Universidade de Aveiro Departamento de Química
2017

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Adaptação *de Saccharomyces cerevisiae* a alta pressão

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Alimentar, realizada sob a orientação do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar e da Doutora Ivonne Delgadillo Giraldo, Professora Associada com agregação ao Departamento de Química da Universidade de Aveiro.

Dedico este trabalho aos meus queridos pais e amigos.

o júri

Presidente

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palavras-chave *Saccharomyces cerevisiae*, fermentação, bioetanol, pressão e adaptação.

resumo

O objetivo do presente trabalho passou pelo estudo da adaptação de *S. cerevisiae* à pressão, usando ciclos consecutivos de fermentação sob pressão em níveis sub-letais. Assim, este trabalho foi divido em duas partes: numa primeira parte, foram aplicadas pressões sub-letais (entre 15-50 MPa) durante o processo fermentativo para determinar as pressões a serem utilizadas na fase posterior; na segunda parte, as culturas de *S. cerevisiae* realizaram fermentação sob pressão ao longo de quatro ciclos consecutivos de fermentação de modo a desencadear um mecanismo de adaptação à pressão. Neste contexto, foram testadas três pressões (15 MPa, 25 MPa e 35 MPa) e duas temperaturas (30 ºC e temperatura ambiente). De modo a monitorizar os processos, foram determinadas as concentrações de açúcares (glucose, frutose e maltose), etanol e ácidos orgânicos (cítrico, málico, succínico e acético). Para além disso, foram realizadas análises microbiológicas para determinar a viabilidade celular e concentração de biomassa. Após cada ciclo a 15 e 25 MPa, tanto o crescimento celular como a produção de etanol mostraram tendência para aumentar, sugerindo a adaptação da *S. cerevisiae* a estes níveis de pressão. Na verdade, no final do 4º ciclo sob ambas as pressões, a produção de etanol foi superior à observada à pressão atmosférica (8.75 $g.L^{-1}$ e 10.69 $g.L^{-1}$ a 15 e 25 MPa, respetivamente, comparando com 8.02 $g.L^{-1}$ à pressão atmosférica). No entanto, quando a pressão aumenta para 35 MPa, o crescimento celular e a produção de bioetanol diminuíram, sendo mínimas após os 4 ciclos de fermentação consecutivos. De um modo geral, estes resultados sugerem que a adaptação a condições sub-letais de pressão (15 e 25 MPa) pode melhorar a produção de bioetanol pela *S. cerevisiae*, podendo esta técnica ser utilizada para aumentar rendimentos e produtividades da fermentação alcoólica.

keywords *Saccharomyces cerevisiae*, fermentation, bioethanol, pressure and adptation.

abstract The objective of the present work was to study the adaptation of *S. cerevisiae* to the pressure, using consecutive cycles of fermentation under pressure at sublethal levels. Thus, this work was divided in two parts: in the first part, sub-lethal pressures (between 15-50 MPa) were applied during the fermentation process to determine the pressures to be used in the later phase; in the second part, *S. cerevisiae* cultures underwent fermentation under pressure over four consecutive fermentation cycles to trigger a pressure adaptation mechanism. In this context, three pressures (15 MPa, 25 MPa and 35 MPa) and two temperatures (30 ° C and ambient temperature) were tested. In order to monitor the processes, the concentrations of sugars (glucose, fructose and maltose), ethanol and organic acids (citric, malic, succinic and acetic) were determined. In addition, microbiological analyses were performed to determine cell viability and biomass concentration. After each cycle at 15 and 25 MPa, both cell growth and ethanol production showed a tendency to increase, suggesting the adaptation of *S. cerevisiae* to these pressure levels. In fact, at the end of the 4th cycle under both pressures, the ethanol production was higher than that observed at atmospheric pressure (8.75 $g.L^{-1}$ and 10.69 $g.L^{-1}$ at 15 and 25 MPa, respectively, comparing with 8.02 $g.L^{-1}$ at pressure atmospheric). However, when the pressure increases to 35 MPa, cell growth and bioethanol production decreased, being minimal after the 4 consecutive fermentation cycles. In general, these results suggest that adaptation to sublethal pressure conditions (15 and 25 MPa) can improve bioethanol production by *S. cerevisiae*, and this technique can be used to increase yields and yields of alcoholic fermentation.

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1. Introduction

1.1. Yeasts – General overview and the particular case of *Saccharomyces cerevisiae*

Yeasts are eukaryotic, unicellular microorganisms classified as members of the fungus kingdom. Although the meaning of the word 'yeasts' is not straightforwardly defined, yeasts are usually recognized as being unicellular fungi. More specifically, yeasts are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, resulting in sexual states that lead to different matte types (Pompon, 1999). Yeasts are of major economic, social and health significance in human culture. They have often been described as mankind's oldest 'domesticated' organisms, having been used to produce alcoholic beverages and ferment bread dough for millennia. Nowadays, yeasts have found numerous other roles besides traditional food fermentations, being some examples represented in (Figure 1). In particular, genetically manipulated yeasts can now be exploited to produce many different biopharmaceutical agents for preventing and treating human diseases (Atkinson & Sherwood, 2014; Fox, Bellini, & Pellegrini, 2014; Pompon, 1999)

One particular genus of yeasts, named *Saccharomyces*, has played a central role in the commercial exploitation of fungi by mankind. These facultative aerobes utilize the Embden-Meyerhof pathway to convert sugars to pyruvate, which results in the production of two molecules of pyruvate. Each of these molecules are then reductively decarboxylated by the enzymes pyruvate decarboxylase and alcohol dehydrogenase to give rise to one molecule of ethanol and carbon dioxide. Therefore, the foundation for two of our major food industries, brewing and baking, was provided by this simple and efficient way of fermenting glucose into ethanol and carbon dioxide (Atkinson & Sherwood, 2014; Molitoris, 1995).

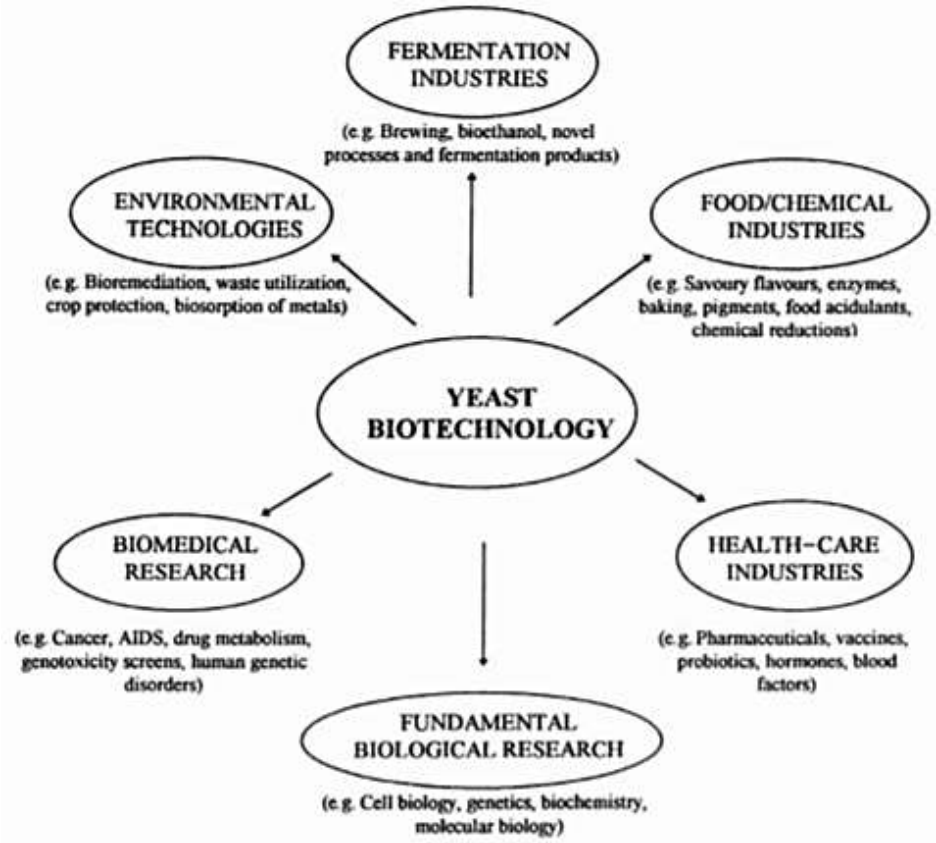

Figure 1 Diversity of channels involving yeasts biotechnology (Walker, 1998a)

1.2.Yeasts Metabolism

Yeasts metabolism refers to the biochemical assimilation and dissimilation of nutrients by yeasts cells. In nutrient assimilation, anabolic pathways involved in the nutrient assimilation are energy-consuming and reductive processes, which lead to the biosynthesis of new cellular material, such as proteins. On the other hand, catabolic pathways are oxidative processes, which remove electrons from intermediates and use them to generate energy, *e.g.* ATP. These reductive and oxidative processes are mediated by dehydrogenase enzymes which predominantly use NADP and NAD, respectively, as redox cofactors. These two processes cannot be independent, having some common compounds between both pathways. Figure 2 shows a general draft of the yeasts carbon metabolism (Walker, 1998b).

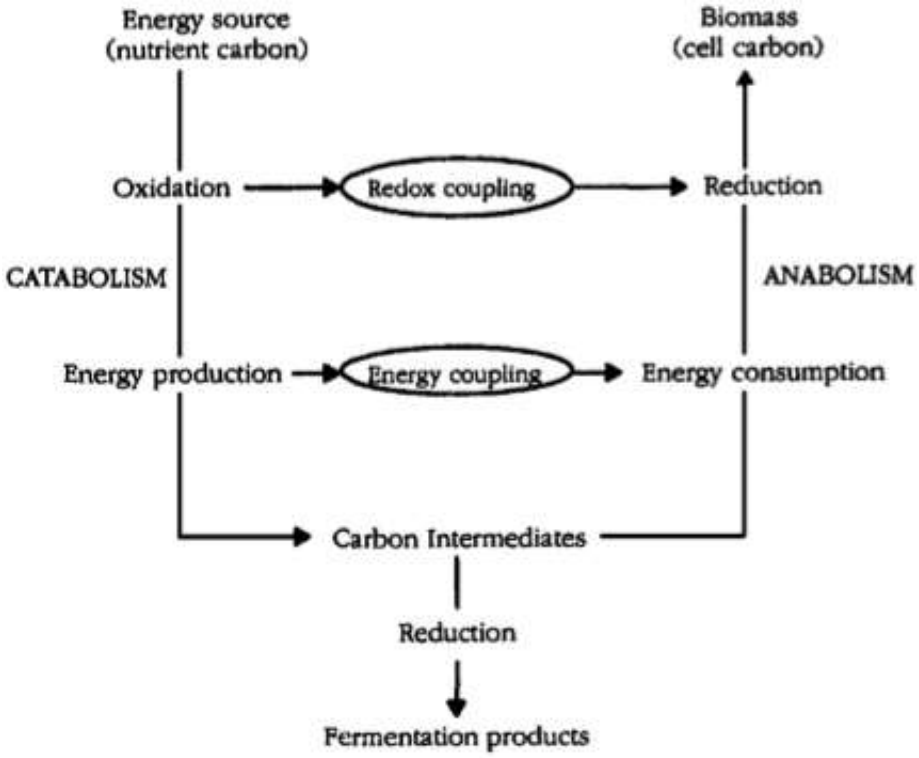

Figure 2 General overview of yeasts carbon metabolism (Walker, 1998b)

Yeasts are chemoorganotrophic microorganisms which derive their chemical energy from the breakdown of organic compounds, in the form of ATP. Despite the similarities between almost every metabolic pathway, there is some diversity in the way which yeasts generate and consume energy from carbon substrates. Depending on the strain, the primary carbon source can be different, since some yeasts use glucose, while others use glycerol, or many other different carbon sources (Walker, 1998b).

In the case of *Saccharomyces cerevisiae,* glucose is metabolized using the glycolysis pathway producing pyruvate as the first product. Pyruvate is then converted into acetaldehyde via enzymatic reaction by pyruvate decarboxylase and this reaction also leads to the re-oxidation of NADH to NAD^+. Finally, the acetaldehyde produced is converted into ethanol via enzymatic catalysis by alcohol dehydrogenase (ADH).

The key regulatory enzymes in the glycolysis pathway, as represented in Figure 3, are phosphofructokinase and pyruvate decarboxylase, whose activity is influenced by numerous effectors, including ATP. The function of these two enzymes is, on one hand, to convert fructose 6-phosfate to fructose 1,6-biphosfate using a molecule of ATP, and,

on the other hand, to catalyse the decarboxylation of pyruvate into acetaldehyde and carbon dioxide, respectively (Walker, 1998b).

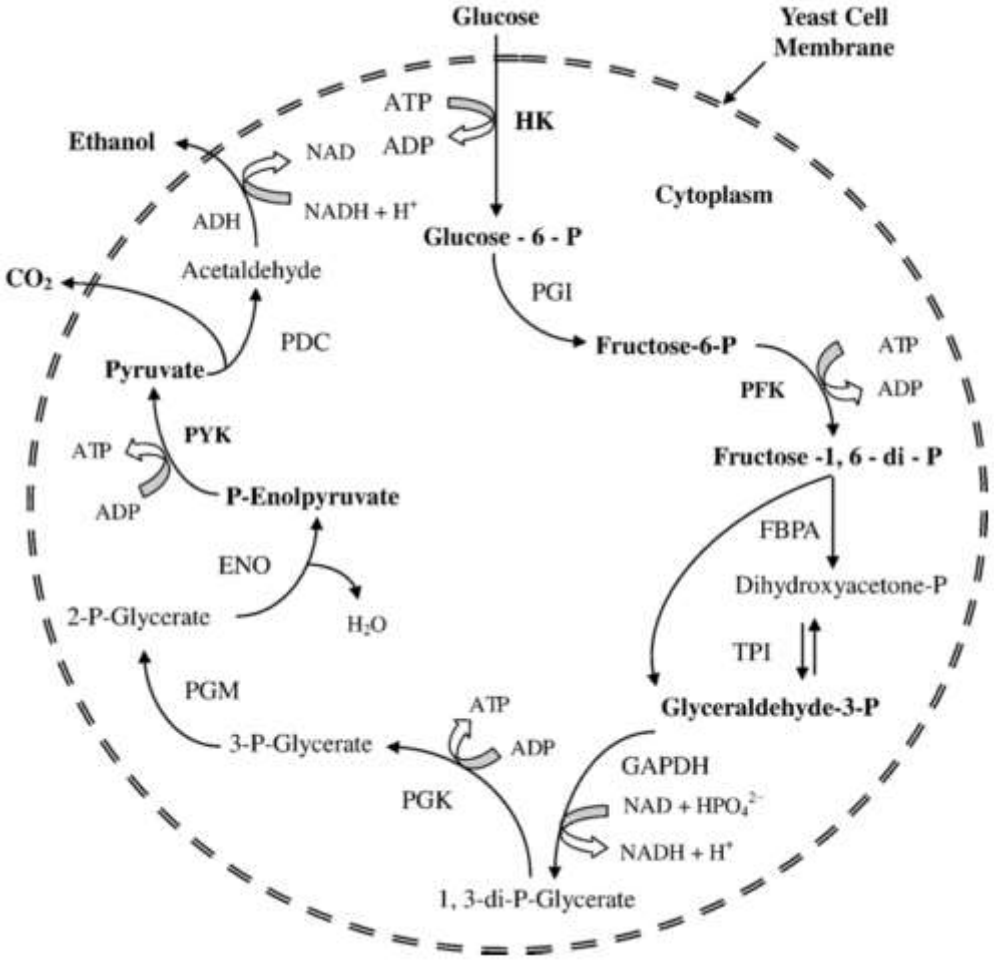

Figure 3 Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase (Bai, Anderson, & Moo-Young, 2008)

In addition, the strains of yeasts are able to transfer pyruvate to the mitochondrial matrix, where is oxidatively decarboxylated into acetyl-coA using pyruvate dehydrogenase. After this, the acetyl-coA can enter on the Krebs cycle and produce a high amount of ATP via aerobic respiration.

1.3. Fermentation processes

The term fermentation is derived from the Latin verb *fervere*, which means "to boil". This boiling appearance is the result of carbon dioxide release, resulting from the anaerobic metabolism of the microorganisms. This process has different meanings to biochemists and to industrial microbiologists: while for the first it means the use of organic compounds to produce energy, for the latter it has a much broader meaning, representing all the respiratory processes performed by cells (Stanbury, Whitaker, & Hall, 2013).

In general, fermentation is the process where substrates are converted into products, as a result of the growth and/or metabolic activities of microorganisms. There is a vast variety of fermentation processes used in food industry and microorganisms responsible for those processes, including bacteria, yeasts, and fungi.

During food fermentation, the growth of some pathogenic and/or spoilage microorganisms is inhibited by the metabolites generated by the fermenting organisms, which consequently extend the shelf life of perishable products. For example, during lactic acid fermentation, lactic acid bacteria may produce several of these metabolites, such as lactic acid, acetic acid, carbon dioxide, ethanol, hydrogen peroxide, bacteriocins, or antimicrobial peptides (Caplice & Fitzgerald, 1999; Di Cagno, Coda, De Angelis, & Gobbetti, 2013). These compounds synergistically suppress the survival and growth of pathogenic and spoilage microorganisms, extending the shelf-life of the fermented dairy products and allowing its preservation (Stanbury et al., 2013; Terefe & Food, 2016). In fact, this was the primary purpose of food fermentation in the past, when the utilization of processing technologies was poorly developed, but nowadays these processes are widely used due to the production of unique flavours, aromas and textures, corresponding to attributes appreciated by the consumer (Caplice & Fitzgerald, 1999).

Fermentation processes are sometimes simple, involving only one substrate component (*e.g.* lactose present in the milk) and one microorganism (*e.g. Lactococcus lactis*), but sometimes these processes can involve a more complex mixture of substrates and a number of microorganisms. For example, kefir, that possesses a wide diversity of microorganisms, namely: several yeasts, lactic acid (LAB), and acetic acid bacteria (Dimitrellou, Kandylis, Kourkoutas, Koutinas, & Kanellaki, 2015). Figure 4 is a general overview of the fermentation processes that are the most used in industry. Lactic acid

fermentation, above mentioned, uses lactic bacteria, *Lactococcus lactis* and *Lactobacillus lactis* and produces most of our dairy products. On the other hand the acetic fermentation uses some bacteria of the genera *Acetobacter* (*Acetobacter oeni*, *Acetobacter pasteurianus*) or *Escherichia coli* to produce vinegar from wine, for example (Gullo & Giudici, 2008; Wu, Ma, Zhang, & Chen, 2012). Propionic fermentation, used to propionic acid production, an important component in Swiss cheese, uses *Propionibacterium freudenreichii ssp. freudenreichii and Propionibacterium freudenreichii ssp. Shermanii,* as the microorganisms responsible for the process (O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016; Salek, Černíková, Maděrová, Lapčík, & Buňka, 2016). Acetone–butanol–ethanol (ABE) fermentation is the process that uses bacterial fermentation to produce acetone, n-butanol, and ethanol from carbohydrates, using bacteria from the genera *Clostridium* (*Clostridium acetobutylicum* and *Clostridium beijerinckii*) (Cai et al., 2016). One other example of a fermentation process is alcoholic fermentation. This process uses some microorganisms, for example *S. cerevisiae*, and sugar to produce ethanol. Ethanol has many uses, being used in food industry to produce alcoholic beverages during alcoholic fermentation, in the medical industry as a disinfectant and it can also be used as bioethanol and produce fuel to be used in automobiles (Bajpai, 2013b; Dussap & Poughon, 2016)

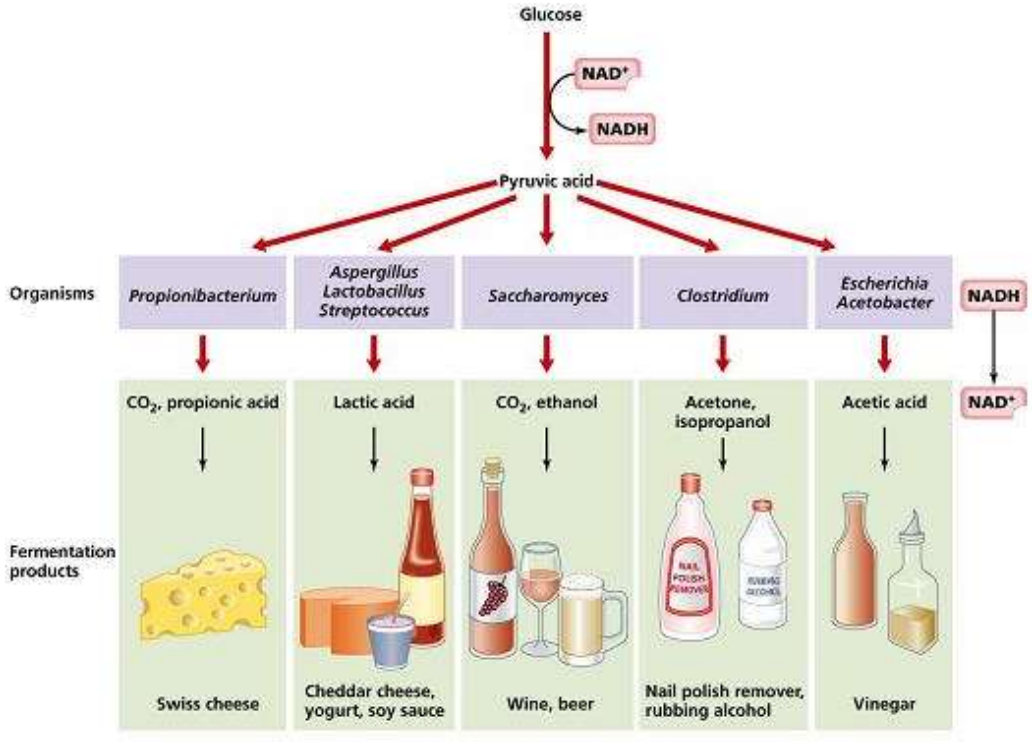

Figure 4 Different examples of fermentations that occur in industry

1.3.1. Bioethanol production

Crude oil has been the major resource to meet the increased world energy demand, but it is not a renewable resource and is related to many environmental issues. However, during ethanol production, huge amount of with very high biological oxygen demand (BOD) and chemical oxygen demand (COD) is produced, which is discharged into the environment without proper treatment. So, the ethanol production using current distilleries is a potential source of environmental pollution and because of this, ethanol production process needs to be improved and optimized for an environment friendly, fast and cheap ethanol production (Arshad, Hussain, Iqbal, & Abbas, 2017). To reduce the dependence of crude oil, the use of bioethanol as an alternative fuel has been steadily increasing around the world (Bajpai, 2013a). Bioethanol is a clean, renewable and sustainable alternative fuel with several advantages and disadvantages when comparing to crude oil, presented in Table 1 (Bajpai, 2013b; Deesuth, Laopaiboon, Klanrit, & Laopaiboon, 2015; Deesuth, Laopaiboon, & Laopaiboon, 2016).

Chemical industry and chemical synthesis was until a few year ago the most common way to produce some compounds, including ethanol, but this industry has many safety and environmental problems. Recently, the chemical industry has been subjected to close scrutiny owing to concerns about its reliance on fossil resources; environmentally damaging production processes that can be unsafe and produce toxic products and waste; products that are not readily recyclable and degradable after their useful life; and excessive regional concentration of production so that social benefits of production are less widely available (Davies & Ni, 2006; Wansink & Kim, 2000). Because of this, the industry has been under increasing pressure to change current working practices in favour of greener alternatives (Davies & Ni, 2006; Hatti-Kaul, Törnvall, Gustafsson, & Börjesson, 2007; Miller & Nagarajan, 2000). One example is green chemistry that is focused on the designing of products and processes that minimize the use and generation of hazardous substances. One example is the utilization of biotechnological processes.

Bioethanol is one of the products that is being produced by industrial biotechnology (OECD, 2001). This product can be produced by biotechnological processes using an enormous variety of raw materials that include not only plants. Initially, the most used substrate was sugarcane and corn, producing the so called first generation bioethanol. However, despite the high bioethanol yield obtained with these

substrates, their use started to be a concern due to the used of arable soil to produce bioethanol (ActionAid, 2010; Berlin (AFP) Staff writers, 2008; Wansink & Kim, 2000). Nowadays, sweet sorghum and waste water have been increasingly used to produce bioethanol (Bai et al., 2008; Deesuth et al., 2015, 2016; El-Dalatony et al., 2016; Tantipaibulvut et al., 2015). Additionally, agricultural, forestry and municipal solid waste can also be used for bioethanol production.

