Chapter 9

High Hydrostatic Pressure Process to Improve Ethanol Production

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http://dx.doi.org/10.5772/intechopen.78712

Abstract

The use of high hydrostatic pressure (HHP) is an interesting approach to optimize the production of both first- and second-generation ethanol. It may be applied on *Saccharomyces cerevisiae* cells to enhance the fermentation pathway and on the lignocellulosic biomass to increase sugar release. HHP has a wide effect on many biological processes, such as growth, division and cellular viability. Actually, conformation, stability, polymerization and depolymerization of proteins are affected by HHP as well as lipid packaging. Moreover, transcriptional profile analysis indicates an activation of the general stress response. In yeast, HHP higher than 100 MPa leads to significant morphological and physiological alteration, and loss of cellular viability occurs over 200 MPa. A yield rate increase in ethanol production occurs at pressures of 10–50 MPa, but over 87 MPa alcoholic fermentation is interrupted.

Keywords: *Saccharomyces cerevisiae*, high hydrostatic pressure, fermentation, stress, ethanol productivity

1. Introduction

Ethanol has a long history as an alternative fuel, and nowadays, it is the most widely used biofuel in the transportation sector [1]. Since the 1980s, the interest in using bioethanol has been increasing, and it is currently used in many countries. Bioethanol can be categorized into three groups depending on the feedstock used to obtain it. First-generation bioethanol is

produced from feedstock rich in sucrose (sugarcane, sugar beet, sweet sorghum, and fruits) or starch (corn, wheat, rice, potato, cassava, sweet potato, and barley). Second-generation bioethanol is obtained from lignocellulosic biomass such as wood, straw, bagasse, grasses and other agricultural residues. Third-generation bioethanol derives from algal biomass including microalgae and macroalgae.

Microorganisms such as yeasts play an essential role in bioethanol production by fermenting a wide range of sugars to ethanol. They have been used for thousands of years for beer brewing and are probably the oldest domesticated organism [2]. Current industrial ethanol fermentation is mainly carried out with the yeast *Saccharomyces cerevisiae* because of its hardiness, low pH, and high ethanol tolerance, thus making the process less susceptible to contamination [1, 3]. Wild *S. cerevisiae* strains are able to survive and dominate alcoholic fermentation vats, which pass through phases of high sugar content, high temperature, CO_2 pressure, being considered, therefore, inhospitable environments [4]. Thus, a yeast strain with multiple stress resistance is a desired attribute [5].

Many microbial communities are adapted to live and survive on extreme environmental conditions including high hydrostatic pressure (HHP). It is known that high hydrostatic pressure induces changes in proteins, enzyme conformation and aggregation, interaction between lipids and proteins, gene expression and cell structures that are composed of lipids such as biological membranes [2]. It has already been shown that high hydrostatic pressure exerts a broad effect in *S. cerevisiae* with results similar to those of other common stresses, such as temperature, ethanol, and oxidative stresses [6]. Moreover, *S. cerevisiae* produces ethanol faster at high pressure when compared to ambient pressure, proving HHP as a tool to enhance ethanol production [7].

In biotechnology industry, one of the oldest and most important fermentation processes used is the ethanol fermentation. Ethanol is the most consumed biofuel in the world, and Brazil was the first country that introduced it in its energy matrix, holding the most economically viable process for its production. It was for decades the largest producer, losing that position to the United States, but it remains the largest exporter of ethanol [4]. About 4.5 billion gallons of ethanol are produced annually from corn and used as a transportation fuel only in the United States. The annual bioethanol production in the U.S. is expected to grow to more than 7.5 billion gallons in the next few years and reach 30 billion gallons by 2025 [3].

This chapter approaches the interaction between HHP and ethanol production by *S. cerevisiae*, describing the main HHP effect in yeast, linking this knowledge to further improvement of ethanol production efficiency.

2. Fermentation process

2.1. Bioethanol production

S. cerevisiae cells under anaerobic conditions undergo alcoholic fermentation; a process that convert monosaccharides (sugars) to ethanol, carbon dioxide and heat. Basically, one molecule

of glucose yields two molecules of ethanol and two molecules of carbon dioxide as shown in **Figure 1** [8]. Baking, brewing and fuel industries rely on this ability of the yeast *S. cerevisiae* to convert glucose into ethanol and carbon dioxide. The fermentation process may present multiple stress conditions such as temperature, ethanol concentration, pressure, desiccation, acidity or alkalinity, osmotic and ionic stress and low oxygen levels (**Figure 2**). Therefore, *S. cerevisiae* has been chosen over the centuries for being physiologically adapted to them [9].

