■).

In samples "without air", cell growth (Figure 7.1.a) was nearly inexistent and substrate consumption was low, with glycerol concentrations (Figure 7.1.b) varying from 18.95 g L^{-1} in the beginning of fermentation to 17.90 g L^{-1} after 72 h. This indicates that *P. denitrificans* was inhibited by the absence of aeration/agitation and confirms that oxygen was required for cell growth, at least when using this culture medium and conditions. In literature, *P. denitrificans* strains are reported to grow both aerobically and anaerobically (Beijerinck and Minkman, 1910), but with specific requirements of culture media composition and culture conditions at each one of these environments (Hahnke et al., 2014; Nokhal and Schlegel, 1983). In the present work, the selected culture medium and conditions did not seem suitable for growth under low oxygen environments. In fact, higher air availability and agitation conditions were more suitable for *P. denitrificans* growth, since the samples "with air" showed pronounced cell growth and substrate consumption over time. In the "24 h with air + 48 h without air" samples, cell growth and glycerol consumption were similar to samples "with air" during the first 24 h, as expected, since the fermentation was performed at the same conditions during that period. Afterwards, when

fermentation was carried out without air, cell and glycerol concentrations remained stable over time, possibly due to inhibition of metabolic activity.

The effects of air availability were also evaluated in terms of production of extracellular compounds by *P. denitrificans* during fermentation. This is a rather unusual approach, since typically only *P. denitrificans* intracellular products are analyzed, in attempt to find biopolymers (Kalaiyezhini and Ramachandran, 2015; Mothes et al., 2007). However, since that was not the purpose of the present work, only the extracellular products were analyzed, in particular alcohols and organic acids. As a result, ethanol, acetate and succinate were identified for each fermentative batch. The variation of these compounds over fermentation time is represented in Figure 7.2, while the respective yields (Y, g g^{-1}) are indicated in Table 7.1. The formation of the extracellular products was profoundly affected by air availability. It is interesting to point out that the conditions "without air", which highly inhibited cell growth and glycerol uptake, were the ones that promoted the formation of extracellular products.

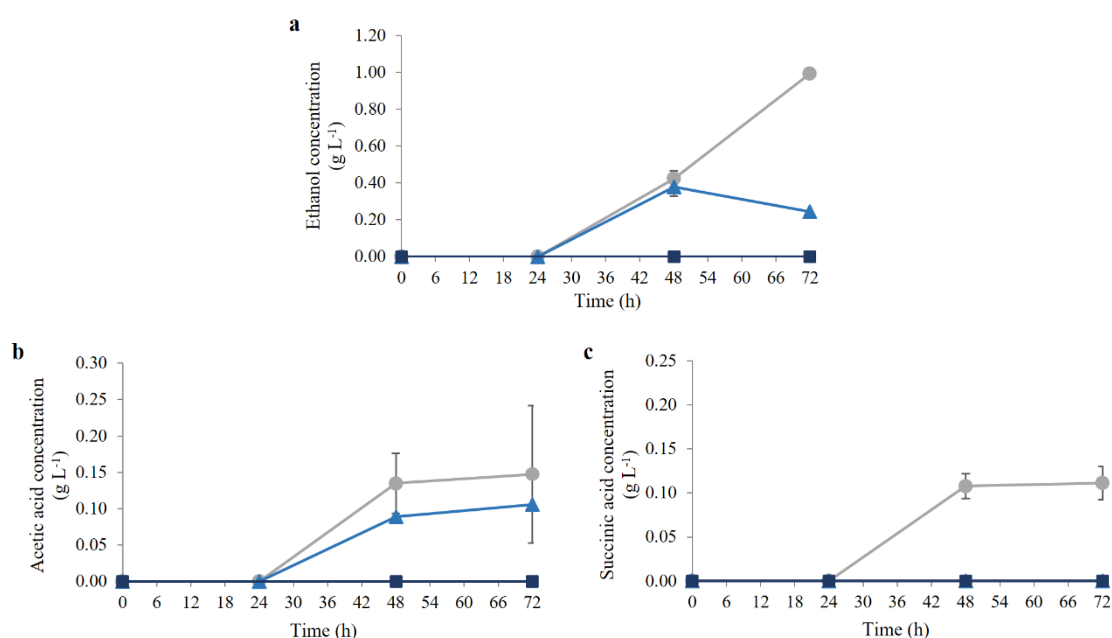

Figure 7.2. Concentrations of ethanol (a), acetate (b) and succinate (c) throughout fermentation, for different air availability conditions: without air during the entire process (●); with air during the first 24 h, and without air during the remaining 48 h (▲); or with air during the entire process (■).

Table 7.1. Yields of biomass ($Y_{X/S}$), ethanol ($Y_{EtOH/S}$), acetate ($Y_{Acet/S}$), and succinate ($Y_{Succ/S}$) on glycerol, for fermentation under different air availability conditions.

Samples	$Y_{X/S}$ (g g^{-1})	$Y_{EtOH/S}$ (g g^{-1})	$Y_{Acet/S}$ (g g^{-1})	$Y_{Succ/S}$ (g g^{-1})
Without air	0.061	0.945	0.140	0.106
24h with air + 48h without air	0.492	0.095	0.028	n.d.
With air	0.221	n.d.	n.d.	n.d.

Yields were calculated from a single time-point corresponding to the end of the experiment (72 h). Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated. N.d. indicates non-detected production of the compound.

While all these compounds were formed in samples fermenting "without air", none of them was detected in samples with high air availability. In the mixed samples, ethanol and acetate (Figure 7.2.a and 7.2.b) were both produced, but only during the period of fermentation without air (24-72 h). It would be expected that the higher cell density accumulated in mixed samples during the first 24 h would result in increased production of extracellular compounds, compared to samples "without air". However, acetate concentration was lower in mixed samples, and succinate (Figure 7.2.c) was not produced, which suggests that both samples developed different mechanisms to survive under low oxygen availability conditions. In addition, the absence of succinate may indicate that this compound was produced by different metabolic pathways, which were differently affected by air availability, relative to ethanol and acetate.

Since the extracellular products were only formed in samples "without air", or during the equivalent period in mixed samples, it is possible to conclude that these products are characteristic of *P. denitrificans* metabolism under lower oxygen availability. A similar behavior was reported for a recombinant *E. coli* strain (de Almeida et al., 2010). In that case, two different agitation speeds were used to provide different levels of oxygen availability, and resulted in variations in the pattern of product formation. In cultures grown with strong agitation, i.e. with higher oxygen availability, there was low production of metabolic products (ethanol, and acetate, formate and lactate) and formation of larger amounts of biomass. In contrast, the reduction in oxygen availability caused a redirection of carbon flow towards the production of acids and ethanol. The authors also observed that this enhancement effect was particularly noteworthy for ethanol production, compared to the formation of organic acids. Similarly, in the present work, there was high ethanol production during fermentation with low aeration (Figure 7.2 and Table 7.1), which

resulted in a concentration of 1.00 g L^{-1} and a yield of 0.95 g g^{-1}, both considerably higher than the values obtained for acetate (0.15 g L^{-1} and 0.14 g g^{-1}) after the same time.

The high value obtained for ethanol yield on glycerol raised the possibility of production of this compound (as well as other extracellular compounds) from carbon sources other than glycerol. In fact, the experiments were performed using complex culture medium, since some ingredients had unspecified chemical composition, e.g. yeast or meat extracts. Therefore, it would be possible to have different carbon sources present in the media. In order to address this issue, the presence of alternative carbon sources was evaluated, and glucose and maltose were detected in the initial samples (0 h), as indicated in Table B.1 (Appendix B). Glucose concentration showed low variation over time, from 1.07 g L^{-1} at 0 h, to 1.03 - 1.66 g L^{-1} after 72 h. In contrast, maltose initially present in the medium (0.26 g L^{-1}) was entirely consumed after 72 h of fermentation. These results indicate that ethanol, acetate and succinate may also be produced from maltose, and not exclusively from glycerol. To understand which substrate is used for the production of each compound, and which are the metabolic pathways used for that purpose, the metabolic profile of *P. denitrificans* should be studied in detail, using specific and suitable metabolomics tools.

In samples "without air", *P. denitrificans* showed metabolic activity, with the production of ethanol, acetate and succinate, but no cell growth over time. In contrast, samples "with air" showed considerable cell growth, but no production of ethanol, acetate or succinate. Samples "24 h with air + 48 h without air" were able to accumulate biomass during the first 24 h, and to produce ethanol and acetate during the period without air. However, it did not achieve the concentrations produced without air, and succinate was not even detected. Overall, the results in this section showed that *P. denitrificans* growth and metabolism were highly affected by air availability. It is also important to consider that the low cell growth under low air availability can represent a serious limitation to achieve reasonable concentrations of fermentation products, due to the reduced number of cells. Therefore, the use of moderate air availability conditions would possibly favor the process, balancing the formation of biomass and the fermentative activity. In addition, these would be the most suitable conditions to perform fermentation under high pressure (HP), due to volume limitations of the HP vessel used at the present work, which does not easily allow high air volumes or agitation. Considering these constrains, two different V_{medium}:V_{air} ratios (1.0:1.8 and 1.0:2.2) were selected, and tested for *P. denitrificans* growth and fermentation during 24 h. As indicated in Table 7.2, specific growth rates (μ, h^{-1}), final biomass concentrations and glycerol consumption percentages were all slightly higher for the 1.0:2.2 ratio, correspondent to higher air availability. It is certain that high air availability and high specific growth rates do not only always translate in higher product formation (de Almeida et al., 2010; Kalaiyezhini and Ramachandran, 2015). However, since the study under HP would be limited by other factors, such

as the lack of agitation, and the stress induced by the pressure itself, the highest ratio (1.0:2.2) was selected to proceed the studies with *P. denitrificans* at HP conditions.

Table 7.2. Specific growth rates (μ), final biomass concentrations and percentages of glycerol consumed, after 24 h of fermentation, for medium:air ratios of 1.0:1.8 and 1.0:2.2.