1.3.1.1. Bioethanol production by *Saccharomyces cerevisiae*

S. cerevisiae is by far the most commonly used microbial species for industrial production of ethanol from sugar- and starch-based raw materials, tolerating a wide spectrum of inhibitors and elevated osmotic pressure. Regarding to ethanol production, it occurs with high yields (Bajpai, 2013b; Martín, Galbe, Wahlbom, Hahn-Hägerdal, & Jönsson, 2002). In that way, *S. cerevisiae* is an efficient microorganism for producing ethanol from hexose sugars. However, during the fermentation process, yeasts cells are exposed to numerous environmental stresses, leading to countless intracellular changes that affect biomass production, fermentation efficiency and cell viability (Teixeira, Mira, & Sá-Correia, 2011). In fact, yeasts undergoing fermentation are challenged with osmotic stress, high temperature and high ethanol concentration (Pataro et al., 2000).

For instance, the initial sugar concentrations typically used in fuel ethanol industry are under normal gravity (NG, *i.e.* less than 180 $g.L^{-1}$ of total sugar) or high gravity (HG, *i.e.* 180–220 $g.L^{-1}$ of total sugar) conditions. But, in order to increase the ethanol fermentation efficiency, very high gravity (VHG) technology may be used, resulting in the improvement of ethanol productivity and consequently increase the cost effectiveness. This VHG ethanol fermentation uses medium containing sugar in excess of 250 $g.L^{-1}$ to achieve over 15% (v) ethanol (Bai et al., 2008). However, the fermentation under high sugar content or VHG conditions may cause adverse effects on yeasts metabolism because of the high osmotic pressure and high ethanol concentrations produced (Pratt, Bryce, & Stewart, 2003).

Figure 5 shows a model flow diagram of a Brasilian sugarcane-ethano producer. In this model sugercane is firstly used to produce sugar and then the sugarcane residues molasses are used to produce ethanol in a integrated manner. This way of obtaing ethanol together with sugar has some advantages compared to the other methods, because less

energy is used, the quality of the products is better, and the yields attained are greater (stoichiometric ethanol yield of 91%), with lower costs (about US$0.20/liter ethanol). Futhermore, this enables the waste reduction of both industries, ethanol and sugar industries (Cortez & Baldassin, 2016; Monteiro Salles-Filho, 2016).

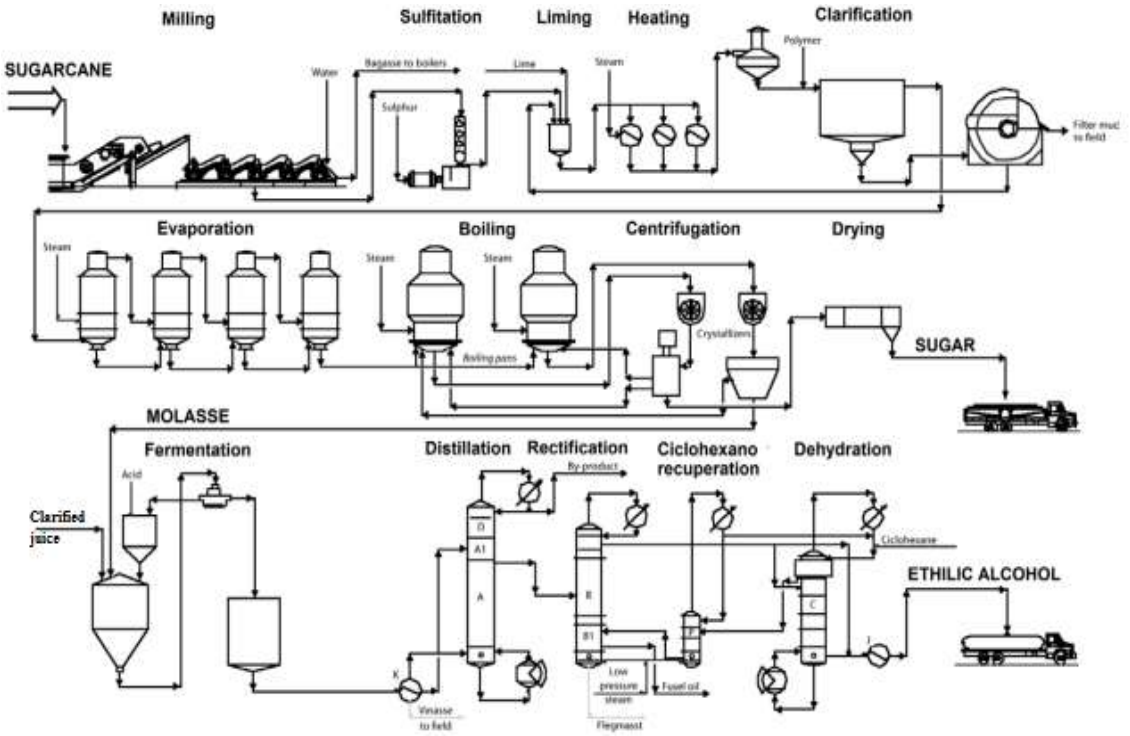

Figure 5 Flow diagram of the sugar-ethanol and electricity industrial production model used in Brazil. Adapted from (Cortez & Baldassin, 2016)

1.4.Stress and adaptation

Stress factors and responses are mechanisms that can reduce or improve the cell viability, respectively. When exposed to stressful conditions microorganisms may produce different reactions. Some are unable to withstand and adapt to these conditions and die; while others are able to survive when less extreme conditions are applied (sub-lethal levels) due to the activation of specific mechanisms of stress response and consequently adaptation to the new conditions (H. Huang, Lung, Yang, & Wang, 2014; Lado & Yousef, 2002).

In this section, some of the most common stress factors that influence cell viability and growth will be discussed, including temperature, pH, water activity, pressure and exposure to toxic substances like antibiotics (Bereksi, Gavini, Bénézech, & Faille, 2002; Imlay & Linn, 1987; Jydegaard-Axelsen, Aaes-Jørgensen, Granly Koch, Stoumann

Jensen, & Knøchel, 2005). In addition, some of the stress responses used by cell to improve their resistance to these stress factors will be also mentioned, including production of heat shock proteins (HSP), modification of fatty acids and modulation of genes.

In general, microorganisms develop different defences to withstand these adverse conditions, increasing their resistance to harsh conditions and sudden environmental changes. Therefore, when bacteria and yeasts are exposed to moderate levels of stress, particular stress responses are triggered, which may involve genetic or physiological changes that allow the increased tolerance when they are subsequently submitted to higher levels of the same stress or even another stresses, like cross-protection and general stress response (GSR). There are many physiological modifications that allow bacteria and yeast to adapt to adverse conditions, such as the modification of membrane fatty acid composition or the production of specific proteins, such as the HSP (Malone, Shellhammer, & Courtney, 2002).

Figure 6 shows some of the most common pathways of stress responses. For each stressful condition, there are activation or deactivation of sets of genes, which makes the yeasts stress response a complex genetic response and not only a physiological response (Rantsiou et al., 2012).

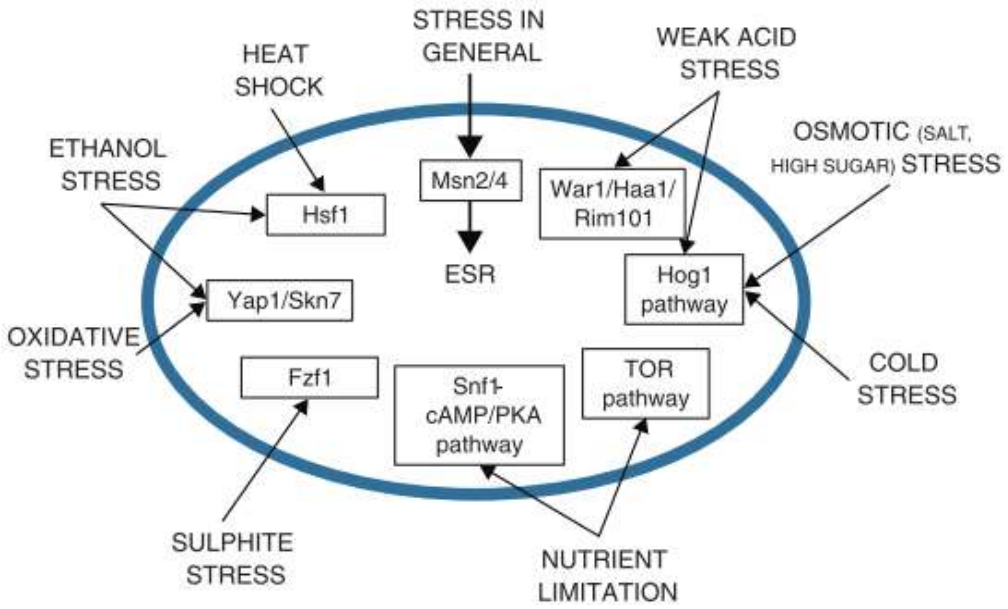

Figure 6 Main signalling pathways controlling the yeasts adaption response to food-relevant stresses. The cross walk between stress responses was not considered (Rantsiou et al., 2012)

1.4.1. Production of heat shock proteins

A wide variety of stresses, such as high temperature, starvation, high pressure, water activity and pH, are conditions that usually repress the synthesis of the most cellular proteins, and in some cases may induce the production of heat shock proteins (HSPs).

Therefore, several of these HSPs play vital roles in cell growth under these conditions as well as in stress tolerance. Despite their diversity in structure, these proteins have a similar functionality: in general, HSPs promote the folding and unfolding of other proteins, the assembly and disassembly of proteins in oligomeric structures, and the degradation of proteins that are improperly assembled or denatured (Aertsen et al., 2004). Regarding HP adaption, many *in vitro* studies with purified proteins and membrane vesicles have indicated protein denaturation may occur due to pressure increase, affecting the membrane fluidity (Balny, Masson, & Heremans, 2002; Heremans & Smeller, 1998).

In a study made by Aertsen and his collaborators (Aertsen et al., 2004), the induction of several heat shock genes after exposure to sub-lethal pressures was demonstrated, which were therefore responsible for the production of HSPs.

1.4.2. Other stresses during fermentation

During fermentation, bacteria and yeasts cells must respond to fluctuations in dissolved oxygen concentration, pH, osmolarity, ethanol concentration, nutrient supply and temperature. Thus the capacity to survive is dependent on their ability to adapt to these changes (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). In Figure 7, some of the stress factors that yeasts cells are subjected during fermentation are summarized.

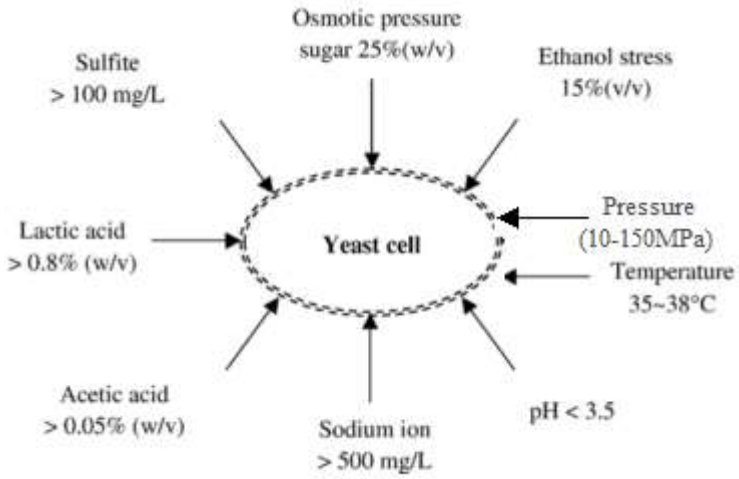

Figure 7 Potential environmental stresses on *S. cerevisiae* during ethanol fermentation. (Adapted from Bai et al., 2008)

Throughout fermentation, *S. cerevisiae* is exposed to stressful conditions and produces a temporary response by reprogramming the cellular activities to ensure its survival in these conditions, protecting the essential cell components and allowing the continuation of the 'normal' cellular activities during its recovery. These responses to environmental stress are complex, involving various aspects: i) cell identification; ii) signal transduction; iii) transcriptional and posttranscriptional control; iv) protein-targeting; v) accumulation of protectants; and vi) increased activity of repair functions (Mager & Ferreira, 1993). These responses are essentially produced by two major stress response pathways. One of those is the production of heat shock response, mediated by the so-called heat shock transcription factor, which is activated essentially by sublethal heat stress but also by different kinds of stresses such as pressure and ethanol stress. The other is the general (or global) stress response which is activated by a number of environmental stresses including oxidative, pH, heat, pressure and osmotic stresses, as well as nitrogen starvation (Chatterjee, Khalawan, & Curran, 2000).

Ethanol is one of the products of the alcoholic fermentation and represents one stress factor for the yeasts cells, affecting the cell viability and growth, cell metabolism and cell structure and membrane function. In Table 1, some of these effects on yeasts physiology are described.

Table 1

Some effects of ethanol on yeasts physiology (Adapeted from Stanley, Bandara, Fraser, Chambers, & Stanley, 2010)

Cell viability and growth

- Inhibition of growth, cell division and cell viability;
- Decrease in cell volume;

Metabolism

- Lowered mRNA and protein levels;
- Protein denaturation and reduced glycolytic enzyme activity;
- Induction of heat shock proteins and other stress response proteins;
- Intracellular trehalose accumulation ;

Cell structure and membrane function

- Altered vacuole morphology;
- Inhibition of endocytosis;
- Increased unsaturated⁄saturated fatty acid ratio in membranes;
- Increase in ergosterol content of membranes
- Loss of electrochemical gradients and proton-motive force
- Inhibition of transport processes
- Inhibition of H^+-ATPase activity
- Increased membrane fluidity

When exposed to ethanol, yeasts cells synthesize a large range of HSPs, which include Hsp104 and Hsp12. These HSPs have been shown to physiologically influence yeasts tolerance to ethanol. Hsp104 acts as a remodelling agent in the disaggregation of denaturated proteins while Hsp12 is a membrane-associated protein that can protect liposomal membrane integrity against desiccation and ethanol. However, there are other studies that report other genes that are highly activated during ethanol stress. (Stanley et al., 2010).

Temperature and pressure are other possible stresses, being some of the effects on cells similar to the ethanol stress ones mentioned above, thus the mechanisms of response are also similar (Gibson et al., 2007).

High osmotic pressure is another stressful condition that results from fermentation causing the decrease of fermentation rate and yield (Casey, Magnus, & Ingledew, 1984).

Osmotic pressure is the force between two solutes of differing concentration separated by a semi-permeable membrane, being higher with the higher difference in the solute concentrations. Yeasts respond to the effects of osmotic pressure in the growth medium in many ways. One of the ways to respond is to alter the cell volume, i.e. decreasing volume in response to hypertonic stress and increasing volume in the presence of hypotonic stresses. In *S. cerevisiae,* the membranes are relatively elastic and weakly shielded against water loss and, therefore, it is expected differences in cytoplasmic volume under external osmotic pressures (Pratt et al., 2003; Rantsiou et al., 2012; Teixeira et al., 2011). When subjected to high osmotic pressure, yeasts cells use the HOG (High Osmolarity Glycerol) pathway in order to preserve its internal volume constant (Teixeira et al., 2011). This pathway mediates the most significant part of the response of yeasts cells to a hyperosmotic shock. For it to work, it is required the stimulated expression of more than 100 genes that include GPD1 and GPP2, which encode enzymes involved in the production of glycerol, the main osmolyte accumulated by yeasts cells (Tamás, Rep, Thevelein, & Hohmann, 2000).

1.4.3. Fermentation using non-conventional conditions

Besides the normal stresses that occur during fermentation some other stresses can be produced depending of the conditions. Those stresses can be produced during fermentation using non-conventional conditions.

Recently, several non-conventional conditions are being tested for the improvement of microbial fermentations and positive results were achieved in the stimulation of microbial growth and fermentation despite the fact that stressful conditions are being used (Chisti, 2003; Mattar et al., 2015; Mota, Lopes, Delgadillo, & Saraiva, 2013; Shikha Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2016). This concept emerged from the need to improve the yield and productivity values of relevant microbial fermentations. Therefore, several emerging technologies are being tested for the improvement of microbial fermentations. The non-conventional conditions already used implicate technologies commonly applied for food pasteurization, including high Pressure (HP), pulsed electric fields (PEF), moderate electric fields (MEF) and ultrasound (US). However, these technologies should be applied at sub-lethal levels, in order to affect the behaviour of microbial strains involved in fermentation, but not causing their destruction (Mattar et al., 2015).

Using these sub-lethal conditions, it is possible the development of specific genetic, physiologic and metabolic stress responses by microbial strains, opening the possibility to obtain fermentation products and processes with different characteristics (H.-W. Huang, Lung, Yang, & Wang, 2014; Mills, Stanton, Fitzgerald, & Ross, 2011). In some cases, these modifications can represent significant improvements, such as increased yields, productivities, and fermentation rates, lower accumulation of by-products and/or production of different compounds (Serrazanetti, Guerzoni, Corsetti, & Vogel, 2009; Shikha Ojha et al., 2016). Therefore, those results are not only relevant for food fermentations (*e.g.* for the production of dairy products, alcoholic beverages, and others), but also may be valuable to industry for production of commodity bio-chemicals (such as acetic acid, citric acid, and ethanol) and high-value bio-products (such as vitamins, antibiotics, and biopolymers (Mattar et al., 2015; Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017; Puértolas, López, Condón, Álvarez, & Raso, 2010; Sinisterra, 1992).

Some studies reported on the literature about this approach are summarized below, including details about the fermentative process, the applied technology and the results obtained in each case. The technology of High Pressure will be discussed in a specific chapter of this work, since is the basis of this thesis.

1.5.High pressure (HP) technology

Pressure, like temperature, is an important thermodynamic parameter that affects molecular systems. According to the Le Chatelier and Braun principle, pressure affects biological and chemical systems towards a volume reduction. Therefore, the reaction equilibriums are shifted towards the most compact state. As a fundamental principle, any change in a biochemical reaction involves a change in free energy. This change in Gibbs energy is a function of pressure and temperature, and is governed by the change in volume and entropy. Since the temperature is assumed to be constant during pressure processing, the pressure dependence of the Gibbs energy is given by the volume change (Bolumar, Georget, & Mathys, 2015; Yaldagard, Mortazavi, & Tabatabaie, 2008).

In this process, all pressure effects arise from a single influence, namely the change in system volume that occur in the environment and that is accompanied by a physiological or biochemical alteration of living cells. High pressure exerts many effects on living organisms, making it difficult to pinpoint the pressure-points in cell growth and

viability. Most *in vivo* observations on living cells exposed to HP can be linked to these two effects: inhibition of key enzymes, and inactivation of cellular structures and processes, including transcription, ribosomes structure, microtubules and membrane proteins, and structural and functional disruption of cell membrane (Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008; Rendueles et al., 2011). Remarkable differences exist in the pressure sensitivity among bacterial species, and several groups have reported the isolation of mutants with acquired HP resistance (Bartlett, 2002a).

1.5.1. Effects of HP on microorganisms

Cell membranes are the primary sites of pressure-induced damage, with consequent alterations of cell permeability, transport systems, loss of osmotic responsiveness, organelle disruption and inability to maintain intracellular pH (Malone et al., 2002; Rendueles et al., 2011).

Together with the alteration of the membrane, protein denaturation and changes in the active centres have also been observed after HP treatments together with changes in enzyme-mediated genetic mechanisms, such as replication and transcription. In Figure 8, some of the effects of HP on cells and cellular components are described.

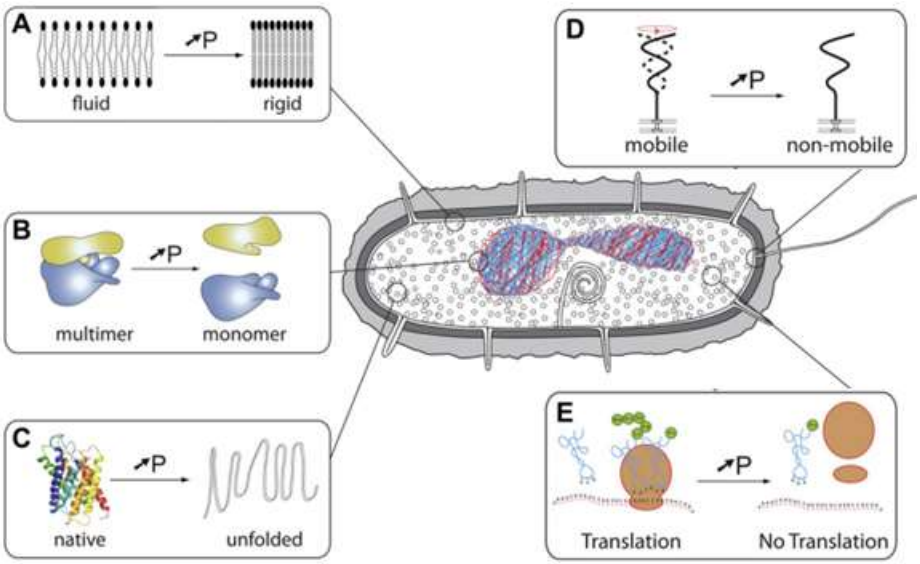

Figure 8 Examples of the effects of high hydrostatic pressure on cells and cellular components. A: lipids in membranes; B: multimeric protein assemblages, C: protein structure; D: cellular motility; E: protein translation by ribosomes (Oger & Jebbar, 2010a)**.**

1.5.1.1. Effects on DNA

Certain levels of HP affect relevant cellular processes, mostly due to changes in DNA structure. For instance, HP stabilizes the DNA hydrogen bonds and assembling interactions. Those "new" and more stable hydrogen bonds increase the duplex to single-strand transition temperature, *e.g.* the melting temperature. As a consequence, DNA is stabilized by increasing the pressure so, the double- to single-strand transition necessary for replication/transcription/translation processes may become more difficult, hindering the development of new molecules and cells (Macgregor, 2002). Moreover, the functionality of genetic materials in microorganisms such as DNA replication and gene transcription is also negatively affected because the activity of the DNA replication and transcription enzymes is reduced. In addition, the chromosomal DNA produced through excision of DNA and nucleic acid enzymes is degraded by pressure as a result of the condensation of the genetic material (H.-W. Huang et al., 2014).

1.5.1.2. Effects on membranes

In membranes, lipids are particularly sensitive to pressure effects because they are compressed more easily than membrane proteins. Therefore, when the pressure increases bacterial membranes compress causing a reduction in the intermolecular distance between acyl chains and membrane lipids. This process causes a leak in the membrane, leading to eventual cell death. Additionally, pressure increases the cell wall hydrolase activity in some microorganisms, leading to an increased permeability that can also lead to cell death (Malone et al., 2002). Besides that, increasing the pressure enhances the order of hydrocarbon chains, raises the temperature of membrane phase transition from the gel state to the liquid crystalline state and increases bilayer toughness by reducing the curvature of acyl chains (Bartlett, 2002a).

Furthermore, several studies have shown that HP changes the membrane fatty acids composition. For example, the increase in pressure in *Photobacterium profundum* strain SS9 increases the proportion of both monounsaturated fatty acid 18:1, *cis*-vaccenic acid, and polyunsaturated fatty acid 20:5, eicosapentaenoic acid (Figure 9).

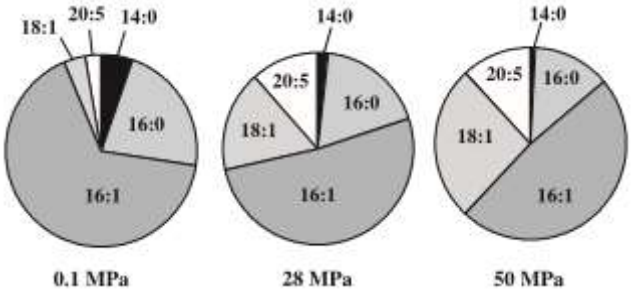

Figure 9 Effect of culture pressure on the proportion of the major fatty acid species in the deep-sea bacterium *Photobacterium profundum* strain SS9 (Bartlett, 2002a)**.**

In addition to membrane lipids, membrane proteins have frequently been implicated as playing an important role in the growth under pressure. ATPase and tryptophan permease were subject to some works that suggested that these membrane protein have a limited the growth in some microorganisms when subject to pressure (Bartlett, 2002b).

At a pressure of 100 MPa, lipid bilayer loses fluidity and occurs a reversible conformational change in transmembrane proteins that leads to functional disorder of membrane bound enzymes. A reversible phase transition in parts of the lipid bilayer (which passed from the liquid crystalline to gel phase) was observed at pressures of 100–220 MPa, as well as dissociation and/or conformational changes in the protein subunits. This could cause the separation of protein subunits and gaps between protein and lipid bilayer, creating transmembrane tunnels. When pressures higher than 200 MPa were applied, an irreversible destruction and fragmentation of the membrane structure occurred due to protein unfolding and interface separation (Campus, 2010).