To enhance yeast growth for fermentation, usually, nutritional salts, vitamins, fermentation inducers and inhibitors, precursors, acids, antifoams are added. Then, in the bioreactor, time is given for yeast duplication until the desired cell concentration is reached. Bioreactor is a tool used in yeast bioprocesses, and it is, often, a stirred tank. It separates the internal environment from the external one so anything entering or leaving the fermentation is monitored. Therefore, its use leads to a higher production and productivity of the intended product due to its capacity to easily control and module the chemical and physical conditions [2].

2.2. Exposure and response to different stresses

Yeasts are free-living microorganisms and therefore need to have mechanisms for rapid adaptation to environmental changes. Upon fermentation in the bioreactor, yeasts are subjected to changes in temperature, ethanol concentration, osmotic pressure, pH, and oxygen level [10, 11]. *S. cerevisiae* presents a general response to changes in the environment disregarding the kind of stress, and there is also a gene expression regulation specific for each stress; therefore, it is controlled by each new condition [12].

Osmotic stresses occur at the beginning of the fermentation and decrease with the gradual reduction of sugar in the medium, leading to alterations in the cell metabolism and viability decrease [13]. Osmotic stress causes a rapid loss of the cell actin filaments, perturbation on the cell membrane structure, permeability and mechanical properties, besides the expected loss

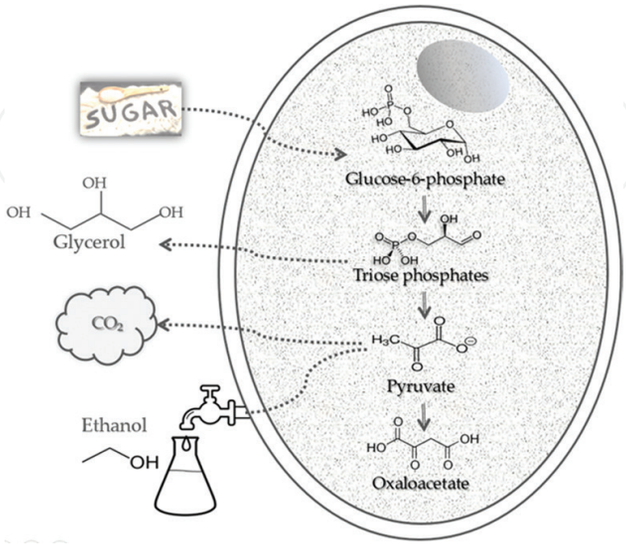

Figure 1. Carbohydrate or sugar or monosaccharide metabolism in yeast under anaerobic conditions.

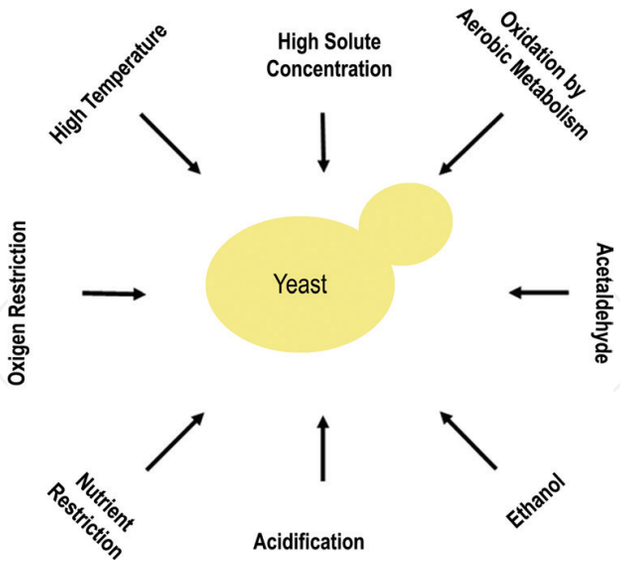

Figure 2. Stresses suffered by yeast during fermentation process.

of water and shrinking of the cell, and, as for most stresses, G1 arrest [9, 14]. Then, during the adaptation phase, these actin filaments are restructured, and the cell is repolarized and starts growing again [15, 16].

Temperature also has a great influence on the metabolic process and can serve as both an activator and a microbial development inhibitor, with lethal implications in some cases. Yeast optimum temperature ranges between 25 and 30°C. When cells are presented to temperatures below optimum they undergo a cold shock, while when grown in higher temperatures than the optimum leads to heat shock. Thermal stress can change proteins properties (chemical and physical), mostly protein aggregation, which triggers malfunctions in all cellular compartments. Cells submitted to thermal stress increase synthesis of the heat shock proteins (HSPs) in order to revert this situation. In *S. cerevisiae*, protein Hsp104 has a decisive role for thermotolerance, acting with Hsp70 and Hsp40 forming a protein complex, which is responsible for induction of partially denatured proteins by high temperatures to return to the native state. Yeast shows intrinsic tolerance when exposed to a sudden thermal shock (50°C) while induced thermotolerance appears when the cells are exposed to an initial moderate thermal shock followed by a severe thermal shock. Others factors can also influence thermotolerance like Ca^{2+} ions, trehalose and cellular growth phase [17].