Samples		μ (h^{-1})	Final biomass concentration (g L^{-1})	Glycerol consumed (%)
0.1 MPa	1.0:1.8	0.098	2.65	28.34
	1.0:2.2	0.104	2.99	33.54

Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated.

7.3.2. Effect of high pressure on *Paracoccus denitrificans* growth and fermentation

The previously discussed experiments provided relevant information about the fermentation process and how it depends on air availability. In the second stage of the work, HP (at 10, 25 and 35 MPa) was applied during the 72 h at 35 ºC of *P. denitrificans* fermentation. Fermentation was also tested at atmospheric pressure (0.1 MPa), to use as control. The pressure effects on cell growth and glycerol consumption (Figure 7.3) showed a clear inhibitory effect, which was more accentuated with the increase of pressure level.

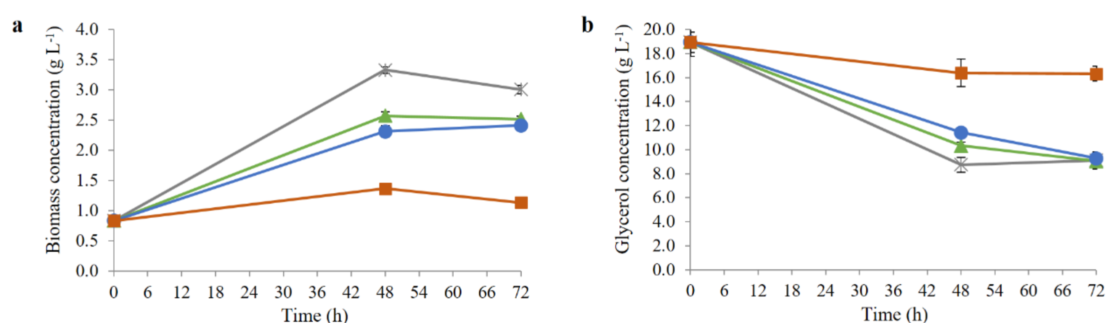

Figure 7.3. Concentrations of biomass (a) and glycerol (b) throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

Similar cell growth profiles (Figure 7.3.a) were observed at 10 and 25 MPa, with similar biomass concentrations reached after 72 h of fermentation (2.52 and 2.42 g L^{-1}, respectively). In both cases, biomass concentration was significantly lower compared to the obtained at 0.1 MPa (3.01 g L^{-1}). At 35 MPa, inhibition of cell growth was even more pronounced, showing only slight

variation over time, which resulted in a final biomass concentration of only 1.14 g L^{-1}. In a study concerning microbial growth under hyperaccelerations (in centrifuges), Deguchi et al. (2011) tested the effects of HP (being the pressure generated by hyperacceleration) on *P. denitrificans* proliferation, and observed that the strain was able to grow at 30 MPa, but was completely inhibited at 40 MPa (at 30 ºC, in LB agar). This negative effect of HP on cell growth was previously reported for other microorganisms, such as *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium lactis*, at 5 and 100 MPa (Mota et al., 2015); for *Gluconacetobacter xylinus* at 100 MPa (Kato et al., 2007); and for *Clostridium thermocellum* at 7 and 17.3 MPa (Bothun et al., 2004).

The inhibitory effect of pressure on cell growth may result from a wide variety of damaging effects. Generally, low pressure levels, such as the ones used in this work, may be enough to impair several cellular processes, such as motility, cell division, nutrient uptake or membrane protein function. A pressure of 50 MPa can inhibit protein synthesis and reduce the number of ribosomes, while 100 MPa can induce partial protein denaturation (Abe, 2007; Huang et al., 2014). However, it is important to take into account that pressure effects on microbial growth are highly variable according to several factors, such as the organisms' degree of piezotolerance, the growth stage, the extent and duration of pressure treatment, as well as other environmental parameters (Mota et al., 2013).

Glycerol consumption (Figure 7.3.b) seemed to be less affected by HP, at least at 10 and 25 MPa: in those cases, glycerol consumption after 48 h was slightly but significantly lower compared to 0.1 MPa, but reached similar concentrations after 72 h (in the range of 9.04 and 9.26 g L^{-1}). In contrast, biomass concentration at these same pressure conditions was always lower compared to 0.1 MPa. This discrepancy between the pressure effects on growth and substrate consumption may be related to the development of stress response mechanisms to ensure cell survival. Under stress conditions, cell growth is usually disregarded, in order to favor other processes more relevant to their survival, i.e. cell maintenance processes. Cell maintenance refers to the fraction of substrate consumed to generate energy for functions other than the production of new cell material (Pirt, 1965). These functions include energy costs of osmoregulation, cell motility, turnover of macromolecular compounds, as well as defense mechanisms (Van Bodegom, 2007). In short, when the energy is used for these maintenance processes, bacterial growth is reduced, even if substrate consumption is maintained. Therefore, it is expectable that under stressful conditions, such as HP, biomass production will be more affected than substrate consumption. However, this effect was not observed at 35 MPa, which showed low glycerol consumption during the entire process, possibly indicating metabolic inhibition at this pressure.

As discussed in the previous section, glucose and maltose can both be found in the culture medium, and its presence (and consumption) may have an impact on *P. denitrificans* metabolism.

Therefore, the concentrations of these sugars were analyzed throughout the fermentation time, at the end of each fermentation condition. In all cases, maltose showed the same behavior, with the 0.31 g L^{-1} of maltose initially present in the medium being completely consumed, regardless of the pressure applied (data not shown). On the other hand, glucose consumption was highly affected by pressure, as indicated in Figure 7.4.

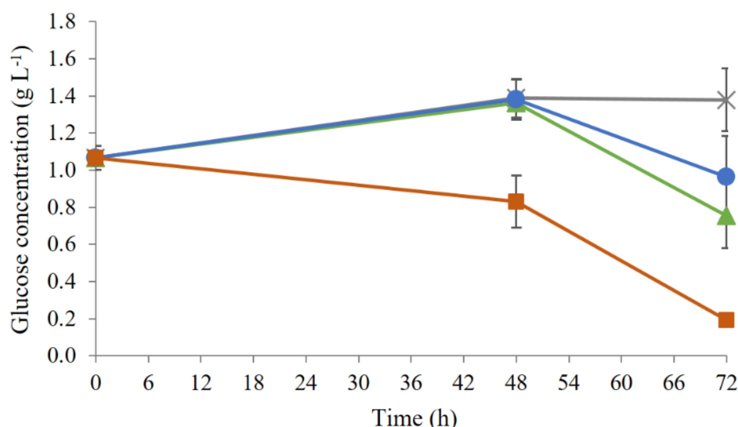

Figure 7.4. Glucose concentrations throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

After 48 h, glucose concentrations were similar at 0.1, 10 and 25 MPa (≈ 1.38 g L^{-1}), but considerably changed at 72 h: *i)* at 0.1 MPa, the concentration remained as 1.38 g L^{-1}; *ii)* at 10 MPa, it decreased to 0.76 g L^{-1}; *iii)* at 25 MPa, it decreased to 0.97 g L^{-1}. This suggests that HP stimulates glucose consumption during fermentation, possibly due to the higher need of substrate and energy to ensure cell survival, with development of general and/or specific stress responses. Interestingly, glucose concentrations at 35 MPa were significantly lower that for all other conditions, after 48 and 72 h (0.83 and 0.20 g L^{-1}, respectively), showing an opposite behavior relative to glycerol consumption. Therefore, fermentation at 35 MPa seems to stimulate glucose consumption, while the same effect was not observed for glycerol. These results suggest specific metabolic changes at this pressure level, which affected differently *P. denitrificans* growth and fermentation compared to the lower pressure levels.

Application of HP on *P. denitrificans* was also evaluated in terms of ethanol, acetate and succinate production (Figure 7.5). Different pressure levels showed different effects on the formation of these compounds. Ethanol production (Figure 7.5.a) was observed for all pressure conditions, with a general increasing trend for the first 48 h of fermentation, and decreasing thereafter.

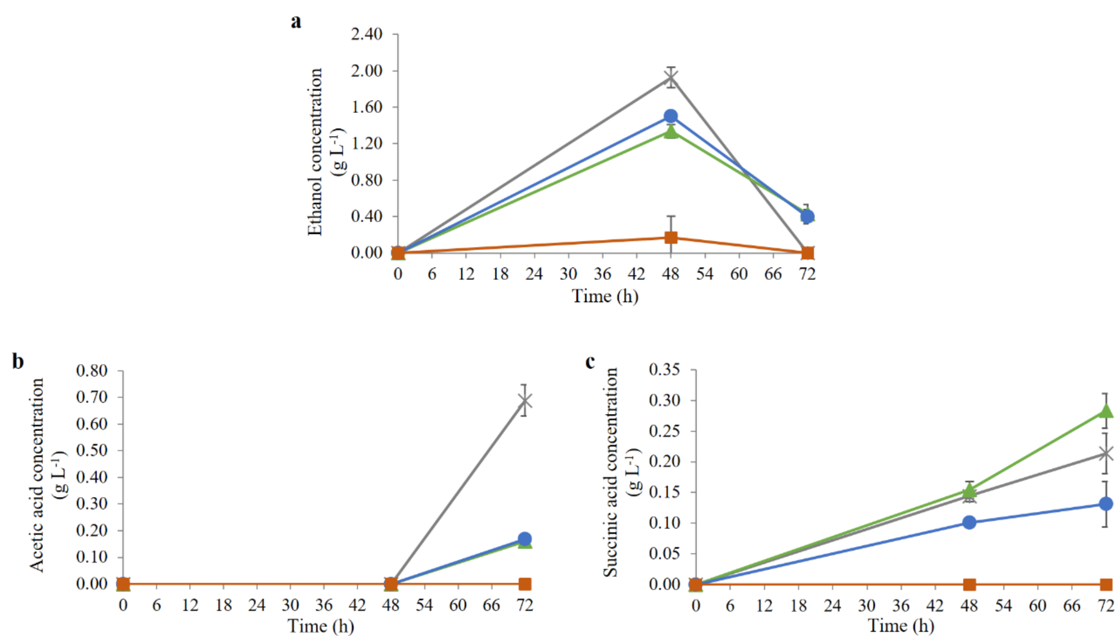

Figure 7.5. Concentrations of ethanol (a), acetate (b) and succinate (c) throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