1.5.1.3. Effects on proteins

HP does not affect the primary structure of proteins (sequence of amino acids) because the ranges of pressure usually applied does not change the energy of covalent bonds. Pressure acts predominantly on the conformation and supramolecular structures of biomolecular systems, and thus, on their functionality in the cells (Oger & Jebbar, 2010b). However, some modifications may occur in the sulphydryl groups and thiol-disulphide interchange reactions (Funtenberger, Dumay, & Cheftel, 1997), and thus, secondary, tertiary and quaternary structures were affected, which have effect on the

protein unfolding and eventually cause denaturation (Campus, 2010; Moreirinha, Almeida, Saraiva, & Delgadillo, 2016).

Studies have shown that HP stabilizes hydrogen bonds and enhances the breaking of salts, which leads to a decrease in volume (Campus, 2010; Norton & Sun, 2008). Also, modifications of the electrostatic and hydrophobic interactions, that are the major forces maintaining the tertiary structure, are accompanied by large hydration changes. This is assumed to be the primary source of the decrease in volume associated with denaturation of proteins (Moreirinha et al., 2016; Norton & Sun, 2008).

There are also some studies that report the possibility of HP activate or inactivate enzymes, depending on the enzyme's inherent ability to withstand pressure stress. The enzymes responsible for the synthesis of ATP, after being deactivated, collapse from the cell membrane, reducing ATP synthesis. In addition, HP can also denature functional proteins and lead to a limited proton flow, reducing the intracellular pH (H.-W. Huang et al., 2014; Tholozan, Ritz, Jugiau, Federighi, & Tissier, 2000).

1.5.2. High pressure adaptation

When the subject is pressure resistance and sensitivity, there are essentially two types of microorganisms. Ones that live in the surface, such as *E. coli* and *S. cerevisiae*, that cannot normally grow at pressures higher than atmospherical pressure, and the others, the piezophiles, which live in HP environments, including the deep ocean, hydrothermal vents, the sub-seafloor and the continental underground, and have optimal growth rates at pressures greater than 0.1 MPa (Oger & Jebbar, 2010b). The ability of piezophiles to grow under these pressure levels (inhibitory to surface organisms), and the inability of obligate piezophiles of growing at atmospheric pressure can be a proof that piezophiles have adapted to HP in the course of their evolution. To explain their ability to grow under HP, three main mechanisms have been proposed: i) fine tuning of overall gene expression to compensate for loss of biological activity; ii) expressing of HP-specific genes; iii) adaptation of the structure of biomolecules to withstand HP (Campanaro et al., 2005; Chilukuri & Bartlett, 1997; Kato & Qureshi, 1999).

Mesophilic microorganisms, such as *S. cerevisiae*, cannot normally grow under pressure due to its sensitivity. However, in some cases these microorganisms are able to grow under these stress conditions due to the development of mechanisms to improve

pressure resistance. Some of these mechanisms are very similar to those used by piezophiles, however without having the same efficiency (Oger & Jebbar, 2010a).

1.5.3. *Saccharomyces cerevisiae* under HP

HP can exert a broad range of effects on microorganisms with similar characteristics to those of other environmental stresses, such as high temperature, ethanol and oxidative stresses. The HP response of wild *S. cerevisiae* shows high correlation with that resulting from increased ethanol concentration or high-temperature stresses (Bravim et al., 2013). For instance, Iwahashi et al (1991) demonstrated that a mild heat shock pre-treatment (43 °C for 30 min) increased the resistance to HP, leading to an increase in cell viability of *S. cerevisiae* at 150 MPa. In addition to prior heat shock treatment, the addition of cryoprotectants (dimethylsulfoxide, Me_2SO) and deuterium oxide may also provide protection for pressure damage. These findings imply that the damage by HP may be similar to that of high temperature (Iwahashi & Kaul, 1991).

Additionally, *S. cerevisiae* cells were submitted to a mild sub-lethal pressure treatment (50 MPa for 30 min) followed by a short recovery at atmospheric pressure (0.1 MPa) and an increase in the tolerance to heat, ultra-cold shock and high-pressure treatments was observed (Palhano, Gomes, Orlando, Kurtenbach, & Fernandes, 2004). After HP treatment (200 MPa for 30 min) *S. cerevisiae* gene expression was profiled, showing that most of the upregulated genes were involved in stress defence and carbohydrate metabolism, while most of the repressed genes were involved in cell cycle progression and protein synthesis. This indicates that pressure application causes a reduction in yeasts cell cycle progression and protein synthesis, causing a loss of cell viability. Still, Miura et al. (2006) demonstrated that upregulated genes are not always responsible for the piezotolerance, which shows the great complexity of this subject (Miura, Minegishi, Usami, & Abe, 2006). One mechanism developed by *S. cerevisiae* is the adjustment of its genomic expression pattern under HP. This effect was studied by Fernandes *et al.,* 2004 using whole genome microarray hybridization and some of the results can be observed in Figure 10. As it can be seen, most of the genes that are overexpressed under pressure are still unknown, however the genes that are repressed are, for example, related to protein synthesis and cell cycle.

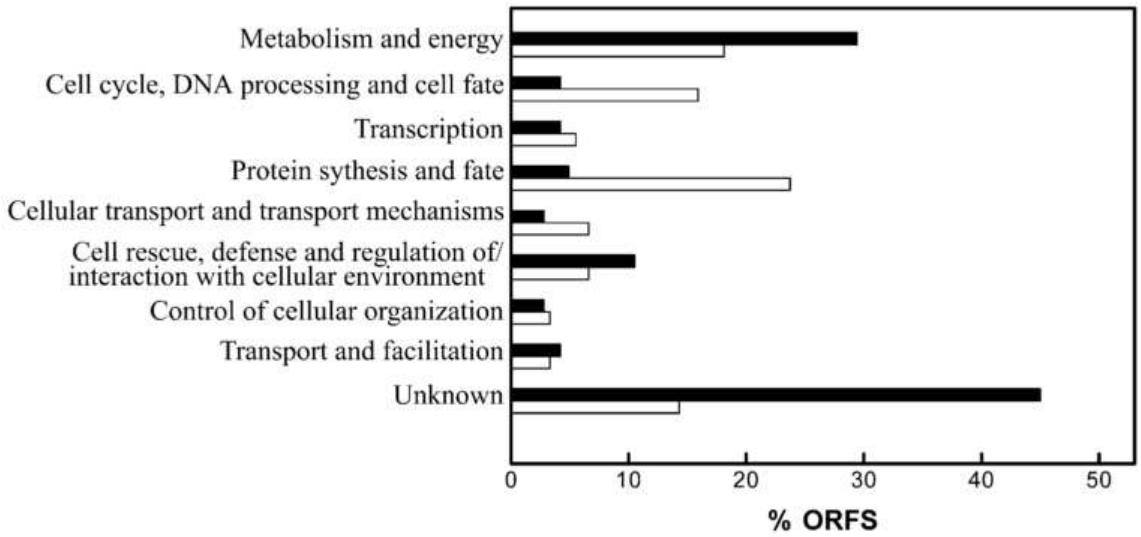

Figure 10 Global gene expression profile in functional categories. Black bars and white bars represent the percentage of induced and repressed genes, respectively (Fernandes et al., 2004)**.**

In another work, fermentation was carried out using a wild-type yeast isolate and the measurement of ethanol production was performed before and after the pressure treatment (50 MPa for 30 min at room temperature), followed by incubation for 5, 10 and 15 min at atmospheric pressure. The HP treatment led to an increase in ethanol content upon fermentation. In parallel, a global transcriptional analysis was conducted to identify genes induced by HP. In this study it was demonstrated that the production of ethanol was enhanced for the wild-type yeasts strain, most likely due to the over-expression of some genes that are responsible for tolerance to stress (Bravim et al., 2013).

In other work, Picard et al. (2007) monitored alcoholic fermentation performed by *S. cerevisiae* under HP in the range of 0.1 and 100 MPa. In all experiments, the stationary phase was reached (Figure 11) and apparently an increasing amount of glucose was being used for cell maintenance and thus less glucose was available for fermentation. Regarding ethanol production over fermentation time, the authors observed that the reaction rate increased significantly as pressure increased up to 10 MPa, with fermentation occurring faster at 5 and 10 MPa than at atmospheric pressure and the ethanol production being slightly enhanced (3–4%). While the estimated final concentration of ethanol produced at atmospheric pressure was 90% of the theoretical maximum, at 5 MPa the fermentation yield was enhanced by 6%, corresponding to the maximal ethanol production. On the other hand, above 20 MPa the process was slowed down with the increasing pressure, and it was estimated that at 87 ± 7 MPa the alcoholic fermentation was interrupted. The

authors suggested that the activity of one or more enzymes involved in the glycolytic pathway may be enhanced at HP up to 10 MPa, becoming progressively repressed with the increasing pressure, until its complete loss of activity. Since phosphofructokinase (a key enzyme in glycolytic pathway) is inhibited due to the pressure induced acidification at ≈50 MPa, some authors predicted that the fermentation would stop at this range of pressures (Abe & Horikoshi, 1995). However, the above discussed study of Picard and collaborators revealed that the alcoholic fermentation is only interrupted at pressures as high as 87 MPa (Picard, Daniel, Montagnac, & Oger, 2007).

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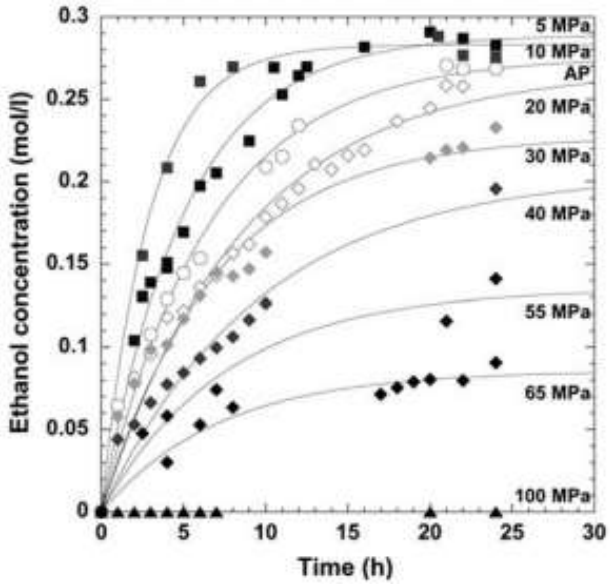

Figure 11 Kinetics of ethanol production as a function of pressure to 100 MPa (Picard et al., 2007)

Another study used a pressure of 50 MPa on alcoholical fermentation to evaluate the prodution of ethanol in these conditions (Bravim et al., 2013). Two different treatments were tested: one of them 50 MPa for 30 min and the other was subjected to the same treatment but then incubated at atmospheric pressure for 15 min (50+0.1 MPa). As a control sample, a non-pressurised sample (0.1 MPa) was used (Bravim et al., 2013). In this work, it was demonstrated that putting the culture medium over 0.1 MPa, after the fermentation and after initial treatment at 50 MPa, enhanced the quantity of ethanol produced. For the short fermentation times (4 and 8 h) that was not verified, obtaining a higher concentration of ethanol in the treatment using only the HP treatment, as shown in Figure 12.

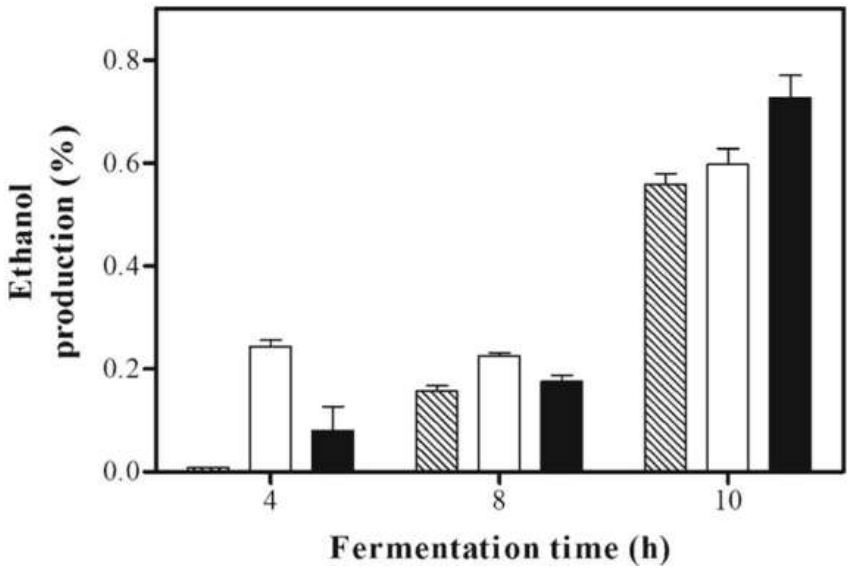

Figure 12 Ethanol production (in percent) after pressure treatment. *S. cerevisiae* cells submitted to a hydrostatic pressure of 50 MPa for 30 min (empty bars) and 50 MPa for 30 min and then incubated at room pressure (0.1 MPa) for 15 min (filled bars), and after that, the fermentative efficiency of this strain was evaluated. A non-pressurised sample was used as a control (striped bars) (Error bars represent the SD of three measurements) (Bravim et al., 2013)

Another study on this subject tested the adaptation of cells to pressures of 150 MPa for 60 min, after a temperature shock using temperatures from 30ºC to 51ºC. Figure 13 shows the effect of the heat shock temperature on the induction of thermotolerance and piezotolerance. Tolerance was greatly increased when the treatment at 40-43°C was applied, with the most effective temperature being 43 °C, in both cases.

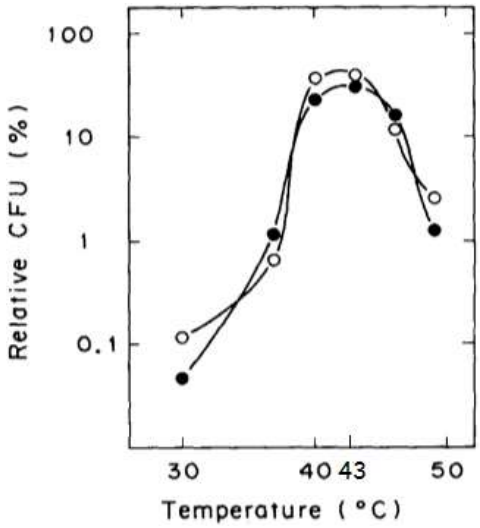

Figure 13 Effects of temperature on the induction of thermotolerance and piezotolerance. Logarithmic phase cells were suspended in fresh YM medium and incubated for 30 rain at various temperatures. Thermotolerance and piezotolerance are shown as % CFU of the untreated control. Symbols: o, incubated for 10 min at 51°C (thermotolerance); ●, incubated for 60 min at 150 MPa (piezotolerance) (Iwahashi and Kaul, 1991)**.**

The results of this study suggest that, when subjected to temperature shocks of 40-43ºC prior to the pressure test, cells exhibit an enhanced piezotolerance, with a more considerable effect at 43ºC (Iwahashi & Kaul, 1991). Those results are confirmed in Figure 14, where a significant increase in the survival of cells was observed when the cells are incubated under HP (100-200 MPa) after the pre-incubation at 43°C for 60 min. The results show that the prior heat shock treatment induces the piezotolerance of the thermally-treated strains, compared to the control ones. This suggests that high pressure and high temperature have similar physiological effects on yeasts and share some of the stress response mechanisms.

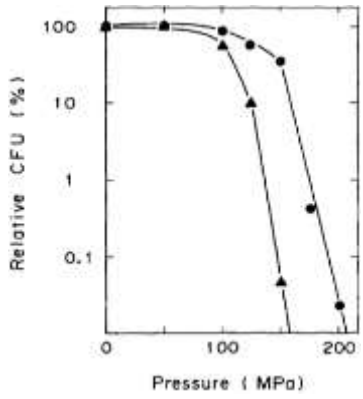

Figure 14 Effects of hydrostatic pressure on cells. Heat-shocked (43 ºC, 30 min) and control (30 ºC, 30 min) cell suspensions in distilled water were subjected to increased pressure for 60 min. Piezotolerance is expressed as % CFU. Symbols: ●, heat-shocked; ▲, control (Iwahashi and Kaul, 1991)

2. Objectives of the work

Considering the potential of HP to improve alcoholic fermentation, the main goal of the present work was to study the adaptation of *S. cerevisiae* to HP and to understand how pressure will affect both cell viability and fermentation. We intended to acquire pressure adaptation using consecutive cycles of fermentation under sub-lethal pressure. In this work, *S. cerevisiae* cultures performed fermentation under pressure and after that first cycle, viable cells were isolated and used as inoculum to carry out other fermentation cycle at the same conditions. As reported in literature, these consecutive cycles of fermentation under HP will be responsible for the development of adaptation mechanisms. For now, the information available in literature about this topic is still very limited and unspecific about the mechanisms of adaptation and the enhanced productivity of bioethanol during the fermentation. Although some studies have already been performed, the objective of this work is not exactly the same, making this a novel topic of study because its objective is not to enhance the fermentation yield in one pressure cycle the others previous projects but is to promote the adaptation of the *S. cerevisiae* to those conditions.

S. cerevisiae was chosen for this study due to its great importance in many industries, including food and fuels industry. In addition, this yeast is one of the most studied microorganism and because of that it can be used as a model of the influence of HP on biotechnological processes relevant to industry.

During this work, only sub-lethal pressures (between 15-50 MPa) were applied to study the effects on growth, adaptation and production of bioethanol without compromising the cell viability. For that, several physical-chemical and microbiological analyses were performed to determine the concentration of cells and monitoring the fermentation, including the measurement of optical density, cell dry weight, and concentrations of sugars (glucose, fructose and maltose), ethanol, acetic acid and formic acid. With the obtained results, different kinetic parameters were determined, including glucose consumption (%), Yield of bioethanol, formic acid, acetic acid and biomass production, in order to better understand and quantify the effects of HP on this fermentation process.

3. Materials and Methods

3.1.Microorganism

S. cerevisiae DSMZ 70468 was chosen due to its highly efficient ethanol production capabilities. A lyophilized culture was bought from DSMZ – a German collection of microorganisms and cell cultures. This strain was cultured according to the manufacturer's instructions, and sub-cultured on Yeast Malt agar plates, subsequently incubated at 30 ºC for 48 h.

3.2.Inoculum preparation

A seed culture was prepared by inoculating a single colony in 100 mL of sterile culture medium (Yeast malt broth) containing 5.00 g/L of peptic digest of animal tissue (peptone), 3.00 g/L of yeast extract, 3.00 g/L of malt extract and 10.00 g/L of dextrose. The culture was incubated at 30 ºC and 150 rpm for 18 h. The inoculum was ready to use when the optical density of the solution was 0.8 at 600nm.

3.3.Fermentation under high pressure

The inoculated medium was homogenized and then transferred to a heat sealed plastic bag (11 cm x 3.5 cm), designed to withstand HP conditions. All these steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination. Fermentation only occurs under oxygen limiting conditions, since *S. cerevisiae* is a facultative anaerobe, which can produce energy in the presence of oxygen, being this the preferable pathway over the conventional respiration. Because of this the samples to be used will be sealed with the minimum level of oxygen possible promoting fermentation over aerobic respiration.

Fermentations were then performed at 15, 35 and 50 MPa, at 30 ºC, for 24h or 48h. These experiments were conducted using the HP equipment with a capacity of 2 L and the other in High Pressure System U33, Unipress Equipment, Poland, own by the Chemistry Department of University of Aveiro. This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature, using a mixture of propylene glycol and

water as pressurizing fluid and to control the temperature in the external jacket. As a control, fermentation was also performed at 0.1 MPa (atmospheric pressure), keeping all conditions equal to the conditions for fermentation under HP. Samples were collected throughout the fermentation time, and each experiment was run in duplicate.

3.4.Consecutive cycles of fermentation under high pressure

Fermentations were performed during four consecutive high pressure cycles (figure 15). The first three pressure cycles had a fermentation time of 72 h and the last one was 24 h longer than the rest, corresponding to a fermentation time of 96 h. These experiments were conducted using 3 different HP equipment's. One with the capacity of 100 mL (System U33, Unipress Equipment, Warsaw, Poland), the other with a capacity of 2 L and the last one with no temperature control for the second experiments SFP FPG13900 (Stansted Fluid Power Ltd, Essex, UK). This equipment consists of 3 vessels, each pressure vessel 37 mm in diameter and 52 cm in height, using as a pressurizing fluid a mixture of propylene glycol and water (40:60). Furthermore, the experiments were performed at different pressure conditions (15, 35 and 50 MPa) and different temperature conditions (at controlled temperature of 30 ºC, and at naturally variable room temperature). As a control, fermentation was also performed at 0.1 MPa (atmospheric pressure), keeping all conditions equal to the conditions for fermentation under HP.

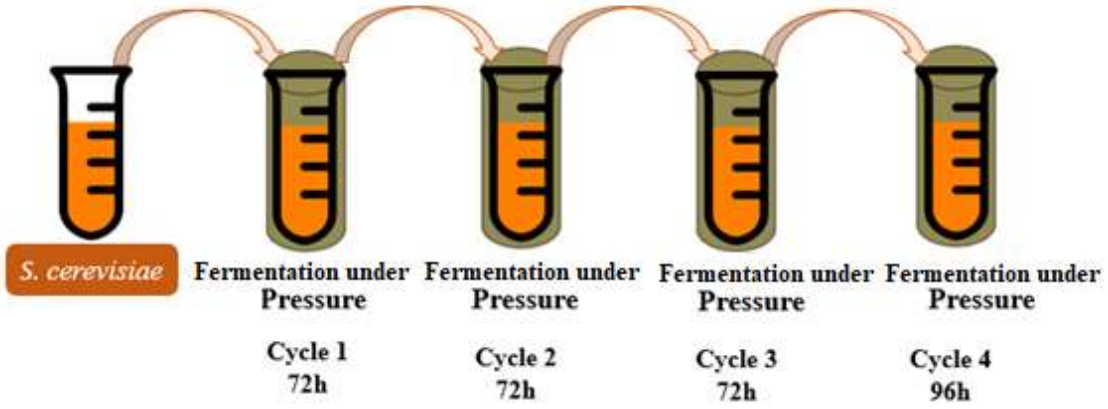

Figure 15 Representation of the four consequitive cycles of fermentaion under pressure

3.5.Biomass concentration

For the determination of biomass concentration, the optical density (OD) of the samples was measured at 600 nm and the cell dry weight was determined after 24—48 h in a freeze dryer. With these results, a calibration curve between those two parameters was determined. In further studies, OD at 600 nm was measured for each sample, and the biomass concentration was estimated using the calibration curve above mentioned.

3.6. Viable cell enumeration

Viable cell enumerations were performed using the pour plate technique. *S. cerevisiae* counts were determined on agar plates of Yeast Malt Agar that were previously sterilized according to the manufacturer's instruction. The cultures were enumerated after incubation at 30 ºC (Dong, Yi, & Li, 2015; Mishra et al., 2015) for 48 h, such as indicated by the manufacture. Plates containing 15–300 colonies were enumerated, and the counts expressed as the $\log_{10}$ CFU mL^{-1} of *S. cerevisiae*.

3.7.Physicochemical characterization

Fermented samples were centrifuged at 10000 rpm for 10 min and the supernatants were collected and filtered through a 0.22 µm filter membrane. The samples were then analysed by high performance liquid chromatography (HPLC) to determine the concentrations of sugars (glucose, fructose), ethanol, glycerol and organic acids (citric, tartaric, malic, succinic and acetic acids). This study was performed using an 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 and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards.

3.8.Kinetic calculations

Taking into account that the results obtained for both conditions (30 ºC and room temperature), different kinetic parameters were determined allow a better comparison between the results. The parameters were glucose consumption (%) (Equation 1) and

yields of bioethanol (Equation 2), formic acid (Equation 3), acetic acid (Equation 4) and biomass (Equation 5) using the formulas presented below. Furthermore the productivity and specific productivity of bioethanol was calculated using the equations 6 and 7.

$$Glucose\ consumption\ (\%) = \left|\frac{[Glucose]f - [Glucose]i}{[Glucose]f}\right|$$

Equation 1. Glucose consumption in percentage

$$Yield\ of\ bioethanol\ production = \frac{[Bioethanol]_{final} - [Bioethanol]_{initial}}{[Glucose]_{final} - [Glucose]_{initial}}$$

Equation 2. Yield of bioethanol production on glucose

$$Yield\ of\ formic\ acid\ production = \frac{[Formic\ acid]_{final} - [Formic\ acid]_{initial}}{[Glucose]_{final} - [Glucose]_{initial}}$$

Equation 3. Yield of formic acid production on glucose

$$Yield\ of\ acetic\ acid\ production = \frac{[Acetic\ acid]_{final} - [Acetic\ acid]_{initial}}{[Glucose]_{final} - [Glucose]_{initial}}$$

Equation 4. Yield of acetic acid production on glucose

$$Yield\ of\ biomass\ production = \frac{[Biomass]_{final} - [Biomass]_{initial}}{[Glucose]_{final} - [Glucose]_{initial}}$$

Equation 5. Yield of biomass production on glucose

$$Q = \frac{[Bioethanol]_f - [Bioethanol]_i}{t_f - t_i}$$

Equation 6. Productivity of bioethanol, Q ($g.L^{-1}.h^{-1}$)

$$q = \frac{Q}{[Biomass]_f - [Biomass]_i}$$

Equation 7. Specific productivity of bioethanol, q ($g.g^{-1}.h^{-1}$)

4. Results and discussion

4.1. Determination of the growth calibration curve

An early stage of the work consisted in the determination of a cell growth calibration curve, which relates optical density (OD) at 600 nm with biomass concentration (estimated by the measurement of the cell dry weight - CDW). The biomass concentration results were helpful to determine the kinetic parameters and, particularly, the yield of production of biomass. **Figure 16** shows the results of OD, and biomass concentration for eight different serial diluted samples, used for determination of the cell growth calibration curve.