Ethanol in low concentrations acts as an inhibitor of cell division, while in high concentrations it may lead to cell death [18]. The structure of the cell membrane is severely affected by ethanol, as well as hydrophobic and hydrophilic proteins and the endoplasmic reticulum [19]. Ethanol also causes changes in cellular metabolism, biosynthesis of macromolecules, increases DNA mutations and leads to intracellular protein denaturation, which in response induces the production of heat shock proteins (HSPs) [3]. Moreover, genes that respond to environmental stresses [environmental stresses response (ESR)] are overexpressed during ethanol stress [20]. Among those genes, a HSP group is positively regulated during ethanol stress, especially HSP12, HSP26, HSP78 and HSP104 [21].

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Cellular response to damages produced by accumulation of reactive oxygen species (ROS) is known as oxidative stress [10]. ROS are produced in larger quantities during mitochondrial respiration process. Lipid peroxidation may lead to a decrease in membrane fluidity and permeability and enzymatic inactivation. Oxidative damage in proteins may lead to formation of hydrogen peroxide and changes in molecular structure by protein aggregation or fragmentation. Another effect is the damaging of DNA structure by ROS, showing a greater influence in mitochondrial DNA [9]. The adaptive response mechanisms to oxidative stress in *S. cerevisiae* are mainly regulated by transcription factors that collectively coordinate appropriate responses to distinct oxidative stresses by repressing or regulating the transcription of specific genes, which are related to antioxidant defenses. These transcription factors are Yap1, Skn7, Msn2 and Msn4 [20, 22–25].

2.3. High hydrostatic pressure and its physical effects

The force applied on a given surface, that is, in an area unit, is called pressure. Thus, the mathematical equation that represents this phenomenon is:

$$P = \frac{F}{A} \tag{1}$$

where P represents the pressure, F represents the normal force applied to the surface, and A is the surface area. Pressure can be determined as static or dynamic. The dynamic pressure is the one in which a super high pressure is applied for a short period of time and can be associated with temperature. On the other hand, static pressure is a constant pressure value maintained for a long time. Pressure can also be classified as isostatic or nonisostatic. In isostatic, the pressure value is the same in all directions of the given space (e.g., hydrostatic pressure) while the nonisostatic pressure corresponds to a gradient of normal forces in response to pressure applied by an equipment or when there is nonuniform compression due to the inhomogeneous composition of the material.

Atmospheric pressure greatly varies on Earth. In terrestrial habitats, pressure value decreases with increasing elevation and it is close to 1 atm (0.101325 MPa) at sea level, while in the oceans at an average depth of 3800 m, pressure reaches approximately 380 atm (~38 MPa). In addition, most living organisms are below 1000 m (**Figure 3**), those organisms tolerant to high pressures are named piezophiles [26, 27].

2.4. How high hydrostatic pressure affects microorganisms?

2.4.1. General effect of HHP in microorganisms

High hydrostatic pressure (HHP) is a unique type of stress since the effect it triggers is caused only by a change in the system volume. Therefore, when compared to thermal stress which involves temperature and volume changes, the results obtained by HHP are simpler. Moreover, it is important to consider that when HHP is applied, biochemical reactions are followed by volumetric changes; therefore, if a reaction is coupled to an increase in volume, it will be inhibited by the HHP, and when associated with a decrease in volume, it can be enhanced [28, 29].