The highest ethanol concentration was reached after 48 h at 0.1 MPa (1.92 g L^{-1}), but this compound was not detected after 72 h at this pressure. Similarly, ethanol concentrations of 1.34 and 1.50 g L^{-1} were observed after 48 h at 10 and 25 MPa, respectively, but these values decreased to 0.43 and 0.40 g L^{-1} at 72 h of fermentation. These results suggest that ethanol formed during the first hours of fermentation was then converted into other products. An option for the ethanol degradation pathway is the oxidation into acetaldehyde, which can be followed by oxidation into acetate. In fact, Felux et al. (2013) indicated that *P. denitrificans* Pd1222 (a derivative of DSM 413) has the genetic machinery to perform these metabolic reactions: a gene that encodes an alcohol dehydrogenase (locus tag Pden_2367) able to convert ethanol into acetaldehyde; and a gene that encodes an NAD^+-dependent aldehyde dehydrogenase (locus tag Pden_2366) that oxidizes acetaldehyde to acetate. This is also supported by the production of acetate (Figure 7.5.b) during the period between 48 and 72 h, with the highest concentration at 0.1 MPa (0.69 g L^{-1}), followed by significantly lower concentrations at 10 and 25 MPa (0.16 and 0.17 g L^{-1}, respectively). However, these acetate concentrations are too low relative to the concentration of ethanol consumed. If ethanol was entirely converted into acetate, ≈ 2.5 g L^{-1} would be obtained at 0.1 MPa, which is quite higher than the concentration actually detected (0.69 g L^{-1}). There may be two possible explanations for this discrepancy: *i)* acetaldehyde (obtained from ethanol) was not entirely converted into acetate and accumulated in the cell, which is unlikely due to the high

toxicity of this compound; *ii)* acetate obtained from this pathway was further converted into acetyl-CoA by an acetyl-CoA synthetase, possibly entering in a wide variety of metabolic pathways, such as the tricarboxylic acid cycle (TCA cycle) or fatty acid synthesis.

Succinate production (Figure 7.5.c) was also detected during fermentation at 0.1, 10 and 25 MPa, but not at 35 MPa. After 72 h of fermentation, succinate concentrations were significantly different for all pressures tested: the highest succinate concentration was achieved for samples at 10 MPa (0.28 g L^{-1}), followed by the ones at 0.1 MPa (0.21 g L^{-1}) and, finally, at 25 MPa (0.13 g L^{-1}). Stimulation of succinate production at 10 MPa could be an interesting outcome of *P. denitrificans* fermentation under HP, since this compound is widely used as a precursor of many industrially important compounds in food, chemical, and pharmaceutical industries (Jiang et al., 2017). However, the concentrations produced by *P. denitrificans* are considerably low compared to other microorganisms typically used for that purpose, such as *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, or *Anaerobiospirillum succiniciproducens*. For those microbial strains, succinate concentrations in the range of ≈ 10 – 83 g L^{-1} are usually reported in literature (Jiang et al., 2017). However, further optimization is highly likely to be possible, and could increase the succinate production by *P. denitrificans*, possibly resulting in titers and yields more similar to the ones reported for other microorganisms. As observed for other results in this section, fermentation at 35 MPa exhibited a different metabolic profile compared to other pressure conditions, with low ethanol production (max. 0.17 g L^{-1}), and no detected production of acetic and succinate. This suggests that HP is inhibiting the formation of these compounds, an effect that was also observed at lower extent for fermentation at 10 and 25 MPa. To clarify this inhibitory effect of pressure, the yields (Y, g g^{-1}) of biomass, ethanol, acetate, and succinate on glycerol were estimated at the end of fermentation (72 h), and are indicated in Table 7.3. In the cases of biomass and acetate, the yields followed a decreasing trend with the increase of pressure, suggesting a negative impact on these features. Ethanol yields were only estimated for fermentation at 10 and 25 MPa, as this compound was not detected after 72 h at the other conditions. Due to the high variation of ethanol concentrations over time, the yields at the end of fermentation did not allow a pertinent evaluation of the HP effects on ethanol formation. In contrast, succinate yields reflected the behavior observed for concentrations over time, with the yield at 10 MPa (0.29 g g^{-1}) being slightly higher than at 0.1 MPa (0.22 g g^{-1}).

Table 7.3. Yields of biomass ($Y_{X/S}$), ethanol ($Y_{EtOH/S}$), acetate ($Y_{Acet/S}$), and succinate ($Y_{Succ/Gly}$) on glycerol, for fermentation under different pressure conditions.

Pressure (MPa)	$Y_{X/S}$ (g g^{-1})	$Y_{EtOH/S}$ (g g^{-1})	$Y_{Acet/S}$ (g g^{-1})	$Y_{Succ/S}$ (g g^{-1})
0.1	0.221	n.d.	0.070	0.022
10	0.170	0.043	0.016	0.029
25	0.163	0.041	0.017	0.014
35	0.121	n.d.	n.d.	n.d.

Yields were calculated from a single time-point corresponding to the end of the experiment (72 h). Values reported in the table represent the mean of two independent biological replicates, and analyzed in duplicated. N.d. indicates non-detected production of the compound.

Overall, HP was found to affect *P. denitrificans* cell growth and metabolism, with different effects on substrate consumption, as well as on production of ethanol, acetic and succinate. These effects varied according to the pressure level, with the lower pressures (10 and 25 MPa) showing a behavior approximate to 0.1 MPa, while the highest pressure (35 MPa) presented a more extensive impact on *P. denitrificans* metabolism. Such as previously reported for other microorganisms (Mota et al., 2018), the application of pressure stresses resulted in particular and interesting effects on *P. denitrificans* growth and metabolism. In this preliminary study, the experiments were performed in a lab-scale HP equipment, designed for pasteurization and food technology purposes, that can also be used for a broader range of applications, including extraction, hyperbaric storage, or microbial growth, but with inherent limitations. In the case of microbial growth and fermentation processes under pressure, the main constrains are related to volume limitations, absence of agitation mechanisms, as well as unpractical oxygen supply. Therefore, it may be worth to perform equipment and process optimization, in order to perform further studies in more suitable and tailor-made systems, able to meet the specifications of these microbial processes. In fact, such type of pressure equipment is now becoming more widely available, making it possible to evaluate the full potential of fermentation under pressure. It should be highlighted that, in this context, this technology is highly versatile, since it can be applied intermittently, as pressure stresses, but can also be maintained during the whole fermentation time, without serious cell loss and no heating effect. Since there is no refrigeration requirement (because the continuous application of pressure does not generate heat), the energetic costs of the fermentation process are lower, and the application of HP to these processes is simpler.

Additionally, it is only necessary to provide energy to generate the pressure (and not to maintain it), and so application of HP stress during the whole fermentation process has minimal energetic costs, which would have a small impact on the integration of high pressure on industrial fermentative processes. Also, as the pressure levels used in these processes are quite lower than those used for food processing, the required equipment could be designed to withstand lower pressures, thus being cheaper than the commercial alternatives currently on the market. Therefore, HP technology can offer a high variety of process possibilities to perform microbial growth and fermentation under pressure.

7.4. Conclusions

The present work intended to study the possibility of applying HP to *P. denitrificans* glycerol fermentation, to stimulate cell growth and/or improve fermentation. However, it was necessary to consider that some of HP systems that may be used for these purposes currently comprise some limitations to aerobic processes, such as the absence of continuous air supply or agitation. To understand if it was possible to perform *P. denitrificans* growth and fermentation under limited-air conditions, the effects of air availability on this process were evaluated. The results showed that growth and metabolism were both highly affected by air availability. With higher air availability, considerable cell growth was observed over time, but no production of ethanol, acetate or succinate. In contrast, without air availability, *P. denitrificans* showed active metabolic activity (with the production of ethanol, acetate and succinate), but no cell growth over time. Therefore, these products seem to be characteristic of *P. denitrificans* metabolism under lower oxygen availability.

To avoid inhibition of both cell growth and formation of extracellular products, fermentation at HP conditions was tested under moderate air availability conditions (V_{medium}:V_{air} ratio of 1.0:2.2). *Paracoccus denitrificans* cells were able to grow under HP, even if at lower extent compared to atmospheric pressure. At 10 and 25 MPa, biomass concentrations were still similar to 0.1 MPa, while a more extensive inhibitory effect was observed at 35 MPa. In fact, this pressure may be enough to impair several cellular processes, resulting in decreased cell growth under these conditions. Application of HP was also found to promote modifications in terms of substrate consumption, and formation of ethanol, acetate and succinate, with the fermentative profile varying according to the pressure level. Generally, it was similar at 10 and 25 MPa, but considerably different at 35 MPa, possibly as a result of metabolic shifts, or even inhibition. The formation of these compounds under HP showed interesting patterns, and confirm that HP has interesting effects on living systems, offering great biotechnological potential (such an example are microorganisms thriving in deep-sea). Therefore, it would be interesting to proceed the studies

on *P. denitrificans* under HP, to further optimize the fermentation process at these conditions, and to improve the titers and yields. As future work, it would also be relevant to study the pressure effects on the production of different compounds, such as the intracellular biopolymers polyhydroxyalkanoates. It would also be important to evaluate in more detail the effects of HP on cell growth and viability, in order to estimate relevant kinetic parameters (that can only be calculated by collecting more samples over time, and thus adding more data points) and modelling these fermentation processes.