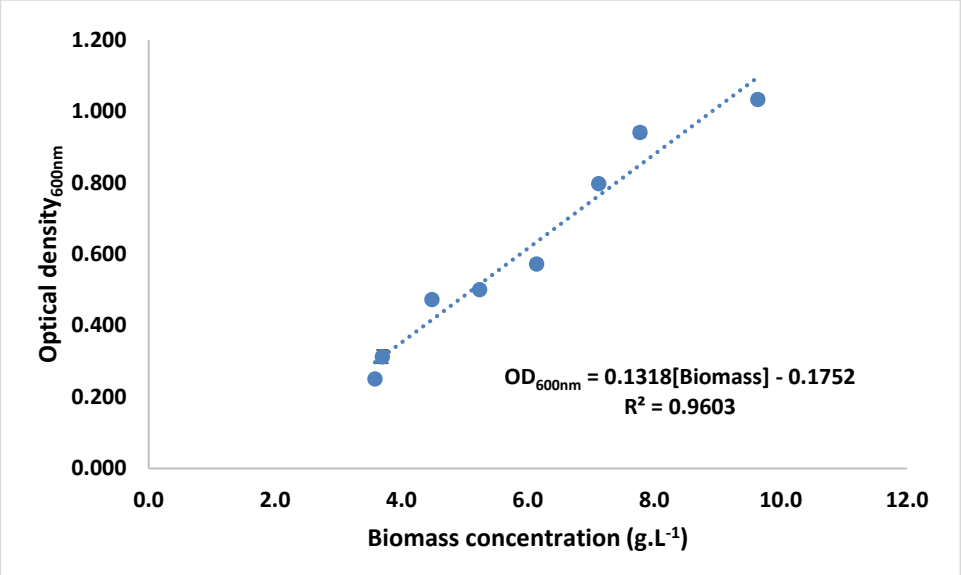

Figure 16 - Calibration curve between $OD_{600\ nm}$ and biomass concentration of *S. cerevisiae*

4.2.Fermentation under HP and selection of the most suitable conditions

As mentioned above, the main goal of the present work is to study the adaptation of *S. cerevisiae* cultures to HP, by performing consecutive cycles of alcoholic fermentation under pressure conditions. This approach may promote the enhancement of ethanol production, due to the improvement of the rates and/or yields of alcoholic fermentation.

In order to determine the most suitable HP conditions to use on these experiments, a first study of *S. cerevisiae* fermentation was tested under different pressure levels (0.1, 15, 25, 35 and 50 MPa). Lower pressures, for example, 5 and 10 MPa had already been

tested and proved to have an important role in the fermentation process (Picard et al., 2007). However, higher pressures were chosen because the biological effects of higher pressures may result in significantly different effects for bioethanol production, and for the adaptation of *S. cerevisiae* (seen in the mRNA expression) (Iwahashi, Odani, Ishidou, & Kitagawa, 2005).

The results of this preliminary study are shown in Figures 17-20, which correspond to the variation of the concentrations of glucose, ethanol, formic acid, and acetic acid, respectively. Furthermore, the cell growth was also analysed by the biomass concentration during the fermentation process (Figure 21).

4.2.1. Glucose consumption

In this part, the consumption of sugars by *S. cerevisiae* during the fermentation is discussed.

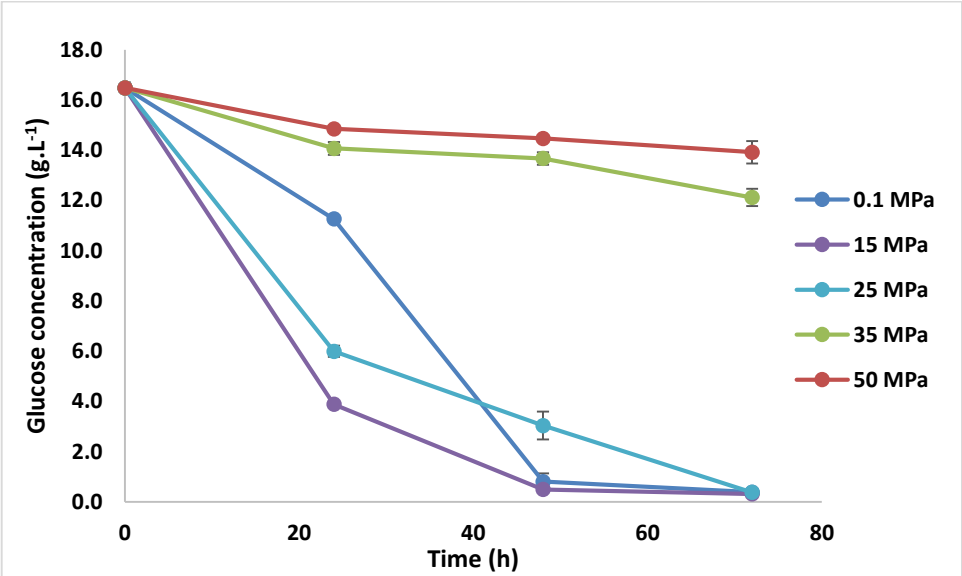

Figure 17. Glucose consumption over time, for fermentation by *S. cerevisiae* under different pressure conditions and at 30 ºC

Figure 17 shows the glucose consumption over the fermentation time under different pressure conditions. A decrease in glucose concentration over time was observed in all cases, even at the highest pressures (35 and 50 MPa), but with lower consumption.

At atmospheric pressure (0.1 MPa), a more accentuated consumption in glucose was noticed during the first 48 h of fermentation, when the concentration of glucose

reached 0.813 $g.L^{-1}$. After that time, the glucose consumption has gradually begun to stabilize, reaching the lowest concentration (0.390 $g.L^{-1}$) at 72 h. It is important to note that the typical fermentation time applied to *S. cerevisiae* at atmospheric pressure is 48 h (Picard et al., 2007), which is consistent with the time period with higher fermentation rate observed in the present work.

Through the analysis of Figure 17 it is also possible to conclude that HP positively affects the glucose consumption over the fermentation time. With the increasing pressure, it was observed a higher glucose consumption variation during the first 24 h for both 15 and 25 MPa. After that time, the glucose concentration was 3.89 $g.L^{-1}$ and 6.00 $g.L^{-1}$ at 15 and 25 MPa, respectively, and only 11.26 $g.L^{-1}$ at 0.1 MPa, indicating a higher and quicker glucose consumption under pressure. However, after 48 h of fermentation, the glucose concentration was similar at 0.1 and 15 MPa, while slightly higher at 25 MPa. Regarding the end of the fermentation time, it was possible to observe that for 0.1, 15 and 25 MPa the glucose consumption was similar (approximately 0.350 $g.L^{-1}$ for every test).

At 35 and 50 MPa, glucose concentration (and possibly fermentation) was inhibited, since almost no variation was observed in glucose consumption over fermentation time. Furthermore, it may be concluded that in these conditions the fermentation process ceases, which probably indicates that *S. cerevisiae* was inhibited or destroyed by HP. For instance, some bacterial strains (e.g. *Escherichia coli*) and *S. cerevisiae* suffer inhibition of several important metabolic and physiological processes in the range of pressures evaluated in this work and may even lose its viability at 100 MPa (Bartlett, 2002a and Picard et al., 2007).

4.2.2. Production of bioethanol

As above mentioned, bioethanol is the main product of alcoholic fermentation by *S. cerevisiae* and its production can be enhanced by the need to produce energy to survive. Figure 18 shows the production of bioethanol under different pressure conditions (0.1-50 MPa). The production of bioethanol during fermentation under pressure is represented by an increase of the rate of bioethanol production at lower pressures (15 and 25 MPa), and no production at higher pressures.

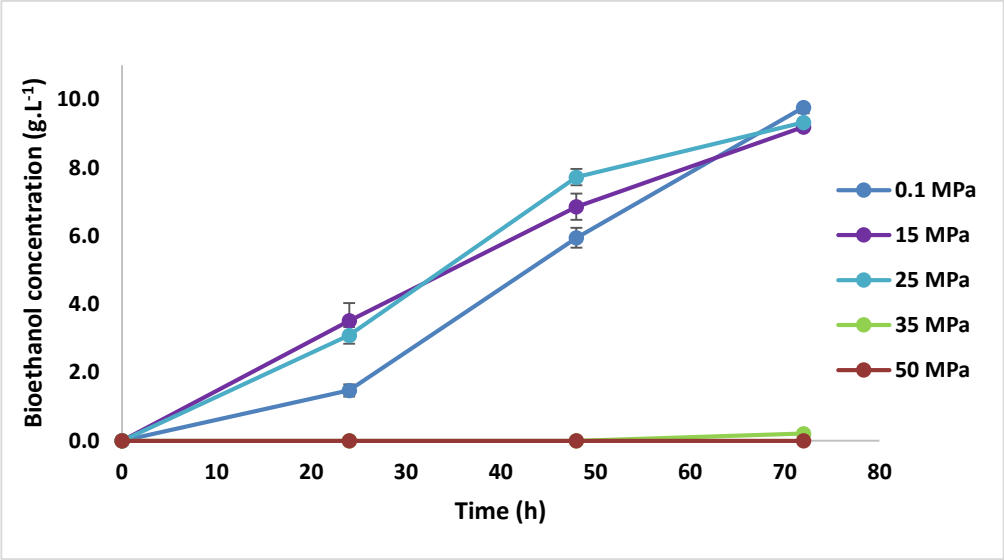

Figure 18 Production of bioethanol over the fermentation time by *S. cerevisiae* under different pressure conditions and at 30 ºC

At 0.1 MPa, 15 MPa and 25 MPa, the concentration of bioethanol reached values close to 10 $g.L^{-1}$ which means that the fermentation was successfully performed. Furthermore, for 15 and 25 MPa, the production of bioethanol was quicker, showing higher concentrations than at 0.1 MPa after 24 and 48 h. However, fermentation at 35 and 50 MPa, revealed almost no production of bioethanol during the fermentation time, which possibly means that the fermentation did not occur. Nevertheless, slight variation of glucose concentration was observed in these conditions (Figure 19), which may indicate that sugars could be used in other metabolic pathways, such as those involved in the maintenance of the cellular viability – a parameter described in the literature as the maintenance parameter.

4.2.3. Consumption different sugars (fructose and maltose)

Although glucose is the main energy source used by *S. cerevisiae*, some other sugars, fructose and maltose were identified in the samples tested. However, since the concentration of these substrates is much lower than glucose results are presented in appendix X (figure X1 and X2).

4.2.4. Production of organic acids

In this section, the production of organic acids of interest in fermentation will be approached. There are numerous organic acids (lactic, succinic, formic, acetic and propionic acids, among many others) present in the fermentation samples but, due to their relevance, only two of them will be discussed (acetic acid and formic acid). Acetic acid acts as a fermentation inhibitor and formic acid is an intermediate in alcoholic fermentation being produced from pyruvate using the pyruvate format liase (Kyong et al., 2016; Keseler et al., 2011 & Olsson & Hahn-Hägerdal, 1996).

4.2.4.1. Production of formic acid

Yeasts may produce formic acid by different ways: i) when subjected to unfavourable environmental conditions; ii) as an intermediate in alcoholic fermentation, being produced from the pyruvic acid, and iii) be obtained from amino acids, ammonia, and also aldehydes (Hohl & Joslyn, 1941).

Figure 19 shows the variation of formic acid concentration over the fermentation time at different pressure conditions. In this case, it is possible to observe that the general tendency corresponds to an increase in formic acid concentration over time, with exception of samples subjected to 50 MPa.

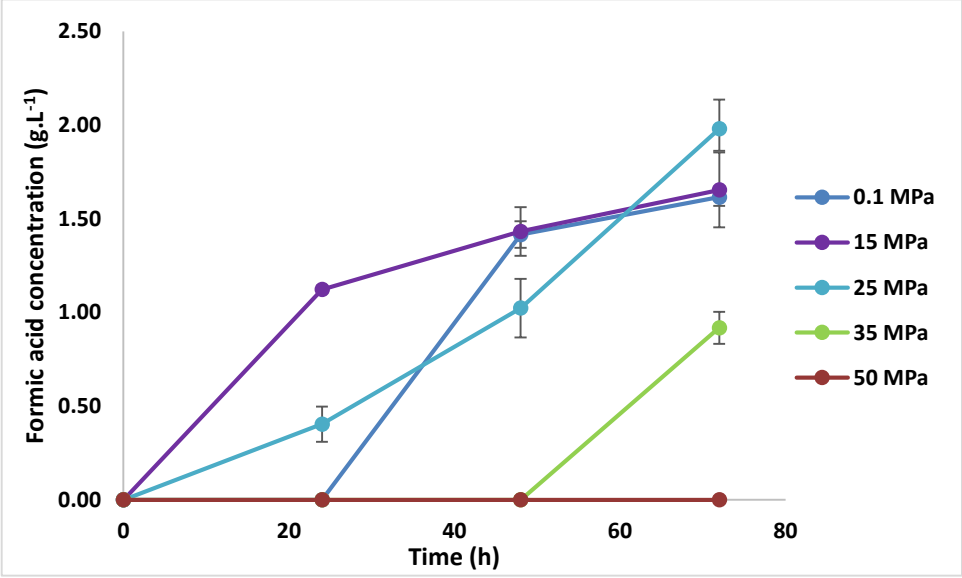

Figure 19 Production of formic acid through the fermentation time by *S. cerevisiae* under different pressure conditions and at 30 ºC

At atmospheric pressure, the production of formic acid only occurs after the first 24 h of fermentation, reaching a maximum at 1.61 $g.L^{-1}$ in the end of the fermentation time. In contrast, the fermentations at 15 and 25 MPa showed a different production profile from the ones verified for atmospheric pressure, since the formic acid production occurred during the first 24 h of fermentation. In fact, at 15 MPa, the highest production rate was observed during these 24 h, reaching a concentration of 1.12 $g.L^{-1}$. After this, the production of this organic acid further increase at a lower rate and in the end of fermentation, a final concentration of 1.65$g.L^{-1}$ was obtained. On the other hand, formic acid concentration increased during all the fermentation time at 25 MPa, reaching the highest final concentration of the conditions tested (1.98 $g.L^{-1}$).

During fermentation at 35 MPa, the production of formic acid started only after the first 48 h, and at 50 MPa, no formic acid was detected during the entire process, which may corroborate with the previous conclusion that fermentation was inhibited, since cells from *S. cerevisiae* were not able to withstand this pressure levels and ferment.

4.2.4.2. Production of acetic acid

Acetic acid is also an important organic acid in the fermentation process working as an inhibitor of this process. It can be produced from acetyl coenzyme A (acetyl-CoA) or be directly produced from bioethanol using the enzyme alcohol-dehydrogenase. Therefore, an increase in the production of this organic acid may be related to a lower production of bioethanol, which is the desired product of the present work (Hopewell, 2014; Müller et al., 2012).

Figure 20 shows the variation of acetic acid concentration over the fermentation time at different pressure conditions. In this case, it is possible to observe that the general tendency corresponds to an increase in acetic acid concentration over time, with exception of samples subjected to 35 and 50 MPa.

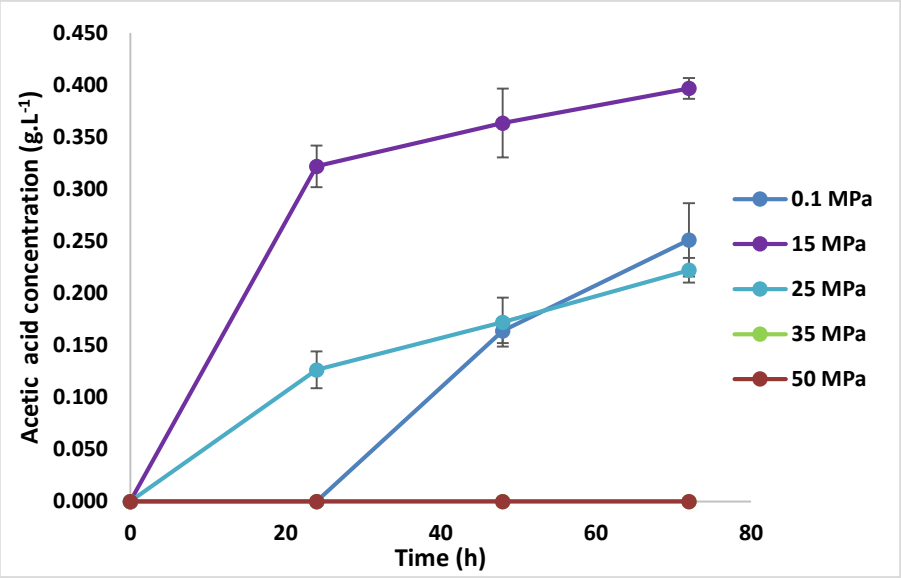

Figure 20 Production of acetic acid through the fermentation time by *S. cerevisiae* under different pressure conditions and at 30 ºC

In general, the production of acetic acid followed a profile similar to the production of formic acid. For instance, at 0.1 MPa, the acetic acid production only occurred after the first 24 h of fermentation, reaching a final concentration of 0.251 $g.L^{-1}$ in the end of the fermentation time. For fermentation at 15 MPa, the highest production rate was observed during the first 24 h of fermentation followed by a lower rate until the end of fermentation with a final concentration of 0.396 $g.L^{-1}$. The final concentration of acetic acid observed at 15 MPa was higher compared to the other conditions. This can represent a disadvantage for the fermentation process, due to the high accumulation of high concentration of an undesirable by-product. At 25 MPa, the acetic acid concentration also increased during the 72 h of fermentation time and a final concentration of 0.222 $g.L^{-1}$ was obtained. For 35 and 50 MPa, no acetic acid was detected, which once more indicate that fermentation was inhibited at these pressure levels.

4.2.5. Biomass concentration

Figure 21 shows the variation of biomass concentration over the fermentation time at different pressure conditions. In this case, it is possible to observe that the general tendency corresponds to an increase in biomass concentration over time, with exception of samples subjected to 50 MPa.

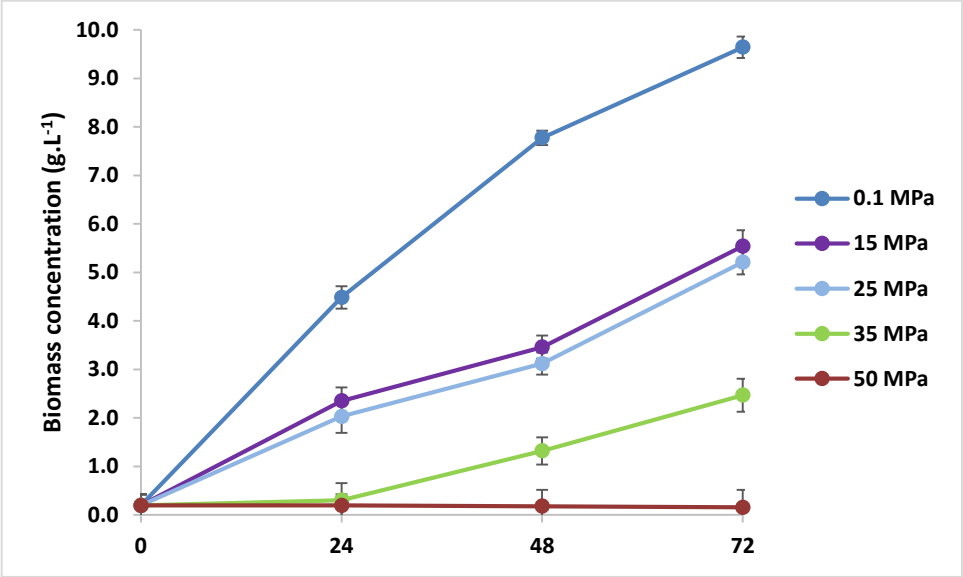

Figure 21 Biomass concentration through the fermentation time by *S. cerevisiae* under different pressure conditions and at 30 ºC

Regarding the control samples (0.1 MPa), a more accentuated increase in biomass concentration during the fermentation time was observed. In consequence, a higher biomass concentration was obtained at the end of fermentation (9.62 $g.L^{-1}$), when compared to the others conditions tested. Similarly, Picard et al., 2007, observed that these results can be explain with the fact that at atmospheric pressure cells grow more easily than exposed to pressures above 10 MPa (Picard et al., 2007). For both 15 MPa and 25 MPa, it can be observed that the biomass concentration was lower than samples at 0.1 MPa, during the complete fermentation time. Furthermore, at these pressures, the biomass concentration was similar in the end of the fermentation time (5.54 $g.L^{-1}$ for 15 MPa and 5.21 $g.L^{-1}$ for 25 MPa), which mean that for those pressures the effect on the growth of *S. cerevisiae* was similar. It is also interesting to note that this considerable decrease in biomass concentration of 15 and 25 MPa, relatively to 0.1 MPa, does not translate into such a marked decrease in ethanol production. This difference may indicate that the medium had fewer cells, but capable of producing the same amount of ethanol.

Analysing the results at 35 MPa, a minor increase in the biomass concentration was observed, having a final concentration of 2.47 $g.L^{-1}$ in the end of the fermentation time. However, this increase in biomass concentration cannot be related to the results obtained for the consumption of substrates and production of the different products of the

fermentation because at 35 MPa the consumption of glucose was low, as well as the production of the metabolism products (ethanol, acetic acid and formic acid), meaning that at this pressure, cells were growing without viability. At 50 MPa, the final concentration in biomass was the lowest (0.184 $g.L^{-1}$), which was the expected considering the results obtained for the others parameters analysed.

After analysing the results of this preliminary test, it was possible to conclude that pressure affects the alcoholic fermentation by *S. cerevisiae* cultures. At 15 and 25 MPa, the production of bioethanol was accelerated compared to atmospheric pressure, but the same did not occur with biomass concentration where it can be observed a lower biomass concentration throughout the entire fermentation time for those pressures when compared to 0.1 MPa. Therefore, it is possible to affirm that 15 and 25 MPa have positive affect on the fermentative process of *S. cerevisiae* enhancing the production of bioethanol without enhancing the biomass concentration. The same results do not occur for 35 and 50 MPa that have a lower production of bioethanol and a lower consumption of glucose. In fact, at 50 MPa there is almost no growth and fermentation, and therefore it is a case with little interest for the study.

4.3.Consecutive cycles of fermentation under high pressure at 30 ºC

Taking into account the results showed in the previous section, the most suitable pressure conditions to attempt the adaptation of *S. cerevisiae* to pressure were selected. The fermentation at 50 MPa was excluded from the study, since almost no growth and fermentation was observed over time. Therefore, 15, 25 and 35 MPa were the pressures selected, and four fermentation consecutive cycles under pressure were performed: the first three pressure cycles with a fermentation time of 72 h and the fourth was 24 h longer, corresponding, therefore, to a fermentation time of 96 h.

4.3.1. Substrate consumption

In this part, fructose and maltose contribute to the fermentation, even if at less extent than glucose, as substrates in the fermentation process, being both consumed during the fermentation cycles as shown in figures A1 and A2 (appendix A).

The values obtained for the glucose consumption during the four fermentation cycles are presented in Table 2.

Table 2 Percentage of glucose consumed by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Glucose consumed (%)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	98.7	85.0	97.6	6.0
Cycle 2	98.6	98.4	98.3	7.9
Cycle 3	98.5	98.5	83.4	15.2
Cycle 4	98.7	98.7	98.4	26.3

As it can be observed in Table 2, the consumption of glucose was higher for the control group and for lower pressures (15 and 25 MPa). In contrast, at 35 MPa the consumption of glucose was considerably lower. Since the results for the lower pressures cannot be properly discussed in this figure, a more zoomed figure was presented in order to facilitate the discussion of these results. In appendix B (Figure B1) the original image of the consumption of glucose is presented.