Overall, the implications of the pressure-promoted changes in *P. denitrificans* growth and fermentation process are still not completely understood, but the results obtained in this work unveil new metabolic features of this bacterial strain, and provide useful information for further studies regarding *P. denitrificans* under pressure. From a more comprehensive view, this study also opens the way for application of this technology to other glycerol fermentation processes, in particular to the ones with high requirements of air availability. It is certain that, in these particular cases, there are more limitations of the process (mostly due to volume constrains) that demand a more complex optimization, but the results obtained so far for *P. denitrificans* confirm that is possible to perform aerobic growth under these lower air availability conditions.

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CHAPTER VIII

Effect of high pressure on *Paracoccus denitrificans* growth and polyhydroxyalkanoates production

Adapted from:

Mota, M.J., Lopes, R.P., Simões, M.M.Q., Delgadillo, I., Saraiva, J.A., Effect of high pressure on *Paracoccus denitrificans* growth and polyhydroxyalkanoates production from glycerol. Submitted in Journal of Industrial Microbiology & Biotechnology.

8.1. Introduction

Biodiesel corresponds to a fuel originated from biomass, and represents one promising alternative to fossil fuels (da Silva et al., 2009). One of the major problems of biodiesel production is the formation of glycerol as a reaction by-product (da Silva et al., 2009; Kolesárová et al., 2011). In consequence, for the effective utilization of the excess glycerol, it became necessary to develop new and sustainable applications, such as the use as substrate for conversion into high-value bioproducts. In the last decades, significant research efforts have been focusing on this subject, aiming the production of several valuable products, including alcohols, organic acids (e.g. citrate, succinate), polymers, among others (Mattam et al., 2013).

Paracoccus denitrificans is a Gram-negative facultative methylotrophic bacterium able to grow in glycerol, as well as in many other carbon sources, including methanol, ethanol, butan-1-ol, and pentan-1-ol (Ueda et al., 1992; Yamane et al., 1996a, 1996b). It exhibits metabolic versatility, and it was shown to grow aerobically and anaerobically. *Paracoccus denitrificans* is reported in literature as a producer of polyhydroxyalkanoates (PHA), a complex class of naturally occurring bacterial polyesters (Ashby et al., 2004). *Paracoccus denitrificans* was found to accumulate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) during growth on pentan-1-ol, with the polymer compositions varying during the fermentation time (Yamane et al., 1996b). *Paracoccus denitrificans* also synthesized co-polymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) when methanol and an amyl alcohol were added together to a nitrogen-limited medium (Ueda et al., 1992). Mothes et al. (2007) firstly reported the production of poly(3-hydroxybutyrate) by *P. denitrificans* using crude glycerol as carbon source. Kalaiyezhini and Ramachandran (2015) have also studied poly(3-hydroxybutyrate) production from glycerol by *P. denitrificans*. In that case, the kinetics of poly(3-hydroxybutyrate) biosynthesis was evaluated in a batch bioreactor, testing different operational parameters, such as nitrogen source, carbon to nitrogen ratio, pH, aeration, and initial glycerol concentration, and the authors observed that the most suitable conditions for bacterial growth were not the same as for PHA production and accumulation.

The performance of fermentation under non-conventional conditions (such as high pressure, electric fields or ultrasounds) is a strategy that is being currently tested for different fermentation processes (Mota et al., 2018). When these stresses are applied at sub-lethal levels, the microbial strains may develop specific genetic, physiologic and metabolic responses, promoting modification of fermentation products and processes. In some cases, these modifications can represent considerable improvements, such as increased yields, productivities and fermentation rates, lower accumulation of by-products and/or production of different compounds. Therefore, the application of these non-conventional conditions to glycerol-based fermentations could introduce significant improvements in these processes, making them more

attractive for industrial production. High pressure (HP) is one of the technologies used for this purpose, with interesting results obtained so far, such as reviewed by Mota et al. (2013). The application of HP allows more possibilities than other technologies (electric fields, ultrasounds) since it can be applied as intermittent pressure stress, or can also be maintained during the whole fermentation time, without serious cell loss and no heating effect. Since there is no refrigeration requirement, the energetic costs of the fermentation process are lower, and the application of HP to these processes is simpler. Additionally, it is only necessary to provide energy to generate the pressure (and not to maintain it), and so application of HP stress during the whole fermentation process has minimal energetic costs. Therefore, the whole fermentative processes can be easily performed under HP stress conditions, differently from other technologies. This new feature might be an advantage for using HP as a non-conventional technology for fermentative processes, while the main drawback is still the high equipment costs.

One of the studies evaluating HP to fermentation used the yeast *Saccharomyces cerevisiae* as a case-study of alcoholic fermentation under HP (5-100 MPa) and observed that fermentation proceeded faster at pressures up to 10 MPa, compared to atmospheric pressure (Picard et al., 2007). Application of HP throughout fermentation can also be used to change product selectivity during the process. For instance, Bothun et al. (2004) applied pressures of 7 and 17.3 MPa on *Clostridium thermocellum*, and observed a shift in product selectivity from acetate to ethanol. At the applied pressures, ethanol:acetate ratio had a 60-fold increase relatively to atmospheric pressure. Another possibility is the application of HP to modify the properties of biopolymers produced during fermentation. For instance, cellulose produced by *Gluconacetobacter xylinus* under HP conditions (30, 60 and 100 MPa) showed several morphological changes (including higher density), compared to the polymer produced at atmospheric pressure. This modification of cellulose morphological properties may promote the acquisition of different functional properties (Kato et al., 2007). Regarding polymer production, Follonier et al. (2012) applied a low pressure level (0.7 MPa) to *Pseudomonas putida* KT2440 and were able to enhance productivity of medium-chain-length polyhydroxyalkanoate production, even with a significant decrease in specific growth rates. The effects of HP have also been evaluated in the context of food fermentation: on lactic acid fermentation, for production of probiotic yogurt (Mota et al., 2015); and in the beginning of malolactic fermentation by *Oenococcus oeni* (Neto et al., 2016).

Currently, there is only one study on literature approaching *P. denitrificans* growth under HP (Deguchi et al., 2011). In this work, a variety of microorganisms were cultured in nutrient media under hyperaccelerations (in centrifuges), to evaluate its ability to grow under these conditions. For instance, in the case of *P. denitrificans*, the cells were able to proliferate even at 403,627 × g. In order to study the role of HP on *P. denitrificans*, the authors evaluated the growth

of this microbial strain at 0.1, 30 and 40 MPa, at 30 ºC, being the pressure generated by hyperacceleration. As a result, they observed that *P. denitrificans* retained the ability to grow at 30 MPa, while it was completely inhibited at 40 MPa. These are interesting but preliminary results regarding the behavior of *P. denitrificans* in response to HP stress. Since this microbial strain has high biotechnological potential and applicability, the present work intended to evaluate the effects of different pressure levels (0.1 - 50 MPa) on *P. denitrificans* growth and fermentation, with special focus on the formation of PHA.

8.2. Material and methods

8.2.1. Microorganism and culture media

A lyophilized culture of *Paracoccus denitrificans* DSMZ 413, obtained from DSMZ, Germany, was used in this study. The strain was reconstituted on nutrient broth according to the manufacturer's instructions. The strain was sub-cultured on nutrient agar plates and incubated at 30 ºC for 24 h and then preserved at 4 ºC for a maximum period of 1 month.

Rich Medium reported by Hori et al. (1994) was used for inoculum preparation. It included polypeptone (10 g L^{-1}), yeast extract (10 g L^{-1}), meat extract (5 g L^{-1}) and $(NH_4)_2SO_4$ (5 g L^{-1}). Mineral medium reported by Mothes et al. (2007) was used for the fermentation experiments. It contained glycerol (20 g L^{-1}), yeast extract (4.5 g L^{-1}), K_2HPO_4 (5 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (20 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}), and trace elements solution (2 mL L^{-1}) for PHB production. The composition of trace elements solution is as follows: $FeSO_4 \cdot 7H_2O$ (4.98 g L^{-1}), $ZnCl_2$ (0.44 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.78 g L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.24 g L^{-1}), $MnSO_4 \cdot 4H_2O$ (0.81 g L^{-1}), dissolved in 1 N HCl solution.

8.2.2. Seed culture preparation

A single cell colony was seeded into 100 mL of rich medium and incubated overnight at 35 ºC for 16 – 20 h, in a shaker (160 rpm).

8.2.3. Fermentation experiments

Mineral medium was inoculated with 5 % (v/v) of standard inoculum. The mixture was homogenized and then transferred to polyethylene bags, with defined air volume: in most cases, with V_{medium}:V_{air} ratio of 1.0:2.2, and in others with V_{medium}:V_{air} ratio of 1:5, as detailed below for each set of samples. All the preparation steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination. Fermentation was carried at 35 ºC under different HP conditions (10, 25, 35 and 50 MPa) for 72 h. The experiments were conducted in a

Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom), with a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature, using a mixture of propylene glycol and water as pressurizing fluid. In parallel, two different control samples were used in this work: one of them at shake-flasks, with V_{medium}:V_{air} ratio of 1:5, and agitation speed of 135 rpm – C1; the other packed at conditions similar to the HP-samples (i.e. in polyethylene bags, with V_{medium}:V_{air} ratio of 1.0:2.2) – C2. The former intended to be a control with high air availability and agitation, while the latter intended to be the control for the experiments under pressure. Both control samples were incubated at atmospheric pressure (0.1 MPa) and 35 ºC. To all pressure conditions (including atmospheric pressure), fermentation experiments were performed in duplicate. Samples were collected over time, and the analyses were also performed in duplicate.

8.2.4. Determination of biomass concentration

Biomass concentration of the samples was determined by optical density measurement at 600 nm, with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA). Cell dry weight (CDW) was routinely determined using a standard curve relating *P. denitrificans* optical density and cell dry weight (CDW).

8.2.5. Determination of viable cell counts

For determination of viable cells, serial dilutions (using Ringer solution) of the culture samples were prepared and aliquots of 1.0 mL of proper dilutions were plated in nutrient agar plates, incubated at 30 °C for 24 h.

8.2.6. Glycerol quantification

Glycerol quantification was performed in the samples supernatants using the Glycerol GK Assay Kit (Megazyme, Ireland), accordingly to the manufacturer's instructions for use in 96-well microplates. The absorbance was measured with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA).

8.2.7. Analysis of polyhydroxyalkanoate formation

After centrifugation, cell pellets were re-suspended in saline solution (0.9 % NaCl), frozen and lyophilized under vacuum for ≈ 24 h. Gas chromatography-based analytical methods require the PHA to be depolymerized and chemically converted into methyl ester derivatives (by methanolysis) prior to analysis. Therefore, a weighed amount (20 mg) of dry cells was combined with 2 mL of acidified methanol (20 % H_2SO_4), and 2 ml of chloroform. Benzoic acid was used as internal standard. For methanolysis, samples and standards were heated at 100 ºC for 3 h Pyrex

test tubes with Teflon-lined caps, to convert the constituents to their methyl esters (Braunegg et al., 1978). After that time, 1 ml of water was added to the reaction mixture, followed by vigorous shaking during 2 min, to induce phase separation. The chloroform layer containing the PHA methyl esters was separated and analyzed by GC-MS (gas chromatography coupled with mass spectrometry) using a Gas Chromatograph Mass Spectrometer (GC-MS Shimadzu QP2010 Ultra) coupled to an AOC 20i autosampler (Shimadzu, Japan) and with the electron impact ionization (EI) at 70 eV. 1 µL of sample was injected automatically with a 10 µL glass syringe into a VF-5ms column (30 m x 0.25 mm with 0.25 µm film thickness; Varian, Inc., USA). Helium was used as the carrier gas and the linear velocity was set at 40 cm s^{-1}. The oven temperature conditions were set at 60 ºC for 2 min, followed firstly by a rise to 150 ºC at a 5 ºC per minute rate, then at 25 ºC per minute until 200 ºC where it stabilized for 3 min. Data were evaluated using the NIST 14 Mass Spectral database, and identifications were validated after injection of standards.

8.3. Results and discussion

Different levels of pressure (0.1 – 50 MPa) were applied to *P. denitrificans* fermentation, and were found to affect different aspects of this process, such as cell growth, substrate consumption and polymer formation. In the first stage of the work, the pressure effects were evaluated in terms of cell growth, through the analysis of biomass concentrations and viable cell counts, as indicated in Figure 8.1.

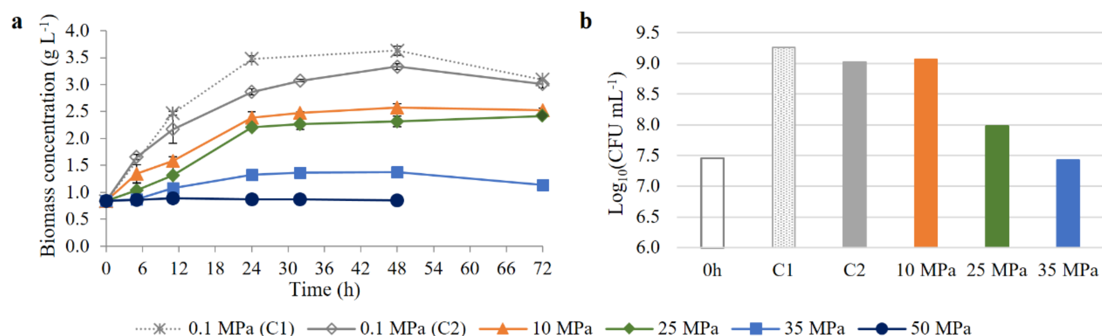

Figure 8.1. Biomass concentration over time and viable cell counts after 72 h, for fermentation at different pressure conditions: 10, 25, 35 and 50 MPa. Control samples, corresponding to fermentation at 0.1 MPa, are also indicated as C1 (higher air availability and agitation) and C2 (lower air availability and no agitation).

Biomass concentration was evaluated over time, at 10, 25, 35 and 50 MPa, and also at 0.1 MPa (C1 and C2). At 50 MPa, fermentation was interrupted after 48 h of fermentation, since biomass concentration remained stable at this pressure, indicating no occurrence of cell growth. Deguchi et al. (2011) have also observed that *P. denitrificans* growth was completely inhibited at 40 MPa, 30 ºC. At lower pressure conditions (in the range of 0.1 - 35 MPa), *P. denitrificans* was able to grow, at more or less extent. The highest biomass concentrations were achieved at 0.1 MPa, in particular for the C1 control (the one with agitation and higher air availability). However, both atmospheric pressure samples showed similar biomass concentrations after 72 h: 3.09 and 3.01 g L^{-1}, for C1 and C2, respectively. Biomass growth was significantly lower under HP conditions, suggesting an inhibitory effect of pressure on *P. denitrificans*, similarly to the previously observed for this microorganism (Deguchi et al., 2011), and for other microbial strains (Bothun et al., 2004; Kato et al., 2007; Mota et al., 2015). At 10 and 25 MPa, similar cell growth profile was observed over time, with biomass concentrations of 2.52 and 2.42 g L^{-1}, respectively, after 72 h. By increasing the pressure to 35 MPa, the inhibitory effect became more pronounced, with low biomass concentrations during the entire fermentation time: after 72 h, a biomass concentration of only 1.14 g L^{-1} was reached. The specific growth rates (μ, h^{-1}), in Table 8.1, showed a similar trend, considerably decreasing with the increasing pressure, from 0.085 h^{-1} at 0.1 MPa/C2, to 0.040 h^{-1} at 10 and 25 MPa, and to 0.020 h^{-1} at 35 MPa.

Table 8.1. Specific growth rates (μ), percentages of glycerol consumed, and yields of biomass on glycerol, after 72 h of fermentation, at different pressure conditions (0.1 – 35 MPa).

Samples	μ (h^{-1})	Glycerol consumed (%)	$Y_{X/S}$
0.1 MPa (C1)	0.135	58.3	0.207
0.1 MPa (C2)	0.085	52.7	0.221
10 MPa	0.040	51.7	0.170
25 MPa	0.040	50.9	0.163
35 MPa	0.020	12.1	0.115

Yields were calculated from a single time-point corresponding to the end of the experiment (72 h). Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicate.

Viable cell counts after 72 h showed a slightly different behavior relatively to biomass concentration (Figure 8.1.b). For instance, cell counts at 10 MPa were closer to 0.1 MPa (both C1 and C2). At 10 and 25 MPa, *P. denitrificans* cells retained their ability to grow, from 7.46 $\log_{10}$(CFU mL^{-1}) at the beginning of fermentation, to 9.07 and 7.98 $\log_{10}$(CFU mL^{-1}), respectively. In contrast, at 35 MPa, viable cell counts after 72 h were similar to those at the beginning of the process (0 h), suggesting inhibition of cell growth by this pressure level, but not cell inactivation (since there was no loss of viability).

The effects of HP on glycerol concentration over time are represented in Figure 8.2, while the percentages of glycerol consumption are indicated in Table 8.1. As expected, substrate consumption by *P. denitrificans* was affected by application of HP during fermentation, but at lower extent compared to cell growth.

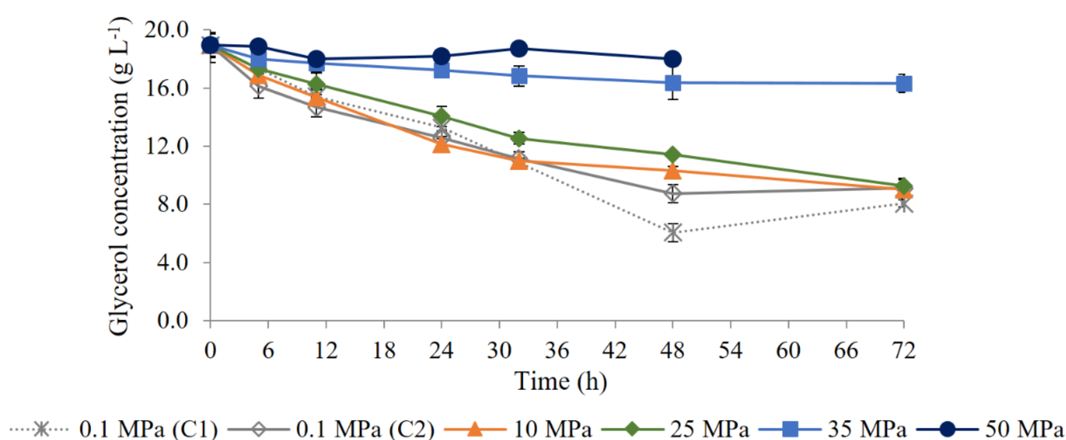

Figure 8.2. Glycerol concentrations over time, for fermentation at different pressure conditions: 10, 25, 35 and 50 MPa. Control samples, corresponding to fermentation at 0.1 MPa, are also indicated as C1 (higher air availability and agitation) and C2 (lower air availability and no agitation).

During the first 48 h at 0.1 MPa, glycerol consumption was faster compared to other pressure conditions, but the consumption decelerated (or even stopped) thereafter. In consequence, at 72 h, glycerol consumption was similar between fermentations at 0.1 MPa/C2 (52.7 %), 10 MPa (51.7 %) and 25 MPa (50.9 %). Such as observed for cell growth, substrate consumption at 35 MPa was considerably affected, corresponding to only ≈ 12 % after 72 h of fermentation. At 50 MPa, glycerol concentration showed almost no variation over time (from 18.95 g L^{-1} at 0 h, to 17.99 g L^{-1} at 48 h), suggesting inhibition of *P. denitrificans* metabolism at this pressure, such as observed for cell growth.