At 0.1 MPa, a similar behaviour was observed in the four fermentation cycles with an analogous final glucose concentration in all cases (below 0.500 $g.L^{-1}$). At this pressure glucose was almost entirely consumed in all of the cycles, with almost 99% of total glucose present in the medium being consumed during the fermentation cycles meaning that cells are growing at the conditions usually considered as optimal for this process, meaning that cells were only subjected to the levels of stress that typically occur during fermentation. Similarly, at 15 and 25 MPa almost all glucose was consumed during fermentation at all cycles. The only exception was in the third cycle of 25 MPa where the concentration is 10-fold higher than the other cycles. These results present may be due to an experimental error.

At 15 MPa, a decrease in glucose concentration was observed after each fermentation cycle: from 0.561 $g.L^{-1}$ at the end of the first cycle, to 0.261 $g.L^{-1}$ at the end of the last one. For this first cycle, the consumption of glucose was 85%, corresponding to the minimum glucose consumption in the four cycles. In the following cycles, the glucose concentration decrease reaching percentages of glucose consumption similar to 0.1 MPa. Similar results were obtained for the cycles at 25 MPa, where, with the exception of the third cycle, a decrease in glucose concentration from 0.500 $g.L^{-1}$ at the

end of the first cycle, to 0.331 $g.L^{-1}$ at the end of the fourth one was observed. Therefore, glucose consumption increased after the consecutive fermentation cycles under pressure, which may indicate that the cells were adapting to pressure during the consecutive cycles and were fermenting more easily in the last cycle.

At 25 MPa, the percentage of glucose consumed is more variable throughout the cycles, having a decrease in consumption in the third cycle that could be a result of an adaptation process or an experimental error. In the other three cycles the percentage of glucose consumed was similar to the one obtained for 0.1 MPa.

Regarding the consumption of glucose when the fermentation cycles took place at 35 MPa, the consumption was lower than the other pressures tested in all cycles. However, consumption increased through the cycles, having a maximum consumption in the fourth one. In this case, approximately 25 % of the total glucose available initially was consumed, contrasting with the 6% in the first cycle. Even though, these results may represent some kind of adaptation to pressure by *S. cerevisiae*.

4.3.2. Production of bioethanol

As above mentioned, bioethanol is the main product of alcoholic fermentation, and the objective of this work is to promote the enhancement of bioethanol production, by carrying out fermentation under some sub-lethal levels of pressure, 15, 25 and 35 MPa.

Figure 22 is a representation of the values obtained for the production of bioethanol under the three pressure throughout the four cycles of fermentation, being the samples subjected to 15 MPa represented by purple columns, the samples subjected to 25 MPa represented by the light blue columns the samples subjected to 35 MPa represented by the green columns and the control samples (at atmospheric pressure) represented by blue columns (the diagram of colours used in this figure will be the same for the entire set of figures below)

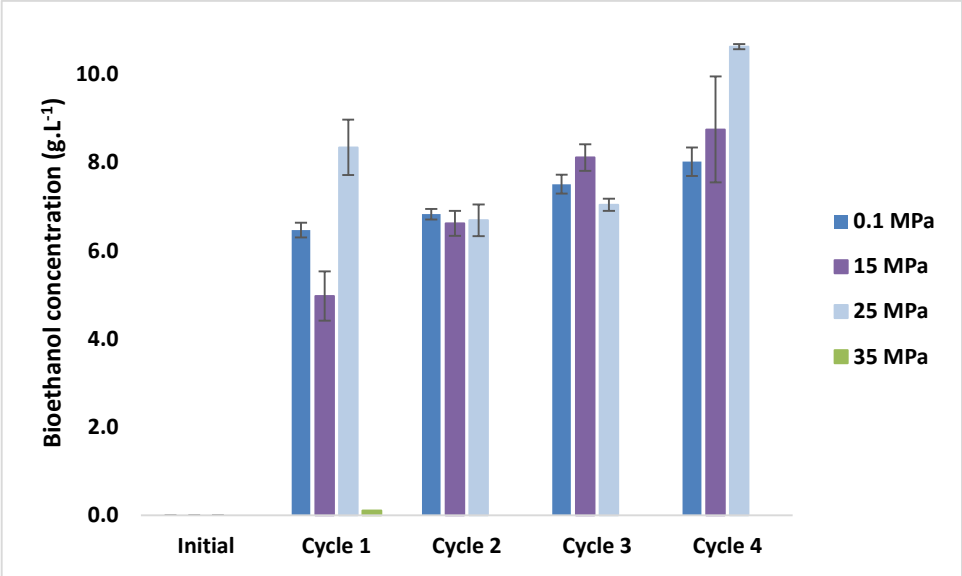

Figure 22 Final concentration of bioethanol after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

At 0.1 MPa, the concentration of bioethanol at the end of fermentation was enhanced from the first to the last cycle, which suggests that *S. cerevisiae* undergoes an adaptive process that enhances the production of bioethanol in each cycle. In this case, adaptation may be related to some of the stresses that the yeast was naturally exposed during fermentation, such as osmotic pressure, reduction of pH, ethanol stress and others.

At 15 MPa, the production of bioethanol in the first cycle was lower than at 0.1 MPa, having a final concentration of approximately 5.00 $g.L^{-1}$, compared to approximately 6.50 $g.L^{-1}$ at 0.1 MPa. However, in the next three cycles, the concentration of bioethanol at the end of the process was enhanced (similarly to what was verified at 0.1 MPa), mainly in the last two cycles. For these two final cycles, the concentration of bioethanol was even higher than at 0.1 MPa, which indicates that the cells were possibly able to adapt to those levels of pressure, which is reflected by a higher concentration of bioethanol produced.

For the pressure cycles at 25 MPa, the values of bioethanol concentration after each fermentation cycle did not follow a clear pattern throughout the cycles. At 25 MPa, it can be observed a decrease in the final ethanol concentration from the first cycle to the second one. Nevertheless, after this decrease in production of bioethanol, the concentration was enhanced at the end of the next cycles, and the maximum bioethanol production was obtained at this pressure level, corresponding to 10.30 $g.L^{-1}$ reached at the

end of the fourth cycle. This value is higher than the observed at the end of the fourth cycle at 0.1 and 15 MPa (8.02 $g.L^{-1}$and 8.80 $g.L^{-1}$, respectively).

In contrast, when a pressure of 35 MPa was applied during fermentation, the concentration of bioethanol was minimal (0.111 $g.L^{-1}$) after the first cycle, and not detectable by HPLC at the end of the next three cycles. These results are in accordance with the glucose consumption at this pressure, which was also minimal during the entire process, possibly meaning that at this pressure cells cannot adapt to the consecutive cycles under 35 MPa.

In general, application of consecutive cycles of fermentation under HP (15 and 25 MPa) may increase the production of bioethanol by *S. cerevisiae*, which is interesting considering that glucose consumption was not considerably affected by these pressures. When compared the results for the production of bioethanol it can be observed an increase of bioethanol production at 15 MPa and at 25 MPa when compared to the final concentration of bioethanol in the three tests. Those results were higher than the ones obtained by Picard at 10 MPa for 24 h where he noticed an increase in 3-4% in the production of bioethanol by *S. cerevisiae*. Even though the fermentation used for this experiments was longer these results may prove that the use of consecutive cycle can enhance the capacity to produce bioethanol thus adapting *S. cerevisiae* to sub-lethal levels of pressure even when higher levels of pressure are used. The calculation of specific kinetic parameters (such as ethanol yields) and the glucose consumption (%) may assist in the clarification of this pressure effect **(sections 4.3.1 and 4.3.6)**.

4.3.3. Production of organic acids

4.3.3.1. Formic acid

As above mentioned, the production of formic acid is relevant because the production of formic acid is directly related to the mixed fermentation itself (Keseler et al., 2011). Figure 23 is a representation of the values obtained for the production of formic acid.

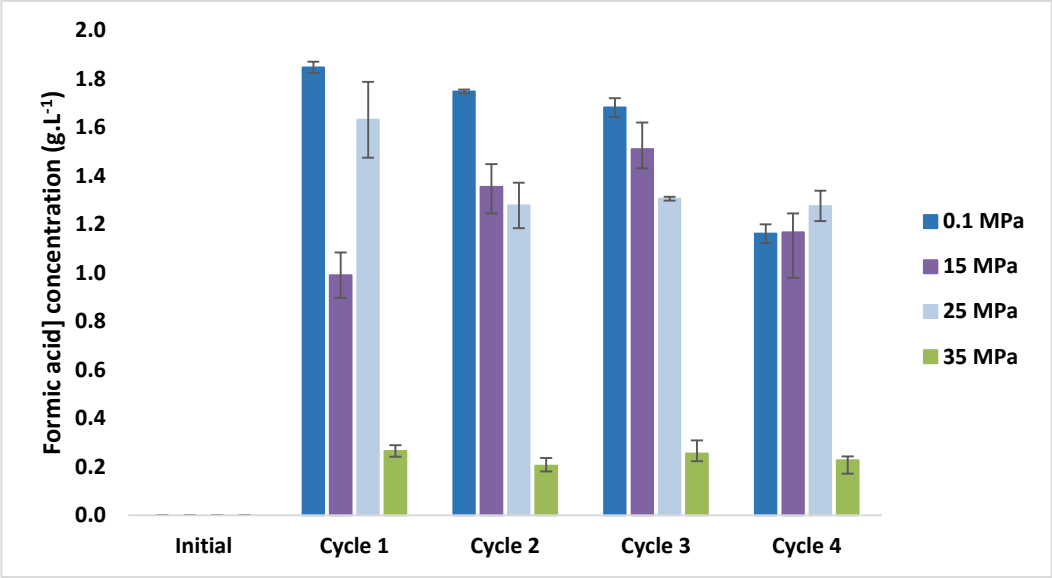

Figure 23 Final concentration of formic acid after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

At 0.1MPa, a decrease in formic acid concentration can be observed through the four cycles of fermentation, with the lowest formic acid concentration registered in the last cycle (1.16 $g.L^{-1}$).

For pressure cycles of 15 MPa, formic acid concentration tended to decrease from the first to the third fermentation cycle. In this case, the concentration of formic acid was lower than at 0.1 MPa, for the first three cycles. However, in the last cycle, formic acid concentration suffered a reduction in concentration when compared to the third cycle. In consequence, at the end of the fourth cycle the concentration of formic acid was similar at 0.1 and 15 MPa (1.16$g.L^{-1}$ and 1.18$g.L^{-1}$, respectively).

Through the analysis of the results obtained for the pressure cycles at 25 MPa, the lowest formic acid concentration was observed at the end of the last cycle, corresponding to approximately 1.25 $g.L^{-1}$. In contrast to the observed at 15 MPa, in the first two cycles at 25 MPa, the formic acid concentration was found to decrease. However, after that, the concentration of this organic acid was almost constant, which may indicate that a pressure of 25 MPa enhances the production of bioethanol (as indicated in **section 4.3.2**) without enhancing the mixed fermentation –responsible for the production of this organic acid.

On the other hand, a lower concentration of formic acid was verified at 35 MPa, with a value of approximately 0.250 $g.L^{-1}$. At this pressure, the production of formic acid

was almost constant in every cycle having a maximum variation of 0.05 $g.L^{-1}$ between the four cycles of fermentation, which indicates that, at this pressure, fermentation was almost stopped, not allowing the formation of this product.

4.3.3.2. Acetic acid

In this work, the production of acetic acid is relevant because an increase in the production of this organic acid is related to a lower production of bioethanol, which, in turn, is the desired product of the present work (Hopewell, 2014; Müller et al., 2012). Figure 24 is a representation of the values obtained for the production of acetic acid.

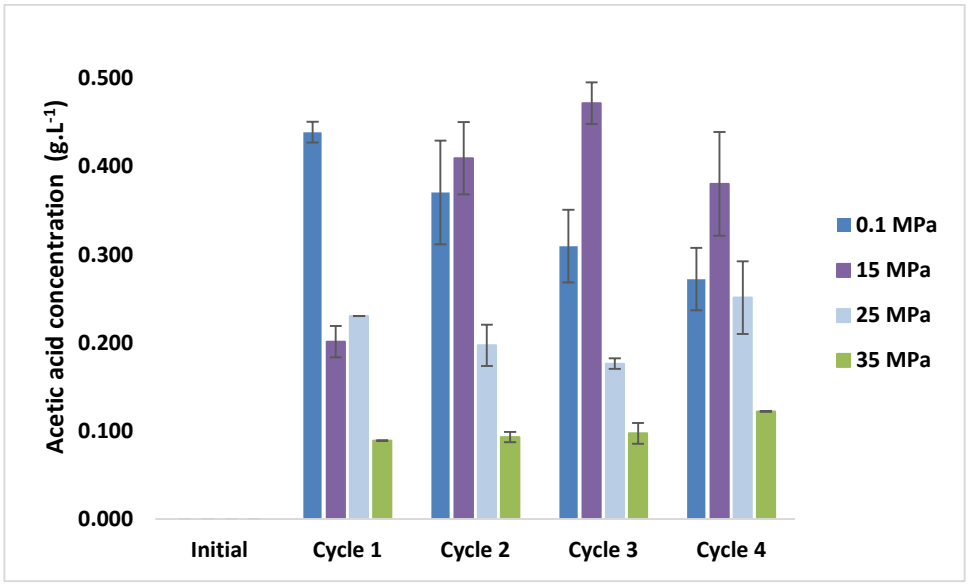

Figure 24 Final concentration of acetic acid after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

For all the studied cases, low concentrations of acetic acid were observed, with final concentrations lower than 0.500 $g.L^{-1}$ for the pressure cycles of 15 MPa, and lower than 0.250 $g.L^{-1}$ and 0.150 $g.L^{-1}$ for the pressure cycles of 25 and 35 MPa, respectively. At 0.1 MPa, acetic acid concentrations were lower than 0.450 $g.L^{-1}$

At 0.1 MPa, a reduction in the production of acetic acid was verified from the first cycle, 0.43 $g.L^{-1}$, to the last, 0.27 $g.L^{-1}$, proving once again the adaptation of *S. cerevisiae* to some of the harsh fermentation conditions (*e.g.* reduction of pH and osmotic pressure). In fact, acetic acid is an inhibitor of fermentation and a decrease of its production enhances de concentration of bioethanol (Xu, Shi, & Jiang, 2011).

In the case of the pressure cycles at 15 MPa, the acetic acid concentration was, in general, higher than at 0.1 MPa, which indicates that this pressure level enhanced the production of different products, possibly by favouring mixed fermentation processes, as previously observed in the results of formic acid production. On the other hand, during the pressure cycles at 25 MPa, the production of acetic acid was lower than at 0.1 MPa for all the four cycles, which indicates that at this pressure level, the production of acetic acid decreased, as it happened for formic acid. This indicates that application of 25 MPa pressure cycles may reduce the production of some of the main alcoholic fermentation by-products, which may represent an interesting improvement for the industrial production of bio-ethanol. A study regarding *Clostridium thermocellum* applied sub-lethal levels of pressure to fermentation and reported the enhancement of bioethanol production, while the production of acetate (as a by-product) was reduced. Therefore, application of HP during fermentation enhanced the ratio bioethanol: acetate, which corresponds to a modification of the metabolic selectivity of the microorganisms. These metabolic changes favouring the production of bioethanol and the inhibition of fermentation by-products appears to be a general effect of pressure on the fermentation processes (Bothun, Knutson, Berberich, Strobel, & Nokes, 2004).

In the case of 35 MPa pressure cycles, the concentration of acetic acid was lower when compared to the rest of the treatments, having a maximum concentration of 0.122 $g.L^{-1}$ in the last cycle. At this pressure it is noticeable that the values of the acetic acid concentration were very similar having a maximum variation of 0.04 $g.L^{-1}$. As it was verified for the production of formic acid (**section 4.3.3.1**) at this pressure, the fermentation process was almost stopped, not allowing the formation of acetic acid.

4.3.4. Biomass concentration

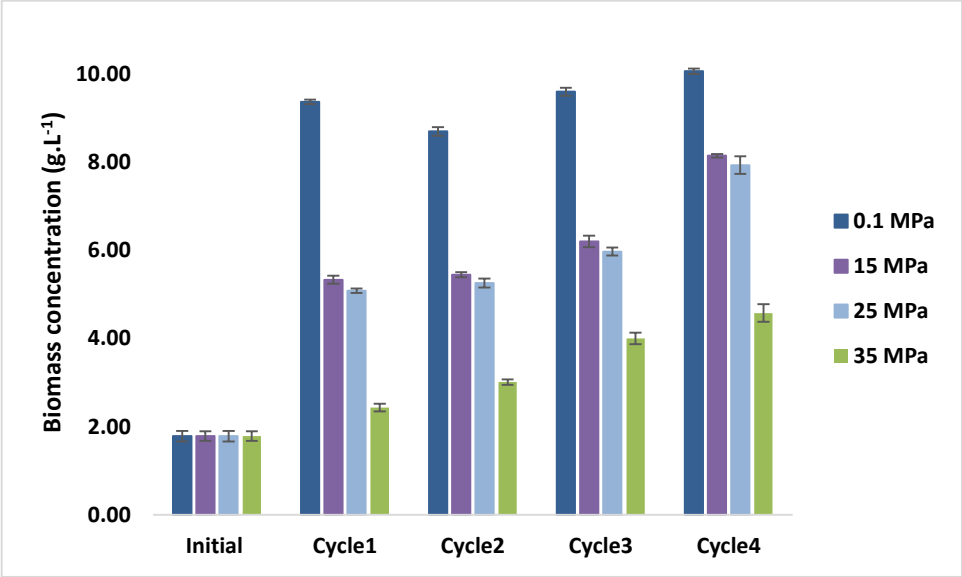

Figure 25 Final biomass concentration after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Analysing the results presented in Figure 25 it can be observed that at 0.1 MPa the biomass concentration is the highest of all the other conditions. At atmospheric pressure, in the second cycle exists a slightly reduction in biomass concentration, from 9.37 $g.L^{-1}$ in the first cycle to 8.70 $g.L^{-1}$ in the second. Despite these slight alterations in the biomass concentration, the production of biomass was almost constant throughout the 4 fermentation cycles.

For all the remaining conditions tested, an increase in biomass concentration was observed over the fermentation cycles, indicating that for all the three pressures tested (15, 25 and 35 MPa), *S. cerevisiae* cells were able to grow. However, the biomass concentration was always lower under pressure, when compared to the atmospheric pressure test. Furthermore, it was possible to notice that at 15 and 25 MPa the enhancement of biomass concentration was higher (from the initial concentration of 1.79 $g.L^{-1}$ to approximately 8.00 $g.L^{-1}$ in both cases) than at 35 MPa, that had a final concentration of approximately 5.00 $g.L^{-1}$. For the lower pressures, 15 and 25 MPa the biomass concentration was almost 2.00 $g.L^{-1}$ lower than samples fermented at 0.1 MPa, but that did not translate in a similar reduction of glucose consumption (has it can be seen in **section 4.3.1**) and in the production of bioethanol (as it can be seen in **section 4.3.2**). For 35 MPa despite the low consume in glucose it can be observed a slight enhancement in the biomass concentration throughout the fermentative cycles, from approximately

2.00 $g.L^{-1}$ in the first cycle to approximately 5.00 $g.L^{-1}$ in the last meaning the at this pressure cells were growing.

However, the biomass concentration evaluated in this section does not provide the number of viable cells in the culture medium, since the total number of cells present in the samples are measured, *i.e*. both viable and non-viable cells. Therefore, to measure the number of viable cells, the culture medium was incubated on solid medium plates and the results will be presented below.

4.3.5. Microbial load

In Figure 26, the values obtained for the variation of microbial load throughout the 4 fermentation cycles are represented

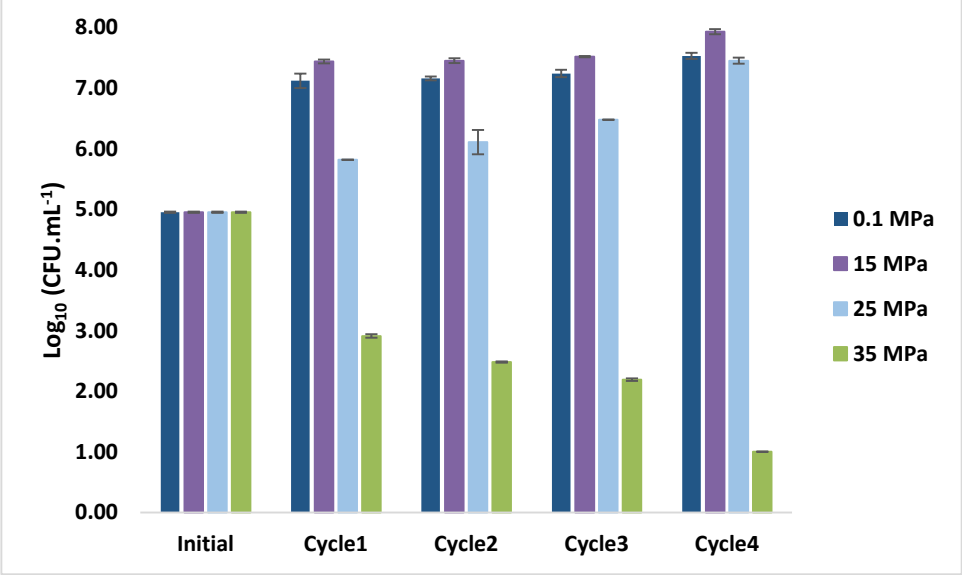

Figure 26 Microbial load after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30ºC

The results at all pressures, except for 35 MPa, show an increase in viable cells from the initial concentration present in the inoculum to the end of the first fermentation cycle. This viability increase corresponds to the typical cell growth that occurs during fermentation processes at suitable conditions. However, cell viability was negatively affected at 35 MPa, possibly because many important cell structures and functions were compromised under such harsh conditions. Throughout the cycles, the number of viable cells at atmospheric pressure was maintained almost constant. Similarly, at 15 MPa the

number of viable cells showed almost no variation throughout the cycles. However, bioethanol production tends to increase after the first fermentation cycle, such as indicated in **section 4.3.2**, suggesting a possible adaptation effect of *S. cerevisiae* to this pressure. These results indicate that the enhancement in the production of bioethanol is not a result of the increase number of viable cells, and may result from an increased ethanol yield, i.e. the cells are re-directing its metabolism and converting a higher amount of substrate in ethanol.

In contrast, when pressure cycles were performed at 25 MPa, there was a clear adaptation of *S. cerevisiae,* with an increase of almost 1.5-log from the first cycle to the fourth one. At this higher pressure, *S. cerevisiae* cells have more difficulties to survive and adapt, but after the first cycle they seem to be able to develop adaptation mechanisms, resulting in an increase in the number of viable cells from the first cycle to the fourth. Differently from the results at 15 MPa, the number of viable cells in the last cycle at 25 MPa was similar to 0.1 MPa. Moreover, at this pressure that the highest production of bioethanol is obtained.

On the other hand, no adaptation of *S. cerevisiae* seemed to occur at 35 MPa, since a reduction in the number of viable cells was observed throughout the four cycles. Probably, at 35 MPa some of the cells could not resist to those levels of pressure and died. The ones that were able to survive, as discussed by Iwahashi et al., 2005 increased in size and complexity in order to try resist to this pressure. Furthermore, specific stress responses are induced in the cells that are able to survive at this pressure, such as the production of certain heat-shock proteins and activation of some genes controlling membrane structure (Iwahashi et al., 2005). Those results can be proven by the reduced consumption of glucose observed in Figure 24, which means that fermentation was almost inhibited and, therefore, no energy is produced for the cells growth.

In general, the results obtained for the tests at 30 ºC revealed that it possible to induce adaptation of *S. cerevisiae* to sub-lethal levels of pressure (particularly, 15 and 25 MPa). This was verified not only for microbial growth and cellular viability, but also for the production of bioethanol. For both pressures, it was observed an increase in the microbial load, as well as in the production of bioethanol, which can be related to the development of an adaptive mechanism.

4.3.6. Kinetic parameters

The determination of kinetic parameters allows a better comparison between the results and a more in-depth analysis of the HP effects on this process. In this section the results obtained for the kinetic calculations of the samples that fermented at 30 ºC will presented.

4.3.6.1. Yield of bioethanol production

Table 3 shows the values estimated for the yield of bioethanol production after each of the four fermentative cycle for each of the pressures tested.