Yields of biomass on glycerol ($Y_{X/S}$, g g^{-1}), indicated in Table 8.1, provide quantitative information about the utilization of glycerol for cell growth. At atmospheric pressure, the yield at 0.1 MPa/C2 (0.221 g g^{-1}) was slightly higher than at 0.1 MPa/C1 (0.207 g g^{-1}), meaning that a higher proportion of glycerol was being converted into biomass when fermentation was performed under more limiting-oxygen conditions. For fermentation under HP, biomass yields were always lower than at atmospheric pressure, and with a gradual decrease with the increase of pressure: 0.170, 0.163 and 0.115 g g^{-1}, for 10, 25 and 35 MPa, respectively. This indicates that, under HP conditions, *P. denitrificans* tends to use a lower proportion of glycerol for biomass production. A possible explanation for this effect relies on the utilization of glycerol for other purposes different than cell growth, such as stress response mechanisms to ensure cell survival, or other stress maintenance processes, which are certainly activated under HP conditions. This can represent a positive feature for fermentation under pressure, since it may be related to the increased production of different valuable metabolites. One class of these *P. denitrificans* metabolites are polyhydroxyalkanoates (PHA), which play a pivotal role in priming microorganisms for stress survival. It promotes the long-term survival of bacteria under nutrients-scarce conditions by acting as carbon and energy reserves. In addition, bacteria that harbor PHA showed enhanced stress tolerance against transient environmental assaults, such as ultraviolet (UV) irradiation, heat and osmotic shock (Tan et al., 2014). Therefore, the effects of fermentation under pressure on PHA production by *P. denitrificans* were evaluated, and the PHA contents, yields ($Y_{PHA/S}$, g g^{-1}) and productivities (Q_{PHA}, mg L^{-1} h^{-1}), after 72 h of fermentation are indicated in Table 8.2. The results for fermentation at 50 MPa were not included, since no PHA production was detected after 48 h.

The polymer concentrations achieved in this work (0.066 – 0.171 g L^{-1}) are low, compared to some other studies on PHA production by *Paracoccus* sp. strains, where optimized conditions were used. For instance, Kalaiyezhini and Ramachandran (2015) and Kumar et al. (2018) achieved PHA titers of 10.7 and 9.5 g L^{-1}, respectively, in batch bioreactor experiments. However, there are other studies about PHA production that report lower titers, in the range of those observed in the present work (< 0.5 g L^{-1}) (Davis et al., 2013; Kenny et al., 2008; Ueda et al., 1992). Nevertheless, it is important to highlight that this work intended to study the effects of pressure, and fermentation was carried out with a different and simpler experimental set-up, with lower volumes more suitable for the HP experiments, due to the limited volume of the pressure vessel and not under optimized conditions.

Table 8.2. Polyhydroxyalkanoate contents, yields and productivities, after 72 h of fermentation, at different pressure conditions (0.1 – 35 MPa).

Samples	PHA (g L^{-1})	PHA (wt%)	$Y_{PHA/S}$ (g g^{-1})	Q_{PHA} (mg L^{-1} h^{-1})
0.1 MPa (C1)	0.171	5.5	0.016	2.37
0.1 MPa (C2)	0.120	4.0	0.012	1.65
10 MPa	0.104	4.1	0.011	1.45
25 MPa	0.105	4.3	0.011	1.45
35 MPa	0.066	5.8	0.026	0.91

Yields were calculated from a single time-point corresponding to the end of the experiment (72 h). Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicate.

In the case of 0.1 MPa/C1, a PHA production of 0.171 g L^{-1} was detected, corresponding to 5.5 % of cell dry mass. On the other hand, at 0.1 MPa/C2, PHA production decreased almost 30 % relatively to 0.1 MPa/C1, showing that lower oxygen availability conditions reduced PHA production. This also reflects on PHA yields and productivities, both lower at 0.1 MPa/C2. These results contrast with some of the studies in literature, which state that lower oxygen availability stimulates polymer production and decreases cell growth. Kalaiyezhini and Ramachandran (2015) observed that higher oxygen availability increased specific growth rates, while moderate oxygen availability promoted PHA production. De Almeida et al. (2010) reported a similar behavior for a recombinant *Escherichia coli* strain: higher oxygen availability resulted in lower production of metabolic products (acids, ethanol and PHA) and formation of larger amounts of biomass; reduction of oxygen availability caused a redirection of carbon flow towards the production of those metabolic products. However, this effect might depend on several aspects, such as the extent of the oxygen-limiting conditions.

Regarding the HP effects, there was a general trend to decrease polymer production with the increasing pressure. Fermentation at 10 and 25 MPa showed similar PHA titers, both of them ≈ 13 % lower than at 0.1 MPa/C2. Polyhydroxyalkanoate production was highly affected at 35 MPa, resulting in a titer 45 % lower relatively to 0.1 MPa/C2. Interestingly, the PHA content in cell dry mass (%) showed an opposite increasing trend with the increase of pressure, indicating that, at HP conditions, *P. denitrificans* favored the polymer production instead of biomass growth. The PHA content in the cells was particularly high for fermentation at 35 MPa, which showed

low biomass accumulation during the 72 h of fermentation. Similarly, the PHA yield at 35 MPa was also high, and even considerably higher than the obtained for all other fermentation conditions. At this pressure, glycerol consumption throughout the process was rather low, but it seems that a high proportion of this glycerol was being directed to PHA formation.

In sum, HP was found to decrease PHA production, resulting in lower titers and productivities; however, the PHA content in cell dry mass tended to increase under pressure, indicating that polymer production was being favored over biomass formation. Follonier et al. (2012) reported a different behavior for *Pseudomonas putida* KT2440 at ≈ 0.7 MPa, with an increase of PHA volumetric productivity under these pressure conditions. In that case, cell growth was not even inhibited, under specific values of dissolved oxygen tension and dissolved carbon dioxide tension. However, that pressure is considerably lower than the pressure levels used in the present work, and, as a result, the extent of stress and damage inflicted to the cells is not comparable.

Another possible effect of HP on PHA production may be related to modification of polymer composition. Polyhydroxyalkanoates are diverse in their chemical composition and material properties, due to the myriad of PHA monomeric units available, as well as the incorporation of these monomers at varying amounts (Tan et al., 2014). Therefore, the monomeric composition (mol%) of PHA produced under different pressure conditions was evaluated, and is represented in Table 8.3. After 72 h at 0.1 MPa/C1, *P. denitrificans* accumulated a homopolymer of 3-hydroxybutyrate, i.e. poly(3-hydroxybutyrate), which is in accordance with the results of Kalaiyezhini and Ramachandran (2015) and Mothes et al. (2007) for PHA production by *P. denitrificans*. Recently, Kumar et al. (2018) reported the production of PHA from glycerol by a culture of *Paracoccus* sp. LL1, isolated from Lonar lake, India, and, in that case, production of a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer was observed. This was already reported for other *Paracoccus* strains, using glucose, methanol, or pentan-1-ol as substrate (Üçisik-Akkaya et al., 2009; Ueda et al., 1992; Yamane et al., 1996b). In the present work, the production of the 3-hydroxyvalerate monomer was assessed with an appropriate standard, but it was not detected in any of the samples.

In the case of fermentation with lower oxygen availability (0.1 MPa/C2, and HP conditions), PHA composition was found to be rather different: the 3-hydroxybutyrate monomer was not produced in these samples, and neither 3-hydroxyvalerate. Instead, medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms) were detected in all cases. Some of these monomers were identified, by comparing the mass spectra with NIST 14 MS database, together with the use of appropriate standards. However, some monomers were not surely identified, and are thus indicated as "unidentified" in Table 8.3.

Table 8.3. Polyhydroxyalkanoate monomeric composition (mol%), after 72 h of fermentation, at different pressure conditions (0.1 – 35 MPa).

Samples	3-OH-C4	3-OH-C12	12-OH-C13	3-OH-C14	Unidentified
0.1 MPa (C1)	100	n.d.	n.d.	n.d.	n.d.
0.1 MPa (C2)	n.d.	5.0	50.2	5.3	39.5
10 MPa	n.d.	9.9	5.7	n.d.	84.4
25 MPa	n.d.	0.2	50.3	5.2	44.3
35 MPa	n.d.	n.d.	3.2	n.d.	96.8

Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicate. 3-OH-C4, 3-hydroxybutyrate; 3-OH-C12, 3-hydroxydodecanoate; 12-OH-C13, 12-hydroxytridecanoate; 3-OH-C14, 3-hydroxytetradecanoate; n.d., non-detected.

The production of mcl-PHA monomers by *P. denitrificans* is, in fact, an interesting feature. Different PHA biosynthesis pathways are possible in bacteria (Suriyamongkol et al., 2007). The production of mcl-PHA seems closely linked to fatty acids metabolic routes: β-oxidation, which is the main metabolic pathway for related substrates (e.g. fatty acids), and *de novo* fatty acid biosynthesis with non-related carbon sources (e.g. sugars) (Huijberts et al., 1992). In the first case, the resulting PHA composition depends on the carbon source, whereas in the second case, there is no relationship between the carbon sources and the resulting PHA composition (Możejko-Ciesielska and Kiewisz, 2016). There are several microorganisms producing mcl-PHA from non-related carbon sources. The most common and well-studied examples are members of the pseudomonas genus (Abe et al., 1994; Huijberts et al., 1992; Kato et al., 1996; Simon-Colin et al., 2008). However, recent studies report the occurrence of this effect on different microbial strains. For instance, Shahid et al. (2013) observed production of mcl-PHA by *Bacillus megaterium* DSM 509 from unrelated carbon sources, such as glycerol, citrate and succinate, under nitrogen depletion conditions. The resulting monomer composition of mcl-PHA related to that of *P. putida* mt-2, which was unexpected considering that PHA synthases of *Bacillus* and *Pseudomonas* sp. belong to distinct classes regarding size, subunit composition and substrate specificities. The authors suggested that poly(3-hydroxybutyrate) initially produced was further degraded, and the energy generated was probably used to drive the metabolic pathways for mcl-PHA production. Ribeiro et al. (2015) have also reported formation of mcl- and lcl-PHA (long chain length-PHA) from non-related sources, by *Cupriavidus necator* IPT 027 and

Burkholderia cepacia IPT 438. The composition of the polymers produced from pure glycerol predominantly consisted of monomers of 11-hydroxyhexadecanoate and 3-hydroxytetradecanoate monomers. The authors stated that formation of building blocks and longer molecular structures of PHA is possibly related to the carbon sources adopted. But the results in the present study suggest that it can also be affected by other operational conditions, such as oxygen availability or pressure.