Table 3 Yield of bioethanol production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Yield of bioethanol production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.319	0.171	0.331	0.0941
Cycle 2	0.337	0.327	0.416	-
Cycle 3	0.371	0.401	0.411	-
Cycle 4	0.395	0.431	0.525	-

The yield of bioethanol on glucose increased throughout the fermentation cycles, at all conditions except 35 MPa. At 15 MPa, the bioethanol yield showed the highest variation between the cycles (from 0.171 $g.g^{-1}$ to 0.431 $g.g^{-1}$) and, interestingly, higher yield were observed for the last two cycles compared to the ones at 0.1 MPa. This indicates that at this pressure (15 MPa) the production bioethanol was considerably improved over the cycles, with the cell producing a higher amount bioethanol per glucose consumed. The comparison between the theoretical yields of bioethanol (0.5136 $g.g^{-1}$) - yield obtained assuming that all the glucose is consumed and transformed into bioethanol- with those bioethanol experimental yields (Table 3) are indicated in the appendix E (Table E1). At 15 MPa, the practical yield was correspondent to only 33.4 % of the theoretical yield after the first cycle, but this value increased to 83.9 % at the end of the fourth cycle, representing an increase of 50.5 %. In contrast, at 0.1 MPa these values ranged between

62.0 % and 76.9 %, corresponding to an increase of only 14.9%. This means that at 15 MPa, *S. cerevisiae* is adapting, possibly by developing HP stress responses, which may be promoting its ability to produce bioethanol from glucose.

The results for 25 MPa are more variable, not following a straight pattern throughout the fermentation cycles. Nevertheless, by comparing the bioethanol on glucose yields at 25 and 0.1 MPa, it can be observed that the values were generally higher at 25 MPa than at atmospheric pressure. The maximum value for the yield of bioethanol on glucose was estimated for the fourth fermentation cycle at 25 MPa, corresponding to 0.525 $g.g^{-1}$. Moreover, the comparison between the experimental/theoretical yields (%), present in the appendix E (Table E1), shows these values increased from 64.5 % in the first cycle to 102.3 % in the fourth cycle, representing an increase of 37.8 %.

Regarding the values of the experimental theoretical yield (%) of 0.1, 15 and 25 MPa it can be observed a slight lower value for the first two cycle of the pressure tests when compared to the control group. However, for the last two cycles, the yield of bioethanol for the pressure tests was higher when compared to 0.1 MPa. In the last cycle it can be seen that at 15MPa the yield is 7% higher than the last cycle of 0.1 MPa and at 25 MPa it is 25.4 % higher. This means that for the pressure tests cells are not only adapting, noticeable by the increase of the yield throughout the cycles, but are also production more bioethanol than the control group. Furthermore, the yield of bioethanol production of the last cycle of 25 MPa is 102.3 % of the theoretical yield meaning that for this pressure S. cerevisiae is using not only the glucose present in the medium but also the other sugars (maltose and fructose). Both 15 and 25 MPa could be used to increase bioethanol productivity, being 25 MPa the pressure that produces bioethanol in more quantity per molecule of sugar consumed.

Finally, for 35 MPa the bioethanol yields on glucose are low (0.0948 $g.g^{-1}$) (or even impossible to estimate), since almost no glucose is being consumed during the fermentation cycles, as well as almost no bioethanol is being produced. Therefore, at this pressure cells seem to be unable to adapt, resulting in inhibition of bioethanol production. Nevertheless, from the results of **section 4.3.1** for the glucose consumption (%) it can be seen that there is an increase in substrate consumption which indicates that the cell is not producing ethanol but is consuming glucose, possibly in an attempt to survive and adapt.

4.3.6.2. Yield of formic acid production

Table 4 is a representation of the yield of formic acid production after each of the four fermentative cycle for each of the pressures tested.

Table 4 Yield of formic acid production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Yield of formic acid production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.101	0.0489	0.0807	0.166
Cycle 2	0.0970	0.0669	0.0746	0.0657
Cycle 3	0.100	0.0744	0.0645	0.0489
Cycle 4	0.100	0.0568	0.0874	0.0110

Regarding the yield of formic acid production for 0.1 MPa, the values are almost constant throughout the fermentation cycles (varying in the range of 0.0970 $g.g^{-1}$ and 0.101 $g.g^{-1}$), indicating that the production of formic acid per unit of glucose is almost constant at atmospheric pressure.

At 15 MPa, there is a general trend for the increase in the yield of formic acid in the three first cycles (from 0.0489 $g.g^{-1}$ in the 1st cycle to 0.0744 $g.g^{-1}$ in the last), while the last cycle had a decrease in this value. However, in general, the yield increased from 0.0489 $g.g^{-1}$ in the first cycle to 0.568 $g.g^{-1}$ in the fourth cycle meaning that at this pressure the adaption of *S. cerevisiae* is being responsible for the production of more sub-products. For 25 MPa, from the 1st to the 3rd the general trend is for a decrease in the yield of formic acid production from 0.0807 $g.g^{-1}$ to 0.0645 $g.g^{-1}$. In the last cycle a considerable increase in the yield of formic acid is obtained contradicting the general tendency of remaining cycles.

Comparing the results of 0.1, 15 and 25 MPa it is noticeable that for the pressure tests the values were lower than 0.1 MPa, which indicates that under HP less glucose is being used for the production of formic. The opposite effect was seen for bioethanol production, *ie* pressure seems to be targeting glucose for ethanol production and not for other products. Less formic acid is being produce from the glucose consumed during fermentation, which can be related to the enhancement in the bioethanol yields, since formic acid is a by-product of bioethanol production.

At 35 MPa in the 1st cycle it was obtained the highest value of the yield of formic acid production 0.166 $g.g^{-1}$. This results can be relate to the fact that the production of

bioethanol was minimal (**Section 4.3.6.1**). From that, a considerable decrease in the yield was observed, being in the last cycle 0.0110 $g.g^{-1}$ that is the minimum value of all the tests. That is, during the cycles at 35 MPa there is a decrease in the use of glucose for the production of formic acid. At this pressure, cells were consuming glucose to try to adapt to those levels of stress and not a considerable percentage of products were formed.

4.3.6.3. Yield of acetic acid production

Table 5 is a representation of the yield of acetic acid production after each of the four fermentative cycle for each of the pressures tested.

Table 5 Yield of acetic acid production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Yield of acetic acid production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.0128	0.0107	0.0097	0.0707
Cycle 2	0.00970	0.0202	0.0114	0.0495
Cycle 3	0.0116	0.0255	0.0116	0.0241
Cycle 4	0.0137	0.0186	0.0137	0.0129

Regarding the results of the yield of acetic acid on glucose, the values varied throughout the fermentation cycles, apparently not following a specific pattern, with exception of the yields at 35 MPa. For this pressure, similar to what happen for the yield of formic acid, the maximum value is in the 1st cycle, 0.0707 $g.g^{-1}$, and from that the yield decreases being minimal in the 4th cycle, 0.0129 $g.g^{-1}$. In other words, during the cycles at 35 MPa there is a decrease in the use of glucose for the production of acetic. At this pressure, cells were consuming glucose to try to adapt to those levels of stress and the glucose consumed is being used for those processes. However, in all cases the yields of acetic acid were very low, indicating that for each of the conditions used (0.1, 15, 25 and 35 MPa) the utilization of glucose for production of acetic acid was very low.

4.3.6.4. Yield of biomass production

Table 6 is a representation of the yield of biomass production after each of the four fermentative cycle for each of the pressures tested.

Table 6 Yield of biomass production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Yield of Biomass production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.358	1.85	1.98	0.319
Cycle 2	0.327	1.98	2.03	0.604
Cycle 3	0.369	2.32	2.45	1.09
Cycle 4	0.403	3.31	3.56	1.38

The general tendency of all the tests is for an increase in the yield of biomass production being that increase more accentuated for the pressure tests (15, 25 and 35 MPa). At 0.1 MPa, the yield of biomass was similar throughout the 4 cycles having increasing from 0.358 $g.g^{-1}$ in the 1st cycle to 0.403 $g.g^{-1}$ in the last one.

At 15 and 25 MPa, the biomass yield showed the highest variations between the cycles, from 1.85 $g.g^{-1}$ to 3.31 $g.g^{-1}$ at 15 MPa and from 1.98 $g.g^{-1}$ to 3.56 $g.g^{-1}$ at 25 MPa, respectively. At these pressures, interestingly, higher yields were observed for all the fermentative cycles compared to the ones at 0.1 MPa. This indicates that at those pressures (15 and 25 MPa) the biomass production was considerably improved over the cycles, with the cell producing a higher amount biomass per glucose consumed. At 15 and 25 MPa, *S. cerevisiae* is adapting, possibly by developing HP stress responses, which may be promoting its ability to grow using glucose as an energy source.

Finally, for 35 MPa the yield of biomass, similar to the other pressure tests, increased from the 1st cycle (0.319 $g.g^{-1}$) to the last cycle (1.38 $g.g^{-1}$). Again, for these pressure higher yields were observed for all the fermentative cycles compared to the ones at 0.1 MPa meaning the biomass production was considerably improved over the cycles, with the cell producing a higher amount biomass per glucose consumed.

4.3.6.5. Productivity and specific productivity of bioethanol

4.3.6.5.1. Bioethanol productivity

Table 7 is a representation of the bioethanol productivity after each of the four fermentative cycle for each of the pressures tested.

Table 7 Bioethanol productivity by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Bioethanol productivity, Q ($g.L^{-1}.h^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.0899	0.0691	0.1160	0.0015
Cycle 2	0.0949	0.0920	0.0929	-
Cycle 3	0.1040	0.1130	0.0978	-
Cycle 4	0.0835	0.0912	0.1110	-

Regarding the bioethanol productivity, similar results can be observed for samples subjected to 0.1 MPa and 15 MPa. For these two conditions, an increase in the productivity of bioethanol can be observed in the first three cycles. However, for the last cycle a reduction in productivity is noticeable for both conditions (from 0.104 $g.L^{-1}.h^{-1}$ to 0.0835 $g.L^{-1}.h^{-1}$ at 0.1 MPa, and from 0.113 $g.L^{-1}.h^{-1}$ to 0.0912 $g.L^{-1}.h^{-1}$ at 15 MPa). Nevertheless, in the end of the four cycles the productivity at 15 MPa was higher than at atmospheric pressure.

At 25 MPa, differently from 0.1 and 15 MPa, it can be observed that in the first cycle the productivity of bioethanol was the highest of the four cycles (0.116 $g.L^{-1}.h^{-1}$). After that, a decrease in productivity occurs in the second cycle, to 0.0929 $g.L^{-1}.h^{-1}$. Furthermore, at 25 MPa, the productivity increased in the last cycle, contrary to what it was verified for 0.1 and 15 MPa. In fact, this productivity value at 25 MPa corresponds to the highest final productivity, compared to all pressures tested. Therefore, in the end of the four cycles, the bioethanol productivities at both 15 and 25 MPa were higher than at atmospheric pressure. These results show that consecutive fermentation cycles under these pressures accelerated bioethanol production, which fulfils the objective of this work.

For 35 MPa, bioethanol was only produced during the first cycle (**section 4.3.2**) and, because of that, it was only possible to estimate productivity for this one. At this

pressure bioethanol productivity was very low (0.0015 $g.L^{-1}.h^{-1}$), which is in agreement with the results obtained above showing that, for 35 MPa, no noticeable bioethanol production was verified.

4.3.6.5.2. Specific bioethanol productivity

Table 8 is a representation of the bioethanol specific productivity after each of the four fermentative cycle for each of the pressures tested.

Table 8 Bioethanol specific productivity by* S. cerevisiae *after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Bioethanol specific productivity, q ($g.g^{-1}.h^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.0097	0.0129	0.0226	0.0006
Cycle 2	0.0110	0.0168	0.0176	-
Cycle 3	0.0111	0.0182	0.0164	-
Cycle 4	0.0084	0.0092	0.0141	-

At 0.1 and 15 MPa it can be observed that in the first three cycles, an enhancement occurs in the specific productivity of bioethanol. However, for the last cycle a reduction in productivity is noticeable for both (from 0.111 to 0.0840 $g.g^{-1}.h^{-1}$ at 0.1 MPa, and from 0.182 to 0.0920 $g.g^{-1}.h^{-1}$ at 15 MPa). Nevertheless, the productivity at 15 MPa, in the end of the four cycles was higher than at atmospheric pressure, which means that at this pressure, more bioethanol was produced from biomass existing in the medium per hour. For 25 MPa it can be observed that in the first cycle the specific productivity of bioethanol was the highest of the four cycles (0.226 $g.g^{-1}.h^{-1}$). From that, a constant decrease in specific productivity may be observed in the remaining cycles, which results in the lowest value at the last cycle, 0.141 $g.g^{-1}.h^{-1}$.

As previously indicated, for 35 MPa the only cycle with bioethanol production was in first one (**section 4.3.2**) and, because of that, only the first cycles shows specific productivity. At this pressure bioethanol specific productivity was very low (0.0006 $g.L^{-1}.h^{-1}$), which indicates that bioethanol production is almost negligible at this pressure.

4.3.7. Ratio of bioethanol:by-products of fermentation

The ratio of bioethanol:by-products of fermentation (formic acid and acetic acid) after each of the four fermentation cycles is represented in Table 9.

Table 9 Ratio of bioethanol: by-products of fermentation after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 ºC

Bioethanol:by-products ratio				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	2.96	4.34	4.61	0.331
Cycle 2	3.36	3.96	4.68	-
Cycle 3	3.91	4.33	4.88	-
Cycle 4	5.85	5.99	7.23	-

Regarding the results of the ratio between bioethanol:by-products it can be observed that the ratios tend to increase over the cycles, at 0.1, 15 and 25 MPa. At 15 and 25 MPa the ratios are always higher than those observed at 0.1 MPa, which indicates under HP there is specificity for the production of ethanol and decrease of the production of the other compounds. Then, at 15 MPa the behaviour is variable over the cycles and consequently the ratio in the last cycle is very similar to that of 0.1 MPa, although slightly higher (5.85 at 0.1 MPa and 5.99 at 25 MPa). At 25 MPa, the ratios increase steadily over the cycles, and there is a considerable increase from the 3rd to the 4th cycle. Thus, in the last cycle the ratio at 25 MPa was much higher than those observed for 0.1 and 15 MPa being 7.23 at 25 MPa. This indicates that after 4 cycles at 25 MPa, the selectivity of the metabolism was modified, leading to the increase of the ethanol production in relation to the by-products.

4.4. Consecutive cycles of fermentation under high pressure at room temperature

In **section 4.3**, *S. cerevisiae* growth and fermentation were performed at optimal temperature reported in literature for this microorganism (30 ºC), but one other objective of this work was to reduce the ecological footprint of this fermentation process. Therefore, we decided to study the adaptation *S. cerevisiae* to sub-lethal levels of pressure at room temperature. In this way, the consecutive fermentation cycles were carried out without

supply of energy for temperature control and an average temperature of 24.5 ºC (temperature varied from 18.2 - 31.3 ºC) was verified being the values of the maximum and minimum temperature presented in Table G1 (**Appendix G**). Some advantages are related with the decrease in the final cost of the product due to the reduction in the consumption of glucose as well as related to the decrease in the ecological footprint, as reported in studies about hyperbaric storage (Freitas et al., 2016; Moreira et al., 2015; Pinto et al., 2017). The results of this study are presented in the sections below.

4.4.1. Substrate consumption

Regarding the values obtained for the glucose consumption during the four fermentation cycles, they are presented in Table 10. Furthermore the results of fructose and maltose are showed in Figures C1 and C2 (Appendix C), being both sugars consumed during the fermentation cycles.

Table 10 Percentage of glucose consumed by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Glucose consumed (%)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	98.7	98.5	93.3	22.1
Cycle 2	98.6	98.5	94.1	25.0
Cycle 3	98.4	98.6	38.9	37.1
Cycle 4	98.4	98.7	82.2	46.2

Samples subjected to lower pressures (15 and 25 MPa) and atmospheric pressure presented a higher consumption of glucose (Table 10), while the consumption was less notorious at 35 MPa. Since the results for the lower pressures could not be properly discussed in the original figure, in the appendix D (Figure D1) the original figure of glucose concentration can be observed.

Similarly to the process at 30 ºC, an analogous final glucose concentration after all cycles at 0.1 MPa was observed with values below 0.250 $g.L^{-1}$. For the lower pressures, no specific correlation was verified, since glucose consumption was variable throughout the process. Additionally, the glucose concentrations after each cycle at 0.1 MPa were lower than the cycles under pressure. This difference is due to the fact that cells at

atmospheric pressure were grown under lower levels of stress when compared to samples subjected to 15 and 25 MPa and fermented more easily while cells under pressure had needed more time to ferment.

At 0.1 MPa as well as at 15 MPa the glucose was almost entirely consumed in all of the cycles being approximately 99% of total glucose present consumed in this test. At 15 MPa, the variation in glucose concentration was minimal, noticing only a small decrease in glucose concentration in the end of each fermentation cycle, from 0.275 $g.L^{-1}$ on the first cycle to 0.249 $g.L^{-1}$ in the last one. For 25 MPa was observed with a maximum level of glucose of 4.58 $g.L^{-1}$ achieved in the third cycle, being approximately 4-fold the concentration obtained in the first two cycles. However, there was a decrease in concentration of glucose to 2.98 $g.L^{-1}$ in the last cycle. Regarding the fermentation cycles at 35 MPa, a lower glucose consumption was observed during the first two cycles. The initial percentage of glucose consumption was low (22.1 %) because this pressure exercised a high level of stress in *S. cerevisiae* almost stopping fermentation. From this first cycle until the last one the percentage of glucose consumed is enhanced being 46.2 %

Comparing the fermentation cycles at room temperature with the ones performed at 30 ºC (**section 4.3.1**), the results are similar in both cases, mainly for fermentation at 0.1, 15 and 35 MPa. In both processes, the minimum concentration of glucose was obtained at 0.1 MPa, which was expected because at this pressure cells are subjected to minimal levels of stress, being able to perform fermentation more easily which consequently decreases more accentually the glucose concentration. Regarding the cycles under pressure at room temperature, similar glucose concentrations were achieved, with exception to the pressure cycles at 25 MPa where higher concentrations were obtained. For instance, in the last cycle, a concentration of almost 10-fold higher was obtained at room temperature when compared to the process at 30 ºC (2.98 $g.L^{-1}$ at room temperature against 0.331 $g.L^{-1}$ at 30 ºC).

4.4.2. Production of bioethanol

Figure 27 represents the values obtained for the production of bioethanol at room temperature.

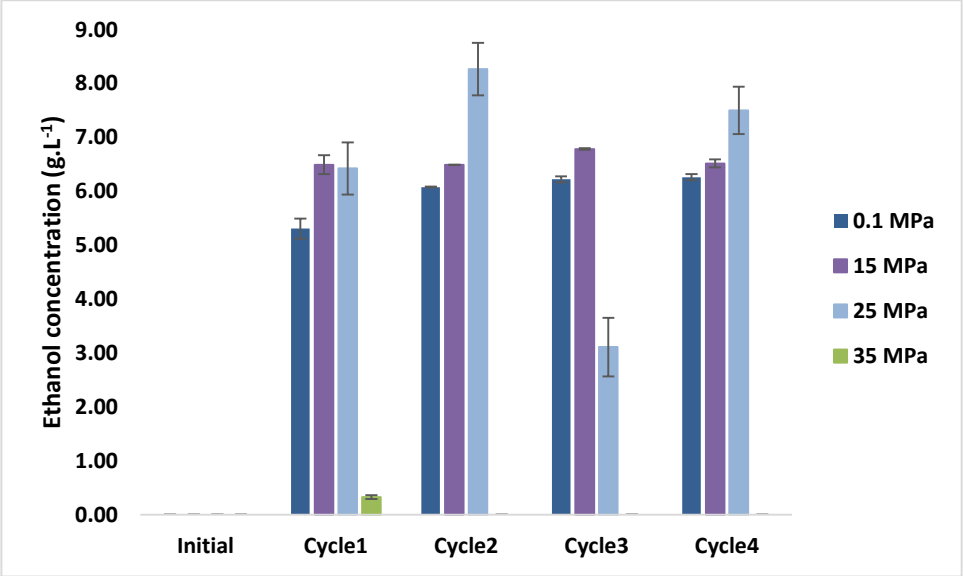

Figure 27 Final concentration of bioethanol after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

At 0.1 MPa, the concentration of bioethanol was enhanced from the first to the last cycle, which may represent an adaptive process of *S. cerevisiae* at atmospheric pressure that enhances the production of bioethanol in each cycle. At 15 MPa, bioethanol concentration after the first cycle was approximately 6.50 $g.L^{-1}$, against to approximately 5.30 $g.L^{-1}$ for 0.1 MPa. In the following cycles, the bioethanol concentration was similar to the 1st cycle and always higher than the respective control sample. Regarding the bioethanol concentration throughout the pressure cycles at 25 MPa, the values were also higher than the respective values for the control sample, with exception of the 3rd cycle where the bioethanol concentration was lower (3.11 $g.L^{-1}$ after the 3rd cycle against 8.27 $g.L^{-1}$ and 7.50 $g.L^{-1}$ after the 2nd and 4th cycles, respectively). However, this pressure level seems to be a good option for the enhancement of bioethanol production by *S. cerevisiae* under pressure. In contrast, at 35 MPa, the production of bioethanol was minimal (0.328 $g.L^{-1}$ for the first cycle), being impossible to detect the bioethanol production after the next three cycles.

Comparing the results obtained for room temperature with the ones obtained at 30 ºC (**section 4.3.2**), similar profiles of the variation of bioethanol concentration throughout

the cycles were observed at 0.1, 25 and 35 MPa. In contrast, when the cycles were performed at 15 MPa, differences between the two situations were verified, since no enhancement in the bioethanol production with the repeated cycles was verified at 15 MPa and room temperature, as occurred at 30 ºC. On the other hand, the values of bioethanol concentration were slightly different between the 2 processes, being predominantly lower in the case of room temperature, with exception of the 1st cycle at 15 and the 2 nd at 25 MPa where the bioethanol concentration was higher.

4.4.3. Production of organic acids

4.4.3.1. Production of formic acid

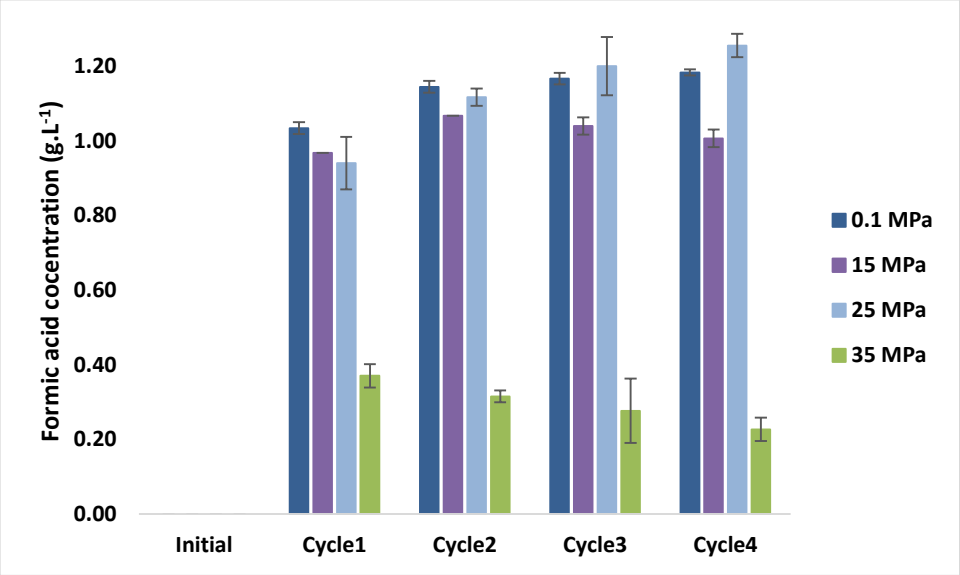

Figure 28 Final concentration of formic acid after each of the four cycles of pressure and the four cycles at 0.1 MPa, room temperature

At 0.1MPa, a slight increase in formic acid concentration was observed, Figure 28, throughout the 4 cycles with the highest concentration after the last cycle (1.184 $g.L^{-1}$). For pressure cycles at 15 MPa, the concentration of formic acid was lower than the control samples in all the fermentative cycles. At this pressure the variation in concentration of this organic acid is so low, that the production of formic acid seems to be not influenced by the consecutive fermentation cycles. In contrast, the formic acid

production increased throughout the pressure cycles at 25 MPa, with a maximum concentration in the last cycle of approximately 1.25 $g.L^{-1}$. Therefore, this increase in formic acid production may indicate that, at room temperature, 25 MPa enhances the mixed fermentation pathway that consequently decreases the alcoholic fermentation pathway. However, from the results obtained that cannot be proved, since the variation in bioethanol concentration at 25 MPa is too irregular to state that it was related to the enhancement in formic acid concentration. In the case of 35 MPa, a reduction in concentration was observed, which can indicate the enhancement of alcoholic fermentation due to the diminution of mixed fermentation, but the concentrations achieved were too low to be sure.

Comparing the results obtained for both temperature tested, an opposite behaviour for samples subjected to 0.1 MPa and 25 MPa was observed, since while the concentration of formic acid decreased throughout the fermentation cycles at 30 ºC, it increased at room temperature. In contrast, a similar behaviour was observed at 15 MPa where formic acid concentration did not follow a straight pattern throughout the fermentation cycles. Finally, regarding the results at 35 MPa, differences were observed in both cases: at 30 ºC, the variation in formic acid concentration was minimal not showing a pattern throughout the cycles, while at room temperature, a slight decrease in formic acid production was verified from the first to the last fermentative cycle.

In general, these results show that variation of both pressure and temperature during the fermentation cycles causes metabolic changes on *S. cerevisiae*, with modification on several fermentation products, including formic acid.

4.4.3.2. Production of acetic acid

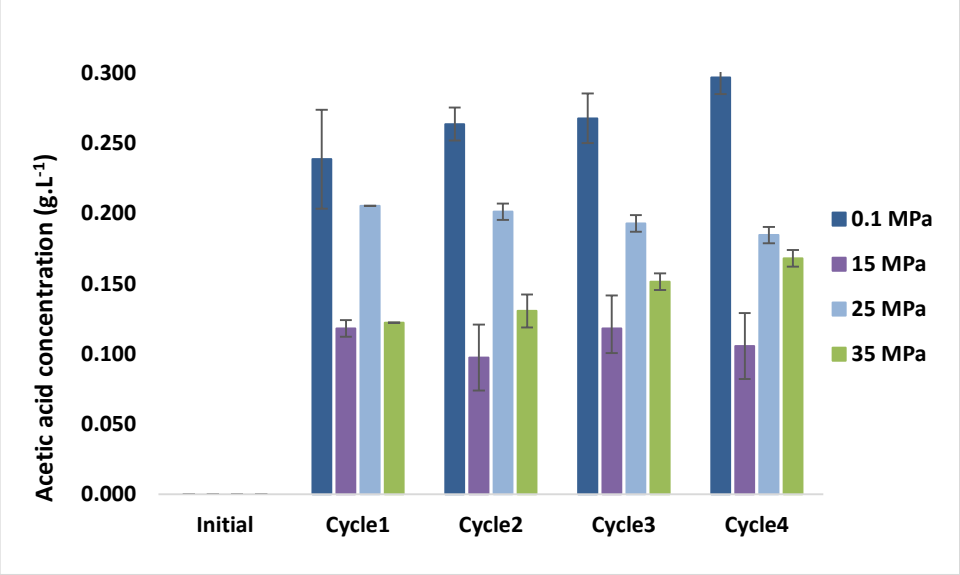

Figure 29 Final concentration of acetic acid after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

For all the studied conditions, low concentrations of acetic acid were observed, with final concentrations lower than 0.150 $g.L^{-1}$ for pressure cycles of 15 MPa and lower than 0.200 $g.L^{-1}$ for the pressure cycles of 25 and 35 MPa (Figure 29). Since the concentration of this organic acid is low for both pressure and control samples, the variations in concentration during the process were not very accentuated. In the case of control samples, a slight increase in the production of acetic acid was verified throughout the cycles and higher values of concentration were achieved in this case. On the other hand, lower concentrations of acetic acid were found for all the cycles under pressure, which may indicate that the production of acetic acid is inhibited under pressure at room temperature.

Comparing the results obtained for both temperatures (30 ºC and room temperature), it can be observed an opposite behaviour for samples subjected to 0.1 MPa. For this group, at 30 ºC, the concentration of acetic acid decreased over the fermentation cycles, while at room temperature the concentration of acetic acid increased. For 15 MPa, a similar behaviour was observed for both tests, not following a straight pattern throughout the fermentation cycles. In both tests, the concentration of formic acid had some variations in concentration between the cycles, being those variations more accentuated at 30 ºC. Similarly, at 25 MPa and 30 ºC, the samples did not had a visible

pattern throughout the fermentation cycles, nevertheless the variations in concentration were minimal. In contrast, at room temperature, samples had a slight decrease in concentration of acetic acid trough out the fermentation cycles, even if these variations in concentration were minimal. Finally, regarding the results at 35 MPa, a slight increase in acetic acid production was verified from the first to the last fermentative cycle at room temperature. At 30 ºC the variation in concentration of formic acid is minimal, but also having a minor increase in concentration though out the cycles. This increase in acetic acid production at 35 MPa indicates that fermentation is not completely inhibited, as can be suggested by other parameters, such as glucose consumption or bioethanol production. In fact, at this pressure *S. cerevisiae* fermentation seems to have metabolic modifications, including the production of acetic acid, which may have some role in the stress response and adaptation of this yeast to HP.

4.4.4. Biomass concentration

Figure 30 is a representation of the values obtained for the biomass concentration after each pressure cycle.

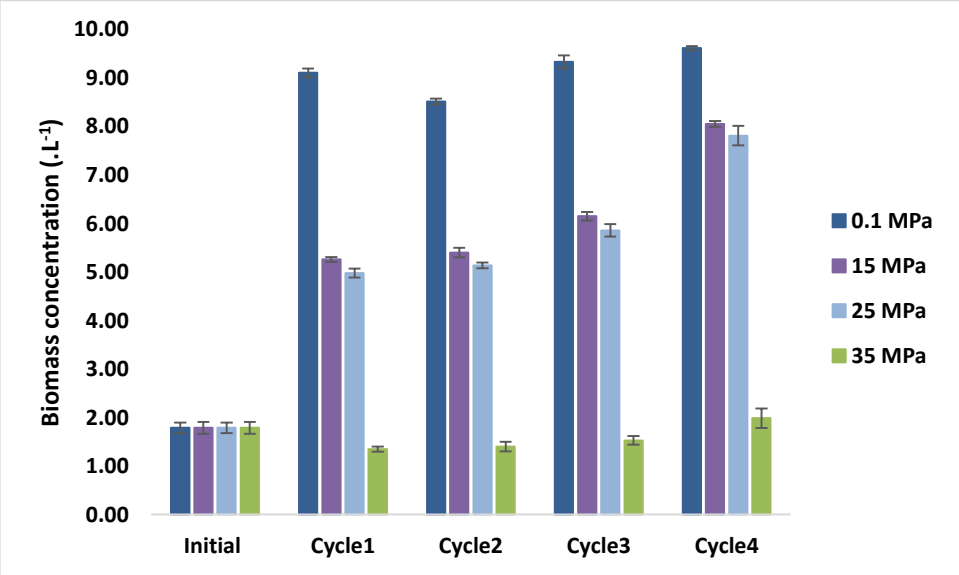

Figure 30 Final biomass concentration after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Analysing the results present in Figure 30, the highest biomass concentration was observed for the cycles at 0.1 MPa. Furthermore, at atmospheric pressure, an increase of

the biomass concentration was verified until a maximum concentration were reached in the final fermentative cycle (9.62 g.L-1). For the lower pressures tested (15 and 25 MPa), an increase in biomass concentration was also verified over the fermentation cycles, possibly indicating that for these two pressures, *S. cerevisiae* was adapting to pressure and grow more easily at these conditions. For 35 MPa, the results for biomass concentration maintain almost the same throughout the fermentative cycles. The increase in biomass concentration over the fermentation cycles at 35 MPa indicate that fermentation is not completely inhibited at this pressure, and cells are still able to grow at some extent, even if ethanol production is not observed.

Comparing the results of biomass concentration obtained for both processes (30 ºC and room temperature), similar results were achieved for 0.1, 15 and 25 MPa, being only approximately 0.25 $g.L^{-1}$ lower at the room temperature. For 0.1 MPa at both temperatures it can be observed a decrease in concentration from the 1st cycle to the 2nd. Furthermore, for both temperatures it can be observed that the control group has the highest biomass concentration throughout the fermentation cycles. For 15 and 25 MPa the results were very similar between pressures, having similar results for both pressures, and between temperatures, having as well similar results for biomass concentration. Some differences in the results obtained for 35 MPa were observed, since while the biomass concentration increased during the process at 30 ºC, it does not vary for room temperature.

4.4.5. Microbial load

Figure 31 is a representation of the values obtained for the biomass concentration after each pressure cycle.

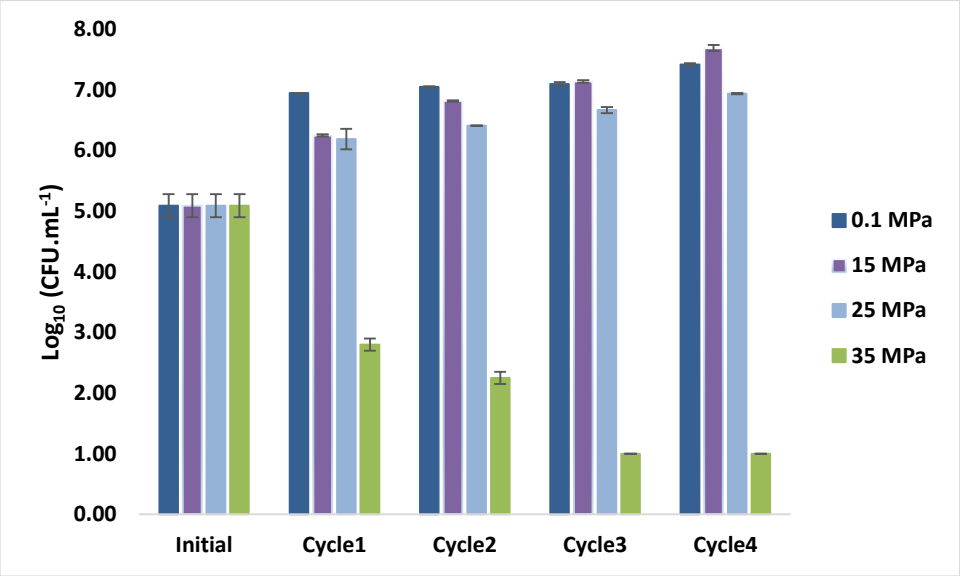

Figure 31 Microbial load after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

An increase of the number of viable cells was verified after the 1st cycle at 0.1 MPa, but it remained almost constant throughout the cycles (from 6.95 $CFU.mL^{-1}$ after the first cycle to 7.42 $CFU.mL^{-1}$ after the fourth cycle). This profile indicates that the possible cell adaptation verified above (due to the enhancement of bioethanol production) was not a result of the increase of number of viable cells but a result of a higher fermentation yield. On the other hand, an adaptation of *S. cerevisiae* appears to be occurring during the pressure cycles at 15 and 25 MPa, since the microbial load increased throughout the fermentative cycles. At 35 MPa, no adaptation of *S. cerevisiae* seemed to occur, since a reduction of the number of viable cells were observed throughout the four cycles.

Comparing the results obtained in the processes at 30 ºC and room temperature, similar results were observed – increase of the number of viable cells for 0.1, 15 and 25 MPa and decrease a 35 MPa. Only one difference was observed for the results at 15 MPa, where the number of viable cells was higher than the control sample since the first cycle at 30 ºC, while this was only observed in the last cycles at room temperature. This

difference may be explained by the lower process temperature that hinders the microbial growth. Furthermore, a lower number of viable cells was achieved for the cycles at room temperature, when compared to the process at 30 ºC.

Therefore, the results obtained for microbial growth along with the ones for bioethanol production at room temperature revealed that it is possible to induce the adaptation of *S. cerevisiae* to sub-lethal levels of pressure (15 and 25 MPa) in these conditions. For both pressures, an increase in the microbial load as well as in the production of bioethanol were observed, which may be related to a possible adaptive mechanism. The results obtained for the production of bioethanol at room temperature were less promising than at 30 ºC, since a lower final concentration of bioethanol was obtained in this case. Nevertheless, since the difference in concentration of bioethanol was near 2 $g.L^{-1}$ at 25 MPa (the pressure with higher concentration of bioethanol), the fact that at room temperature the process possesses less energy consumptions could be beneficial in the end of the process.

4.4.6. Kinetic parameters at room temperature

4.4.6.1. Yield of production of bioethanol

Table 11 is a representation of the yield of bioethanol production after each of the four fermentative cycle for each of the pressures tested at room temperature.

Table 11 Yield of bioethanol production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Yield of bioethanol production				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.355	0.353	0.368	0.0794
Cycle 2	0.426	0.368	0.470	-
Cycle 3	0.473	0.354	0.427	-
Cycle 4	0.490	0.352	0.488	-

Regarding the yield of bioethanol production for 0.1 it can be seen that it enhances throughout the fermentative cycles from 0.355 $g.g^{-1}$ in the 1st cycle to 0.490 $g.g^{-1}$ in the last meaning that, for these groups, *S. cerevisiae* is adapting it capability to produce bioethanol enhancing its production with the diminution in substrate consumption.

Comparing the results of the experimental yield with the theoretical yield for the last cycle of 0.1 MPa it was obtained a 95.4 % yield meaning that almost all the glucose consumed was used to produce bioethanol. For 15 MPa the yield of bioethanol production is lower than the control group, 0.352 $g.g^{-1}$ at 15 MPa compared to 0.490 $g.g^{-1}$ at 0.1 MPa, meaning that at this pressure it is necessary use more substrate to produce the same concentration of bioethanol produced at 0.1 MPa. At 15 MPa the yield of bioethanol was similar for the four cycles, having its maximum in the 2nd cycle of 71.7 % (**Appendix F, table F1**). For 25 MPa from the first to the last cycle it can be observed a 23.3 % increase of bioethanol production which is lower when compare to the increase of 26.2 % obtained for 0.1 MPa. However, in the last cycle the yield was very similar, 95.0 % for 25 MPa compared to 95.4 % for 0.1 MPa. Since the productivity of bioethanol was similar to the one for 0.1 MPa, 25 MPa is the most suitable pressure to enhance the capacity of *S. cerevisiae* to produce bioethanol at room temperature. Finally, for 35 MPa the results are near zero since almost no glucose is being consumed as well as almost no bioethanol is being produced. At this pressure, cells are unable to adapt and die.

Comparing the results of the yield of bioethanol production at room temperature with the results at optimal conditions (30 ºC) it can be observed that the results were quite similar, this is, for both temperature tested, at 0.1 MPa the yield enhances thought out the cycles, for the lower pressures (15 and 25 MPa) the results were more variable not following a straight pattern and for 35 MPa for both temperatures it can be observed a decrease in the yield of bioethanol production. Despite the similarities it can be observed that the results for optimal conditions, as it was expected, were higher meaning that for the same pressure and same fermentation cycle at 30 ºC more bioethanol is produced per molecule of glucose. Furthermore, the results for the production of bioetanol presented above give similar conclusions once the final concentration of bioethanol is higher at 30 ºC making this the more indicated temperature when the objective is to enhance bioethanol production. Nevertheless, it cannot be discarded the utilization of room temperature since this test shows promising results and its more environment friendly.

4.4.6.2. Yield of production of formic acid

Table 12 is a representation of the percentage of the yield of formic acid production after each of the four fermentative cycle for each of the pressures tested at room temperature.

Table 12 Yield of formic acid production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Yield of formic acid production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.083	0.053	0.053	0.079
Cycle 2	0.085	0.058	0.154	0.046
Cycle 3	0.090	0.056	0.078	0.027
Cycle 4	0.093	0.054	0.067	0.012

Regarding the yield of formic acid production for 0.1 MPa and 15 MPa it can be seen that the results are almost constant meaning that for this two tests the production of formic acid per unit of glucose is almost constant (a slight increase is verified is the atmospheric group), not being observed a significant adaptation for the production of formic acid. At 25 MPa, the results of the yield of production of formic acid at this pressure were lower for three of the fermentative cycles when compared to the control group. The second yield may result from an experimental error. At this pressure, less formic is being produce for molecule of glucose consumed (when compared to the control group which could result in an increase in the production of bioetanol). Having in consideration the results of 15 and 25 MPa it can be said that lower levels of pressure can decrease the production of formic acid when compared to atmospheric pressure. For 35 MPa, the yield of formic acid is decreasing, from 0.079 $g.g^{-1}$ in the first cycle to 0.012 $g.g^{-1}$ in the last cycle. That is, during the cycles at 35 MPa there is a decrease in the use of glucose for the production of formic acid.

Comparing the results for the yield of production of formic acid at both temperatures the results were very similar. For both temperatures at each pressure the patterns followed were the same and since the yield is minimal it can be concluded that the production of formic acid per molecule of glucose thus not represent an inhibitor of alcoholic fermentation.

4.4.6.3. Yield of production of acetic acid

Table 13 is a representation of the yield of acetic acid production after each of the four fermentative cycle at room temperature for each of the pressures tested.

Table 13 Yield of acetic acid production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Yield of acetic acid production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.0129	0.0064	0.0117	0.0281
Cycle 2	0.0143	0.0053	0.0143	0.0225
Cycle 3	0.0146	0.0064	0.0146	0.0191
Cycle 4	0.0160	0.0057	0.0160	0.0128

Regarding the results of the yield of acetic acid production it can be observed for 0.1 and 25 MPa a slight increase from 0.0129 $g.g^{-1}$ to 0.0160 $g.g^{-1}$ at 0.1 MPa and from 0.0117 $g.g^{-1}$ to 0.0160 $g.g^{-1}$ at 25 MPa. For 35 MPa a minor decrease in the yield of acetic acid from 0.0281 $g.g^{-1}$ to 0.0128 $g.g^{-1}$ can also be observed. The results found for 15 MPa were more variable not following a specific pattern. Nevertheless, for every condition testes the yield of production of acetic acid as very low in every cycle being lower for 15 MPa than the rest of the tests with a maximum of 0.064 $g.g^{-1}$, meaning that the production of acetic acid per unit of glucose is very low.

Regarding the results for both temperatures it can be observed that they are very similar. For all of the pressure tested the results for each of them were similar between temperatures being the yield of production of acetic acid minimum not presenting that way an inhibitor of alcoholic fermentation.

4.4.6.4. Yield of biomass production

Table 14 is a representation of the yield of biomass production after each of the four fermentative cycle for each of the pressures tested.

Table 14 Yield of biomass production by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Yield of Biomass production ($g.g^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.381	1.63	1.57	0.102
Cycle 2	0.351	1.71	1.65	0.0802
Cycle 3	0.394	2.06	2.01	0.0414
Cycle 4	0.409	3.03	3.01	0.0201

About the results of yield of biomass production for 0.1 MPa it can be observed, with the exception of the second cycle, an increase in the yield of biomass from 0.381 $g.g^{-1}$ in the 1st to 0.409 $g.g^{-1}$ in the last. A similar tendency to increase was obtained for the lower pressure tests (15, 25 MPa). For this two pressures the yield of biomass production was much higher when compared to the control group. These results allow to conclude that for each of these pressure tests (15 and 25 MPa) through the fermentative cycles the cells from *S. cerevisiae* produces a higher concentration of biomass per molecule of glucose consumed which represents an attempt of this microorganism to survive and adapt to the new conditions. For 35 MPa, the results are very different from the rest of the tests. At this pressure it can be verified a decrease in the yield of biomass production from 0.102 $g.g^{-1}$ in the 1st to 0.0201 $g.g^{-1}$ in the last. At this pressure and temperature *S. cerevisiae* was not able to produce a significant amount of biomass.

Taking into account the results for the yield of production of biomass for both temperatures, similar results were obtained at 0.1, 15 and 25 MPa. For 35 MPa the results were opposite. At 30 ºC an increase in the biomass yield of notice and at room temperature the biomass yield decreased.

4.4.6.5. Productivity and specific productivity of bioethanol

4.4.6.5.1. Bioethanol productivity

At 0.1 MPa, in the first three cycles, it can be observed an enhancement in the productivity of bioethanol being at the third cycle the highest (0.0864 $g.L^{-1}.h^{-1}$) (Table 15). However, for the last cycle a reduction in productivity is noticeable for both (from 0.0864 $g.L^{-1}.h^{-1}$ to 0.0652 $g.L^{-1}.h^{-1}$). For both 15 and 25 MPa, differently to what was verified for 0.1 MPa, the highest productivity was in the second cycle (0.0942 $g.L^{-1}.h^{-1}$ for 15 MPa and 0.115 $g.L^{-1}.h^{-1}$ for 25 MPa). From that, at 15 MPa it can be observed a decrease in productivity having the lowest value at the end of the four cycles of 0.0676 $g.L^{-1}.h^{-1}$. At 25 MPa, the productivity increased contrary to what it was verified for 0.1 and 15 MPa having the maximum productivity been obtained in the last cycle of all the pressure tested. Finally, the productivity for both the lower pressures (15 and 25 MPa), at the end of the four cycles was higher than at atmospheric pressure, which means that at this pressure, more bioethanol was produced per litter of medium per hour. As previously indicates, for 35 MPa the only cycle with bioethanol production was the first one (**section 4.3.2**) and, because of that, only the first cycle shows specific productivity. At this pressure bioethanol specific productivity was very low (0.000456 $g.L^{-1}.h^{-1}$).

Table 15 Bioethanol productivity by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Productivity of bioethanol, Q ($g.L^{-1}.h^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.0737	0.0902	0.0892	0.00456
Cycle 2	0.0844	0.0942	0.115	-
Cycle 3	0.0864	0.0905	0.0432	-
Cycle 4	0.0652	0.0676	0.0781	-

4.4.6.5.2. Specific bioethanol productivity

The results of bioethanol specific productivity at room temperature (Table 16) showed similar behaviours when compared to the results of bioethanol productivity (**section 4.4.6.6.1**). The results of specific productivity for 15 MPa were, in every cycle, higher than the control group, meaning that at this pressure, per hour, more bioethanol is produced per gram of biomass. For 25 MPa the lower result was obtained in the third

cycle (0.00723 $g.g^{-1}.h^{-1}$), which can be related to the lower glucose consumption verified (**section 4.4.1**). In the last cycle, it can be noticed an enhancement in the specific bioethanol productivity at room temperature compared to the third cycle. Comparing the results of the last fermentative cycle at 0.1, 15 and 25 MPa, it can be observed that 25 MPa possesses the higher specific productivity. For 35 MPa the only cycle where bioethanol was produced was in first one (**section 4.3.2**) and because of that only the first cycle possesses productivity results.

Comparing the results of the specific productivity of bioethanol at room temperature with the results at 30 ºC similar variations were obtained. Furthermore, at room temperature it can be observed a slightly lower value in the specific productivity for all tests meaning that at optimal conditions a higher quantity of bioethanol was produce per molecule of glucose. However, the differences obtained for this specific productivity were low meaning that the use of room temperature is a possibility to enhance the productivity of bioethanol.

Table 16 Bioethanol specific productivity by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, room temperature

Specific productivity of Bioethanol, q ($g.g^{-1}.h^{-1}$)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	0.00799	0.0168	0.0174	0.00177
Cycle 2	0.00983	0.0172	0.0217	-
Cycle 3	0.00914	0.0146	0.00723	-
Cycle 4	0.00659	0.00683	0.0100	-

4.4.6.6. Ratio of bioethanol:sub-products of fermentation at room temperature

Regarding the results of the ratio of bioethanol:sub-products of fermentation at 0.1 MPa it can be observed that they were similar throughout the fermentation cycles having a maximum of 4.53 in the 3rd fermentation cycle (Table 17). The results at both 15 and 25 MPa were more variable. For the first pressure, it can be observed that the highest ratio is in the 1st cycle. For 25 MPa, the maximum value for the ratio is 6.50 and it was obtained for the second cycle. For 35 MPa the only cycle where bioethanol was produced was in first one (**section 4.4.4.2**). At this pressure the ratio was lower than 1

(0.331) which means that for this pressure more sub products were produced when compared to the production of bioethanol.

Comparing the results of the ratio of bioethanol: sub-products of fermentation at room temperature with the results at optimal conditions (30 ºC) it can be seen that for room temperature the results were more variable which could be expected since no temperature control was applied and as indicated before, temperature varied from 18.2 to 31.3 ºC. It can be observed that the results for optimal conditions, as it was expected, were higher meaning that for the same pressure and same fermentation cycle at 30 ºC more bioethanol is produced. Furthermore, the results for the ratio of bioethanol: sub-products of fermentation presented above (**section 4.3.2**) give similar conclusions since the final concentration of bioethanol is higher at 30 ºC making this the more indicated temperature when the objective is to enhance bioethanol production. Nevertheless, it cannot be discarded the utilization of room temperature once this test shows promising results and its more environment friendly.

Table 17 Ratio of bioethanol: sub-products of fermentation after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

Bioethanol ratio at room temperature				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	4.36	6.13	5.85	0.71
Cycle 2	4.51	5.94	6.50	-
Cycle3	4.53	5.76	2.30	-
Cycle 4	4.43	5.96	5.36	-

5. Conclusions

Currently, there are no published studies concerning the effect of consecutive cycles of sub-letal levels of pressure on *S. cerevisiae* and its effect on adaptation and the production of bioethanol by this microorganism under pressure. On this regard, the present work analysed several physicochemical and microbiological parameters, in order to observe the effect of temperature (30 ºC and room temperature) and pressure (15, 25, 35 and 50 MPa) in alcoholic fermentation.