In the 0.1 MPa/C2 samples, the PHA polymer produced was mainly composed by 12-hydroxytridecanoate, as well as unidentified monomers. Minor components included 3-hydroxydodecanoate and 3-hydroxytetradecanoate. By applying HP during fermentation, the PHA composition was found to vary according to the pressure conditions. At 25 MPa, the monomeric composition was similar to 0.1 MPa/C2, while the same was not observed at 10 MPa. In the latter, unidentified monomers were the major PHA components, with a minor contribution of 3-hydroxydodecanoate and 12-hydroxytridecanoate. For some reason, 3-hydroxytetradecanoate monomers were not even detected in polymer produced at this pressure. When performing fermentation at 35 MPa, the accumulated polymer was mainly composed by the unidentified monomers, with a minor proportion of 12-hydroxytridecanoate. With this information, it is not clear why the polymer composition was modified at 10 and 35 MPa, but not at 25 MPa, since a progressive and more consistent effect of HP on polymer composition would be expected. In their study regarding PHA formation by *P. putida* KT2440, Follonier et al. (2012) observed no changes in PHA composition when fermentation was carried out at ≈ 0.7 MPa, compared to the polymer produced at atmospheric pressure. However, it is important to note that the pressure applied in that work was considerably lower than the pressure levels that we were dealing in the present study. By using pressure levels in the range of 30 – 100 MPa, Kato et al. (2007) reported an example of polymer modification under HP conditions, for bacterial cellulose produced by *Gluconacetobacter xylinus*. Therefore, polymer production at higher pressure levels seems more prone to promote changes in polymer composition and morphological properties.

Overall, PHA production and composition was highly dependent on the pressure applied on *P. denitrificans* growth and fermentation. On the one hand, HP decreased polymer titers, but increased the PHA content in cell dry mass (%), indicating higher ability to accumulate these polymers in the cells. On the other hand, some levels of HP affected the PHA monomeric composition, mainly at 10 and 35 MPa. Although the HP effects on PHA properties were not evaluated in the present work, it is possible to foresee that the changes in polymer composition will certainly affect its mechanical properties. For instance, a complex organization of building blocks with medium and long chains is usually correlated with PHA polymers with higher molecular weight, and may contribute to their minimal crystallinity and higher industrial applicability (Laycock et al., 2013; Ribeiro et al., 2016; Simon-Colin et al., 2008). However, in

the particular case of polymers produced under HP, it was not possible to get a specific pattern of monomer modification, and thus, the effects on mechanical properties are not predictable. Therefore, future work must focus on further evaluation of the composition of PHA produced under pressure, as well as determination how it impacts its physical and mechanical properties. In order to do that, a higher amount of PHA polymers should be obtained, by optimizing fermentation under HP conditions, and thus increasing the scale of the process to higher volumes, using a higher volume pressure vessel. In the present study, the experiments were performed on a HP equipment suitable for pasteurization and food technology purposes, which can also be used for a wider range of applications, but with some inherent constrains, such as volume limitations and absence of agitation mechanisms. Therefore, further optimization studies should be performed in tailor-made HP equipment able to better meet the specifications required for these microbial growth and fermentation studies, such as agitation. It should be highlighted that such type of pressure equipment is now becoming available.

8.4. Conclusions

Paracoccus denitrificans growth and fermentation were both affected by HP, with the effects varying according to the pressure level applied. Despite of the negative impact on cell growth, interesting metabolic features were observed under HP conditions. For instance, biomass yields were always lower than at atmospheric pressure, and with a gradual decrease with the increase of pressure. This indicates that, under HP conditions, *P. denitrificans* decreased the utilization of glycerol for purposes of cell growth, suggesting the formation of different metabolic products. In fact, the PHA content in cell dry mass (%) tended to increase with the increasing pressure, suggesting higher ability to accumulate these polymers in the cells. High pressure was also found to promote changes in PHA composition, with the polymer produced at 10 and 35 MPa showing considerable differences relatively to the ones obtained at atmospheric pressure (C1 and C2). The effects of these modifications on PHA physical and mechanical properties have not yet been disclosed, and neither the potential improvements arising from that. In any case, the results obtained from this work demonstrated the possibility of applying HP technology to this type of fermentation processes, without compromising the production of PHA by *P. denitrificans*. It also showed that the PHA produced under HP had different monomeric composition, which may lead to a polymer with different properties. These results display another example of interesting effects of pressure on living systems, with possible biotechnological potential. Under the same reasoning, some pharmaceutical companies are investigating microbial growth under deep-sea like environments (at HP conditions), aiming to obtain novel compounds with different biological activities.

To better understand the potential of using HP to change the chemical composition and mechanical properties of bio-polymers, in particular those produced by *P. denitrificans*, more information must be disclosed on this subject. The present study will certainly be helpful in further optimization studies, as well as in scale-up to higher volumes. In order to do that, tailor-made HP equipment will be needed, to meet the specifications of these microbial growth and fermentation processes, which require particular system features, such as the possibility of agitation and air supply. This type of HP equipment is now becoming more widely available, and allow to easily apply pressure to any microbial system, during the entire time of growth and/or fermentation, and with low energetic costs.

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CHAPTER IX

Conclusions and outlook

Application of sub-lethal HP has a tremendous potential to modulate (and potentially improve) glycerol-based fermentation processes. In the present work, the effects of pressure were evaluated on two microbial strains, involved in two different fermentation processes. The most relevant distinct features between them relied on their oxygen requirements and on their HP tolerance. Regarding the oxygen requirements, the results showed that it was possible to perform fermentation under pressure at both anaerobic and aerobic conditions, such as observed for *L. reuteri* and *P. denitrificans*, respectively. However, many high pressure equipments currently available (including the one used in the present work) are not adapted to allow continuous air supply and agitation. Therefore, this approach is generally more suitable for anaerobic processes, which do not have high oxygen requirements, and, because of that, most studies on this field are carried out with anaerobic or facultative anaerobic microbial strains. The other distinctive factor between the two microbial strains was their tolerance to HP. *Lactobacillus reuteri* and *P. denitrificans* were both able to grow at low pressure levels (e.g. 10 or 25 MPa), but showed different tolerance to higher pressure levels. While *P. denitrificans* was still able to grow at 35 MPa, *L. reuteri* was already inhibited at this pressure. This may indicate that both strains have different response mechanisms to HP and/or that their cellular and molecular structures have different resistance to pressure. Despite all the differences between the fermentative strains and respective processes, sub-lethal HP was successfully applied throughout fermentation. By optimization of pressure and general fermentative conditions, the microbial strains were able to grow and retain the fermentative activity, while promoting interesting metabolic changes in both cases.

For the studies with *L. reuteri* under HP, some of the most important findings are indicated as follows:

- High pressure (10 – 35 MPa) affected *L. reuteri* growth and fermentation, with the effects varying according to the pressure level and the initial acetate content. In general, fermentation was less inhibited by HP when acetate was present, indicating that acetate enhances the resistance of *L. reuteri* to pressure.
- Production of 1,3-PDO was stimulated at 10 MPa, leading to higher titers, yields and productivities, compared to 0.1 MPa. In fact, fermentation at 10 MPa promoted a metabolic shift, with modification of product selectivity towards production of 1,3-PDO, and general reduction in the formation of by-products.
- Application of consecutive fermentation cycles increased 1,3-PDO production, especially at 10 MPa, in samples without added acetate. In that case, considerable improvements of 1,3-PDO titers were achieved relatively to the “conventional approach”, i.e. without the fermentation cycles and at 0.1 MPa.

- At 25 MPa, 1,3-PDO production was also improved between the first and the fourth cycles, but the increment was less pronounced and highly variable, showing an “improvement and decline” trend. This suggests that *L. reuteri* was not able to adapt (at least not steadily) to a pressure of 25 MPa, within the number of cycles studied.
- The comparative metabolomic study between *L. reuteri* fermentation samples at 0.1 and 10 MPa showed a modification in metabolic profiles throughout the cycles. This effect was considerably more accentuated at 10 MPa. One of the metabolites characteristic of fermentation cycles at 10 MPa was 2,3-BDO, which showed higher relative abundance at this pressure, relatively to the respective cycle at 0.1 MPa.

The most relevant observations concerning the effects of air availability and HP on *P. denitrificans* are also summarized below:

- *Paracoccus denitrificans* growth and metabolism were affected by air availability. At higher air availability, considerable cell growth was observed, but no production of ethanol, acetic or succinic acids. Without air availability, *P. denitrificans* showed active metabolic activity (with production of ethanol, acetic and succinic acids), but no cell growth.
- *Paracoccus denitrificans* was able to grow at 10 and 25 MPa, with biomass concentrations similar to 0.1 MPa. A more extensive inhibitory effect occurred at 35 MPa, while no cell growth was observed at 50 MPa. HP also affected substrate consumption, as well as the formation of ethanol, acetic and succinic acids, with the fermentative profile varying according to the pressure level.
- High pressure decreased PHA titers, but increase the PHA content in cell dry mass, suggesting higher ability to accumulate these polymers in the cells. In addition, HP promoted changes in PHA monomeric composition, with the polymer produced at 10 and 35 MPa showing considerable differences relative to the ones obtained at atmospheric pressure.

Overall, the present work indicates that HP can be applied to different microorganisms, with different process specificities. The combination of these studies provide a general overview of the main potentialities and limitations of HP application to glycerol-based processes. The final titers of 1,3-PDO and PHA obtained with this strategy were low compared to some other studies in literature, and far from those required for industrial application. However, these results unveil relevant information regarding the adaptation of mesophilic microorganisms to sub-lethal HP, and give a perspective on how these mechanisms can be used to stimulate or inhibit specific metabolic pathways, similarly to genetic engineering approaches, but with a more general and

less targeted approach. Moreover, the insights provided by this work pave the way for further optimization studies, with HP equipment suitable for these microbial growth and fermentation approaches. This will promote the use of these type of strategies on a wide range of microbial processes, with potential application in the food, pharmaceutical and energy industries.