Firstly, in order to determine the most suitable HP conditions to use on these experiments, a first study of *S. cerevisiae* fermentation was tested under different pressure levels (0.1, 15, 25, 35 and 50 MPa). The results of this preliminary study demonstrated that pressure levels of 15 and 25 MPa had a similar consumption in glucose and production of bioethanol and that for 35 and 50 MPa both consumption of glucose and production of bioethanol were minimal.

After this, it was decided to analyse the same fermentation parameters using two different temperatures of fermentation: the temperature stated as optimal in literature (30 ºC); and room temperature. Furthermore, samples were also subjected to four consecutive fermentative cycles of fermentation in order to infer about the possible adaptation of *S. cerevisiae* to those sub-lethal levels of pressure. Those four consecutive cycles were used in order to promote a particular stress level within each cycle allowing in some cases adaptation. For both temperatures used, it was concluded that the fermentation monitoring parameters (consumption of glucose and other substrates, production of bioethanol, formic acid and acetic acid, biomass and microbial load) were clearly affected by HP and the use of consecutive fermentative cycles. With the increasing pressure, a lower variation in the consumption of glucose was observed over time being minimal at 35 MPa. Regarding the production of formic acid, acetic acid and bioethanol the results proved that some levels of pressure can enhance their production. At 15 MPa and 25 MPa for the first cycles it was noticed that the fermentation rate was lower when compared to atmospheric pressure. However, for both pressure tests, in the last cycles the fermentation rate was higher than at 0.1 MPa and, in consequence, the consumption of glucose and the production of bioethanol were higher which means that at 15 and 25 MPa the use of consecutive cycles of fermentation allows the adaptation of this microorganism to those levels of stress, and, furthermore, it enhances production of bioethanol. At higher

pressures, 35 MPa (and 50 MPa used in the preliminary tests) it became harder to adapt and ultimately the fermentation process ceased. These results show that lower levels of pressure (15 and 25 MPa) enhanced the fermentative process and the production of bioethanol while higher levels caused the slowdown of alcoholic fermentation, probably due to the inhibition of metabolic activity of *S. cerevisiae*. Finally, in the end of each of the fermentative cycles the number of viable cells was determined and it was verified for both the temperature tested an enhancement in the number of viable cells for 0.1, 15 and 25 MPa which means that *S. cerevisiae* was able to resist to those levels of pressure, adapt and grow increasing even the number of viable cells. For 35 MPa the number of viable cells in both temperatures decreased meaning that for this pressure cells were not able to adapt and died.

Through the calculation of fermentation kinetic parameters such as the glucose consumption (%), the yield of production of bioethanol, formic and acetic acid and biomass and the productivity and specific productivity of bioethanol, it was possible to confirm the results obtained for the previous tests. Analysing the results of those yields it was possible to conclude that, in general, the reactions involved in alcoholic fermentation are slowdown by higher pressures (35 MPa) and accelerated by lower pressures (15 and 25 MPa). Those results are supported by the increase in the yield of bioethanol production and by the increase in the productivity and specific productivity of bioethanol that occur throughout the four fermentative cycles for those lower pressures. In general, it was noticeable that for the 1st fermentative cycle the kinetic parameters were lower for the pressure tests when compared to 0.1 MPa. However, throughout the fermentative cycles the kinetic parameters increase for lower pressures, with exception of the yield of formic and acetic acid, that decrease (as it was desirable), having in the last cycle higher values than atmospheric pressure. For 15 and 25 MPa, the production of sub-products of fermentation is slowdown as it was observed for the yield of formic and acetic acids and for the ration bioethanol: sub-products of fermentation. Finally, for samples fermented at 30 ºC, comparing the values of the theoretical yield of bioethanol with the experimental it can be observed that for both 15 and 25 MPa the results were higher than the control group. For atmospheric pressure the yield in the last cycle as 76.9 % of the theoretical yield while for 15 MPa was 83.1 % and for 25 MPa was 102.3 % of the theoretical yield.

Additionally, as it was mentioned above, this study was performed at two different temperatures. Using room temperature during fermentation would eliminate the need to

provide energy during the process reducing the ecological foot print of the process. Bioethanol itself can be considered a “clean” source of energy due to its reduction in the final carbon dioxide production when compared to fossil fuels. However, if this compound could be produced in higher amounts using HP and not needing to use additional sources of energy to control the process temperature it would be an important improvement for the process. Taking into account the results obtained for the room temperature tests it can be concluded that this condition was not as effective as the fermentation cycles at 30 ºC, however the results are still promising for fermentation at room temperature at both 15 and 25 MPa. For these pressures the production of bioethanol in the end of the fourth cycle was higher than for the control group, resulting in higher bioethanol yields and productivities.

6. Future prospects

The results obtained in the present work allowed a detailed analysis of the effects of the use of consecutive fermentative cycles under pressure and temperature in the alcoholic fermentation process in *S. cerevisiae*, however, new combinations of pressure and temperature could be tested in order to carry out a more complete kinetic analysis and to ascertain the which of the combinations of pressure and temperature would better improve the adaptation and having a higher production of bioethanol. Furthermore, different fermentative cycles could be also used, *i.e* more cycles or longer cycles. In an upcoming phase, to corroborate the results obtained during this study it could be used the new adapted strain of *S. cerevisiae* and perform a new fermentation under the same fermentative cycles conditions to confirm if it could be obtained better results for 25 MPa at both 30 ºC and room temperature.

It will be appropriate to carry out a study of the metabolome in order to understand the effects of the used of consecutive fermentative cycles under pressure on *S. cerevisiae* since this microorganism may use different metabolic pathways in an attempt to adapt to pressure, producing different metabolites during the process. These possible changes in the metabolic profile of the fermentative strain when compared to the one obtained for the use of one fermentative cycle under pressure and, in turn, it will have an impact on the alcoholic fermentation as well as in the final products present in the medium. Furthermore, perform microscopic analysis (infrared microscopy, scanning electron microscopy and transition electron microscopy) in order to verify the morphological alteration in *S. cerevisiae* after the consecutive cycles under pressure as well as perform genetic analysis in order to better understand the effects of pressure in the genome of *S. cerevisiae* more specifically, better understand the effects of this levels of pressure after consecutive cycles in some important enzymes during fermentation (*e.g.* phosphofructokinase and alcohol dehydrogenase).

7. References

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8. Appendix

8.1. Appendix X

8.1.1. Fructose consumption

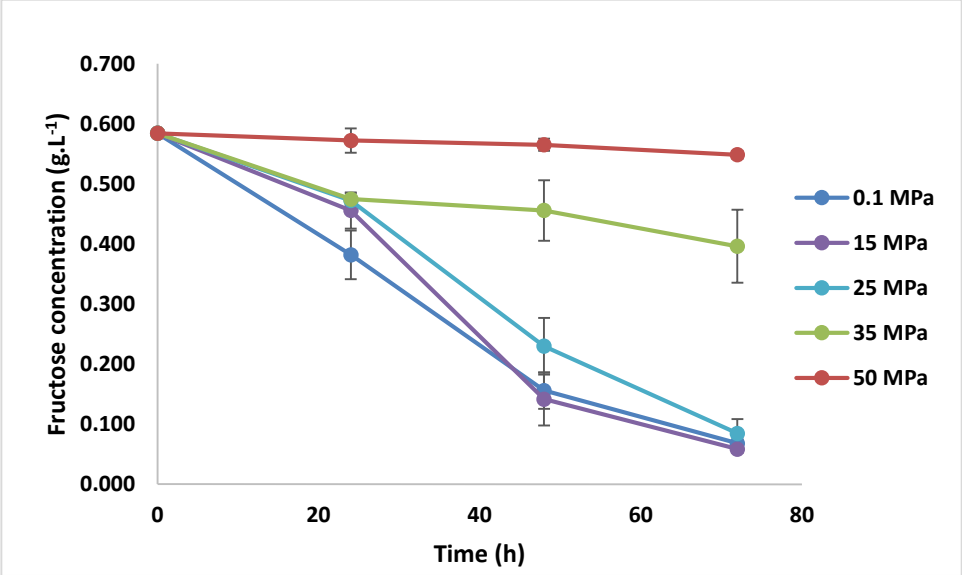

Figure X1 Fructose consumption over time, for fermentation by *S. cerevisiae* under different pressure conditions and at 30 ºC

In this case, it was possible to observe that the general tendency of the results was the decrease of fructose concentration over time, with exception of fermentation at 50 MPa, where no variation occurs. These results are consistent with those observed for the consumption of glucose (Figure 17). With the increasing pressure, the fructose consumption tended to decrease, suggesting, once more, that pressure leads to a slower fermentative metabolism. For instance, at 35 MPa, the variation of fructose concentration over time was much lower than at atmospheric pressure, thus the final concentration of fructose was markedly different in both cases. As previously stated, no fermentative activity was registered at 50 MPa with fructose concentration remaining practically stable over time, indicating, once more, that *S. cerevisiae* was inhibited or destroyed by the harsh conditions. In contrast, a similar decrease in the concentration of fructose over time was observed for fermentations at 15 and 25 MPa and at atmospheric pressure.

8.1.2. Consumption of maltose

Like fructose, maltose is not the main energy source of *S. cerevisiae* during fermentation, being also present in low quantities in the samples tested. Figure X2 shows the variation in the concentration of maltose over the fermentation time. In this case, the general tendency of the results also corresponds to a decrease in maltose concentration over time, with exception of samples subjected to 35 MPa and to 50 MPa.

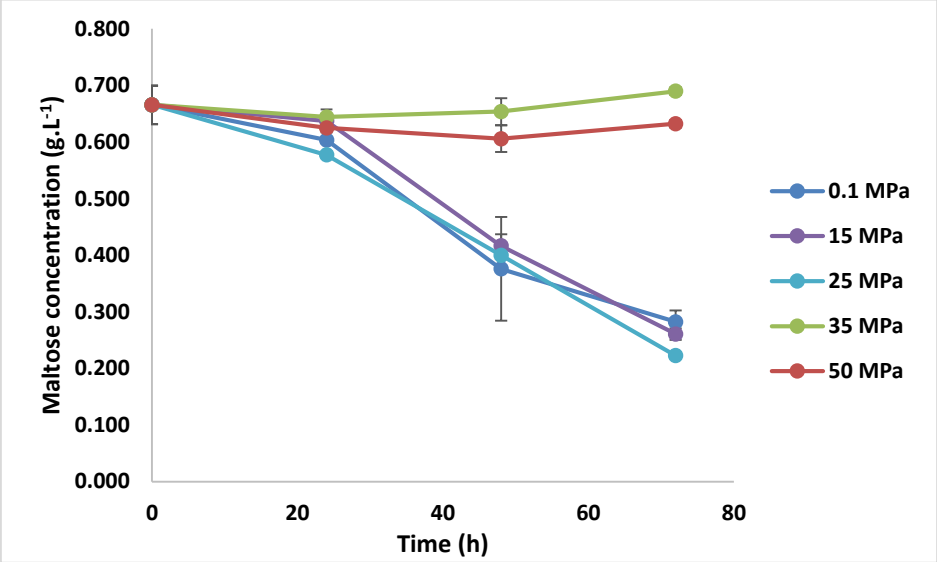

Figure X2 Maltose consumption over time, for fermentation by *S. cerevisiae* under different pressure conditions and at 30 ºC

Through the analysis of Figure X2, it is also possible to observe that the variation in the concentration of maltose is very similar for fermentations at 0.1 MPa, 15 and 25 MPa, which seems to indicate that those pressure levels don't have a considerable effect in the consumption of this sugar by *S. cerevisiae*. For both 35 and 50 MPa the final concentration in maltose is similar to the initial concentration, which indicate, once more, that the fermentation process was inhibited at these higher pressures and no consumption of substrates occurred.

8.2. Appendix A

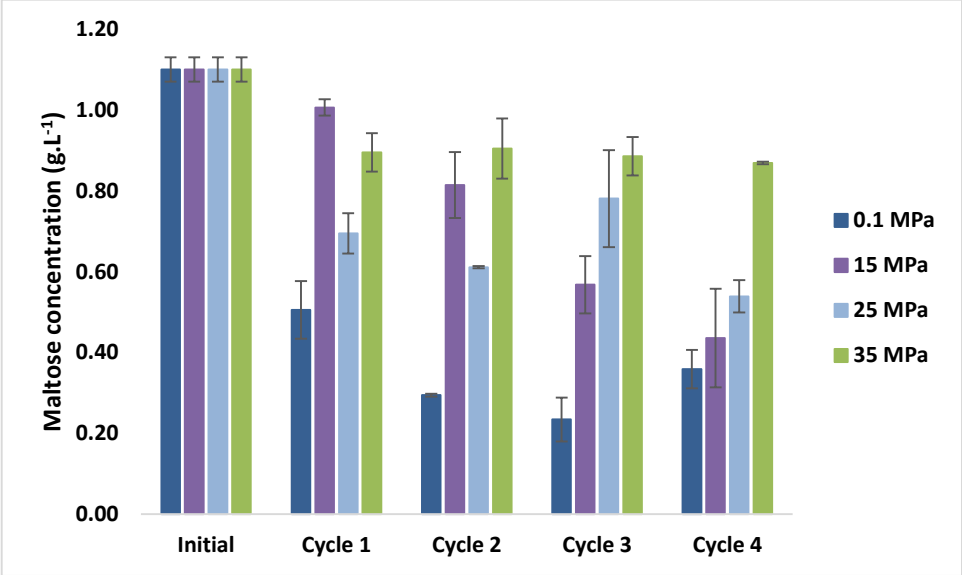

Figure A1 Final concentration of maltose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30ºC

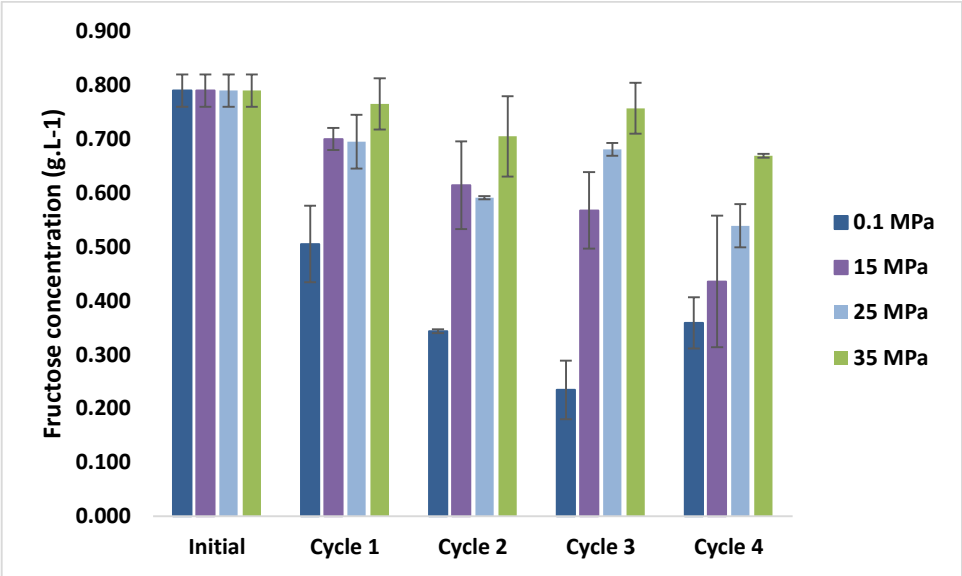

Figure A2 Final concentration of fructose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30ºC

8.3. Appendix B

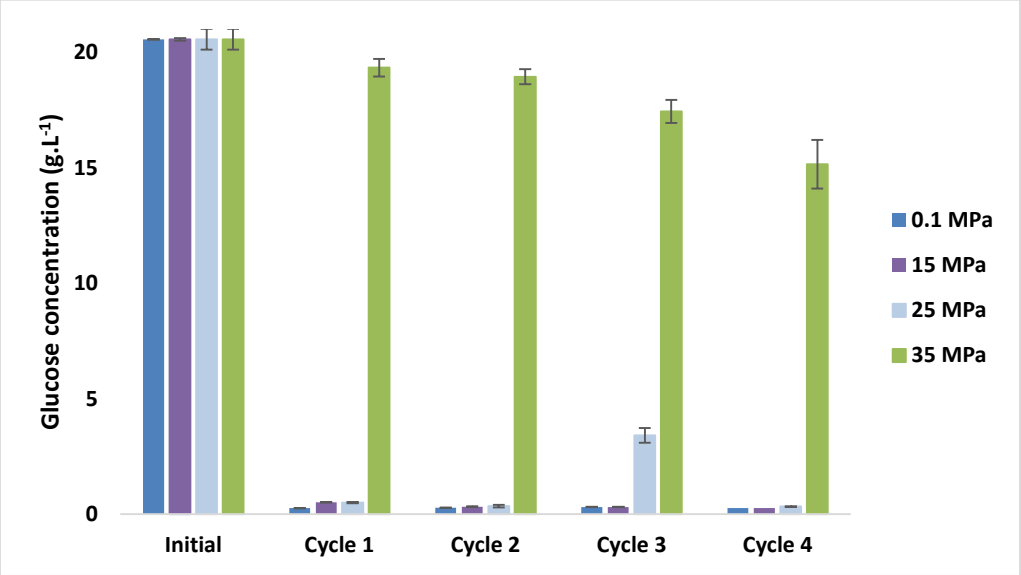

Figure B1 Final concentration of glucose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30°C

8.4. Appendix C

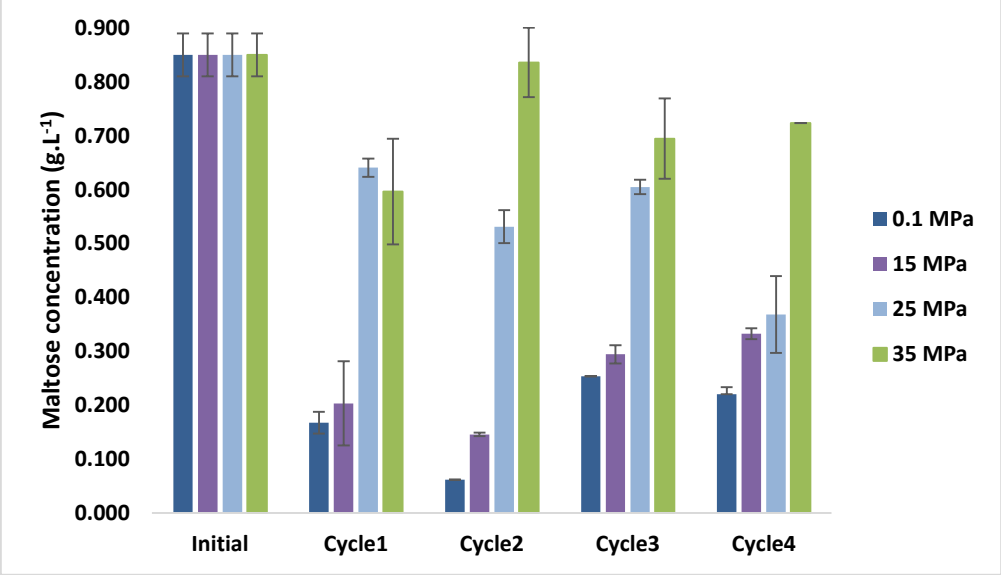

Figure C1 Final concentration of maltose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

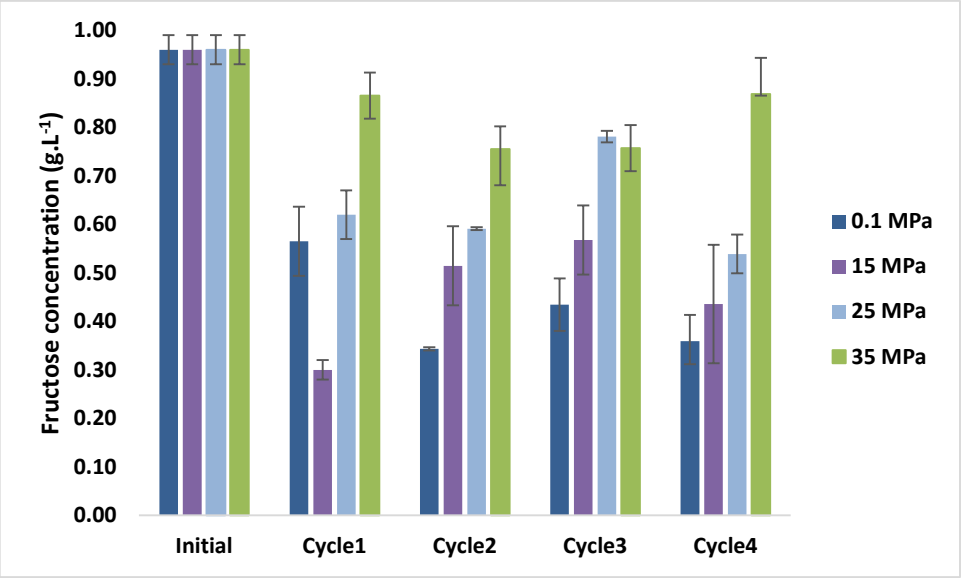

Figure C2 Final concentration of fructose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

8.5. Appendix D

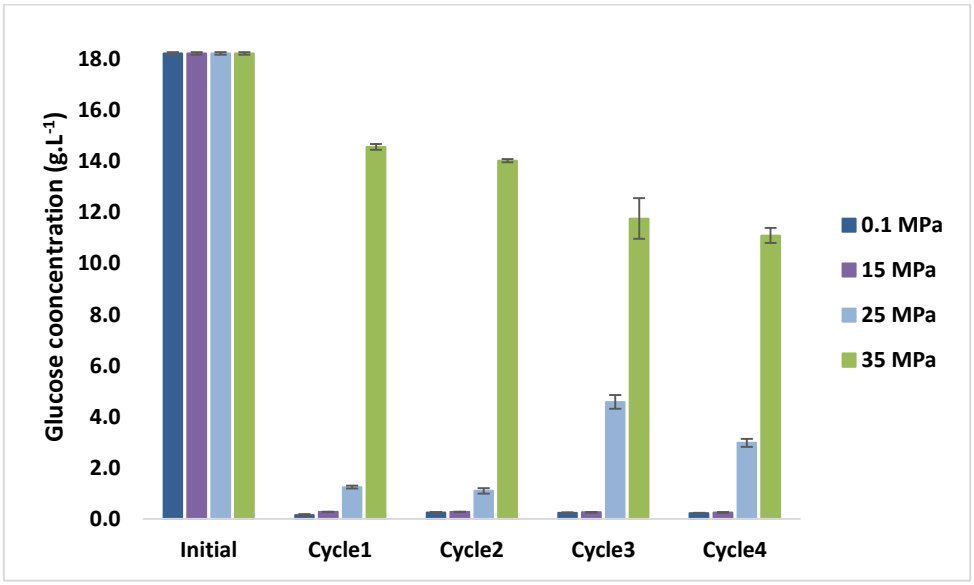

Figure D1 Final concentration of glucose after each of the four cycles of pressure and the four cycles at 0.1 MPa, at room temperature

8.6.Appendix E

The maximum theoretical ethanol yield from glucose was calculated according to the stoichiometric relationship represented by equation F1, *i.e.*, 100 g of glucose could theoretically produce 51.1 g of ethanol (Zhang et al., 2015).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (1)$$

Table E1 Comparison between the experimental yield of bioethanol production with the theoretical yield (%), at 30 ºC

Experimental/ theoretical yield (%)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	62.0	33.4	64.5	18.3
Cycle 2	65.6	63.7	81.0	-
Cycle 3	72.2	78.0	80.0	-
Cycle 4	76.9	83.9	102.3	-

8.7. Appendix F

Table F1 Comparison between the experimental yield of bioethanol production with the theoretical yield (%), at room temperature

Experimental/ Theoretical yield (%)				
	0.1 MPa	**15 MPa**	**25 MPa**	**35 MPa**
Cycle 1	69.2	68.6	71.7	15.5
Cycle 2	82.9	71.7	91.4	-
Cycle 3	92.2	68.8	83.1	-
Cycle 4	95.4	68.5	95.0	-

8.8. Appendix G

Table G1 Maximum and minimum temperatures reached in the room temperature test (Max- maximum temperature and Min- minimum temperature)

Temperatures for the room temperature test (ºC)								
	0.1 MPa		15 MPa		25 MPa		35 MPa	
	Max	Min	Max	Min	Max	Min	Max	Min
Cycle 1	28.0	18.2	28.0	19.2	29.4	19.1	29.9	20.1
Cycle 2	29.2	20.6	29.2	18.6	31.3	20.2	30.8	19.5
Cycle 3	30.7	18.4	30.7	19.4	30.9	18.9	29.4	18.4
Cycle 4	29.4	19.1	29.4	19.1	29.6	19.2	29.5	19.4