APPENDIX A

Additional information supporting

CHAPTER VI

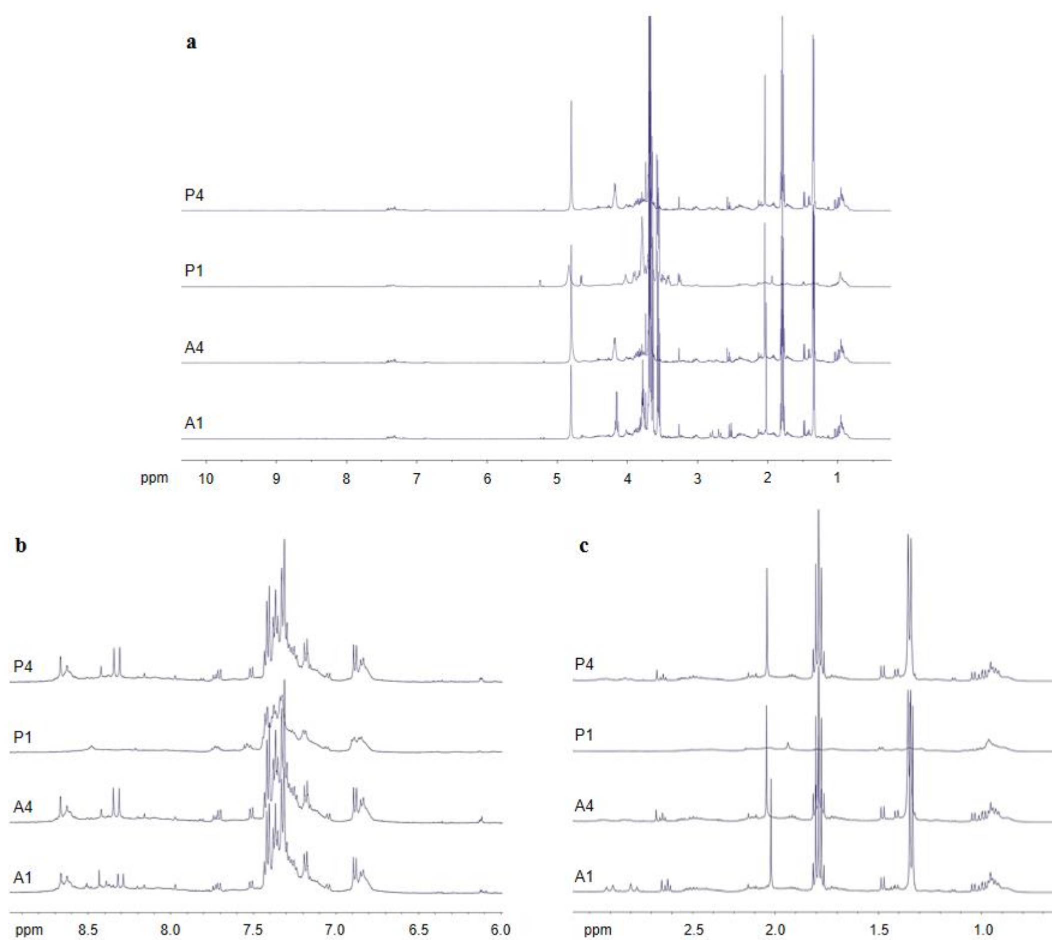

Figure A.1. Examples of ^{1}H NMR spectra of fermentation samples after one and four cycles at atmospheric pressure (C1 and C4, respectively), and at 10 MPa (P1 and P4, respectively): (a) full spectra; and expansions for (b) aromatic region (6.0-9.0 ppm) and (c) aliphatic region (0.6-3.0 ppm).

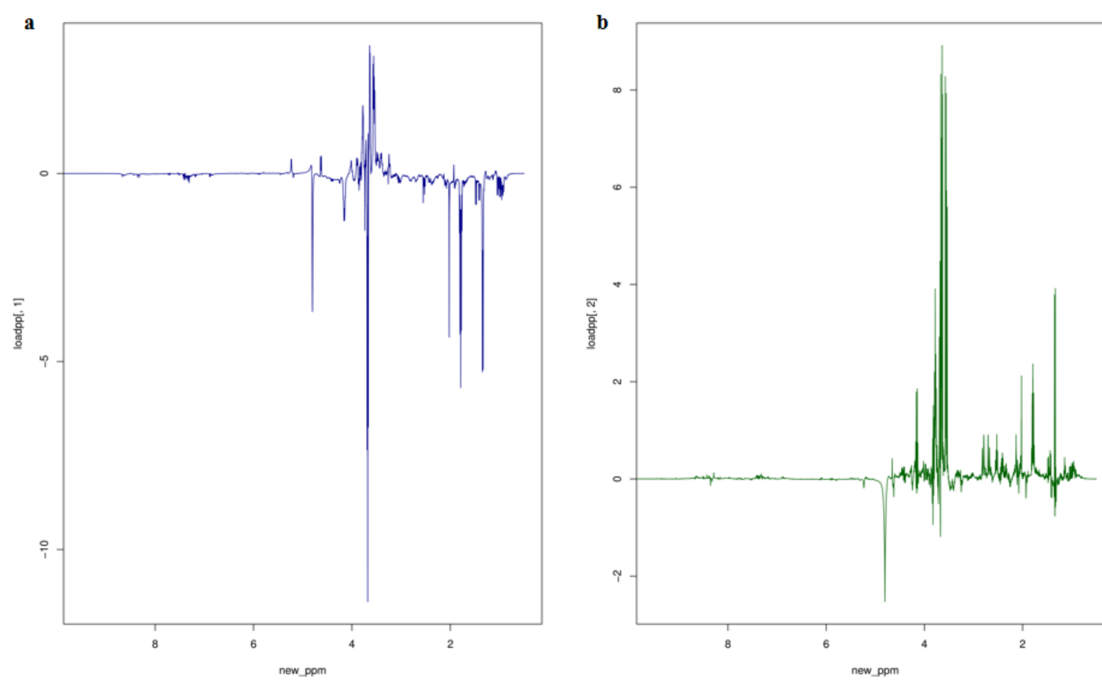

Figure A.2. Principal component analysis (PCA) loading plots PC1 (a) and PC2 (b) of *L. reuteri* fermentation metabolites, obtained by 1D ^{1}H NMR.

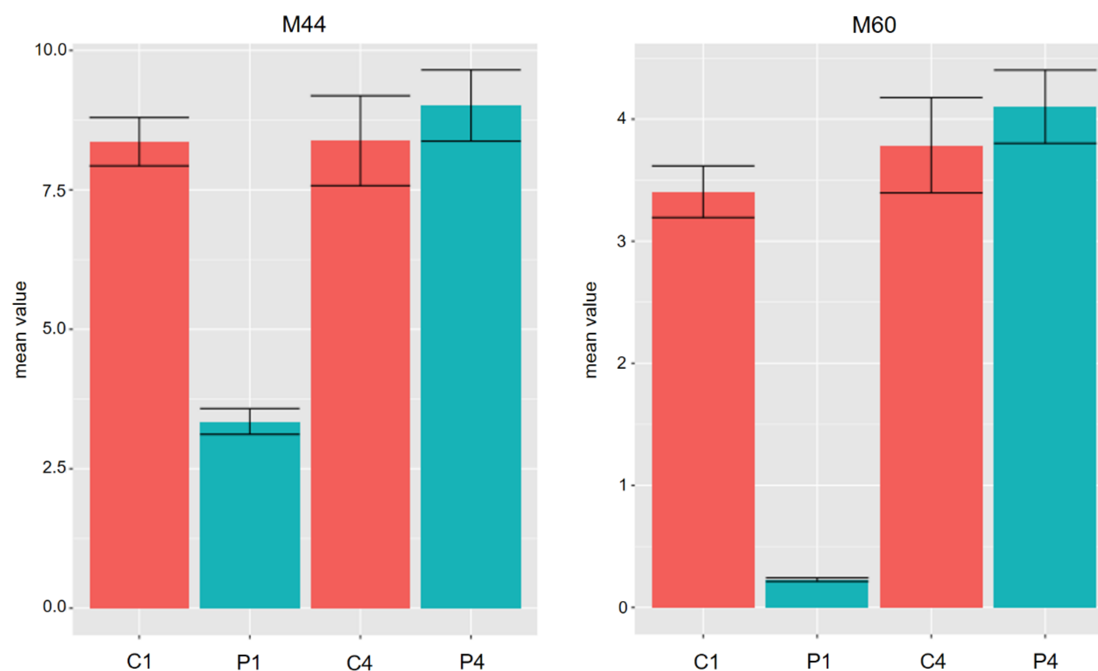

Figure A.3. Metabolite plots showing the abundance of 1,3-PDO (for M44 and M60, both signals corresponding to this metabolite) in samples at 0.1 MPa (C1 and C4, for the first and fourth fermentation cycles, respectively) and at 10 MPa (P1 and P4 samples, for the first and fourth fermentation cycles, respectively).

APPENDIX B

Additional information supporting CHAPTER VII

Adapted from:

Mota, M.J., Lopes, R.P., Pinto, C.A, Sousa, S., Gomes, A.M., Delgadillo, I., Saraiva, J.A., The use of different fermentative approaches on *Paracoccus denitrificans*: Effect of high pressure and air availability on growth and metabolism. Submitted in Biotechnology Progress.

Table B.1. Glucose and maltose concentrations, at 0 h and 72 h, for fermentation under different air availability conditions.

Samples	Maltose concentration (g L^{-1})		Glucose concentration (g L^{-1})	
	0 h	**72 h**	**0 h**	**72 h**
Without air		n.d.		1.126
24h with air + 48h without air	0.256	n.d.	1.066	1.037
With air		n.d.		1.155

Values reported in the table represent the mean of two independent biological replicates, analyzed in duplicated. N.d. indicates non-detected production of the compound.