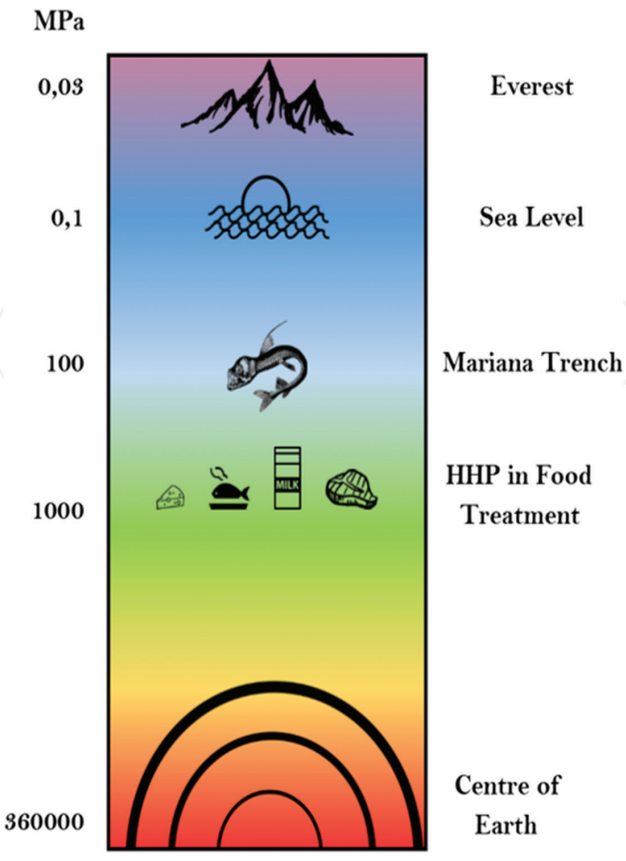

Figure 3. Pressure on Earth—variation and biotechnological use.

Studies on the effects of HHP in microorganisms mostly use the yeast *S. cerevisiae* (**Figure 4**) and the bacteria *Escherichia coli* as models. Yeast cells that are on stationary phase are more resistant to pressure when compared to cells on proliferative phase [30]. This response also occurs in prokaryotic organisms, since *E. coli* presents a 70% resistance in stationary phase after 200 MPa when compared to cells on exponential phase, which present a decrease up to 0.01% of cellular viability under the same amount of HHP [31]. High pressure induces many physiological changes in *E. coli*, such as lag phase extension, cellular filamentation and DNA, RNA and protein synthesis interruption [10, 32–34]. Both in yeast and *E. coli*, changes in membrane lipids occur, as well as the reduction of its fluidity [35].

The wide effects of HHP influence many processes in biological systems, such as growth, division and cellular viability. Depending on the amount and time that HHP is applied, the pressure acts inhibiting or retarding cytokinetic and mitotic activities in dividing cells. The conformation, stability, polymerization and depolymerization of mitotic proteins are affected by high pressure. It also induces lipid packaging, which leads to a reduction in membrane fluidity [36].

2.4.2. HHP effect on Saccharomyces cerevisiae cells

The yeast *S. cerevisiae* is a unicellular fungus that can undergo asexual and sexual reproduction. The asexual reproduction is carried out through budding and the sexual reproduction is

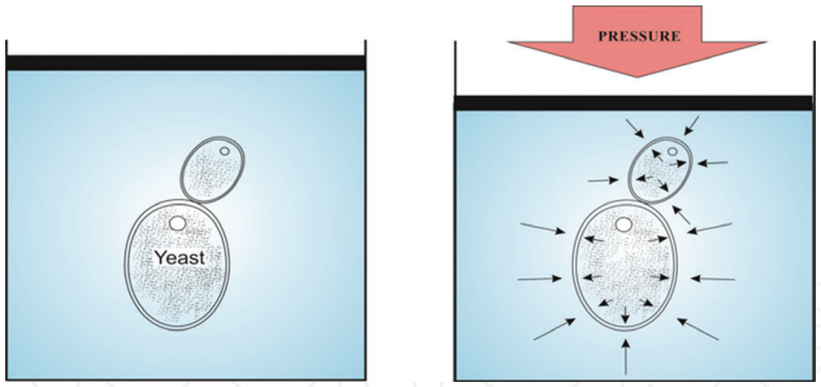

Figure 4. Yeast under high hydrostatic pressure.

through mating between cells of opposite mating type, *a* and α. Cells can grow as haploids or mate and grow in a vegetative form as diploid, or even form spores (meiosis) generating haploid gametes. *S. cerevisiae* was the first eukaryote to have its genome completely sequenced, generating the possibility to study many aspects of life [36].

In yeast, significant morphological and physiological effects are observed in pressures higher than 100 MPa, and loss of cellular viability occurs over 200 MPa [37, 38]. At 50 MPa, cell cycle arrest is induced, but it is a sublethal effect and does not affect the morphology of the cell [6, 39–41]. A yield rate increase for ethanol production is observed after 10 MPa pressurization, but higher pressure can lead to an opposite effect, interrupting the alcoholic fermentation when pressures over 87 MPa are applied (**Figure 5**) [42].

2.4.2.1. Yeast morphology under pressure

Pressure presents an interference in the structure of the cell by directly affecting the cell wall (**Figure 6**), cell membrane and its fluidity, as well as other intracellular organelles [44]. The yeast cell wall is conformed by polysaccharides (80–90%), mainly glucans and mannans and

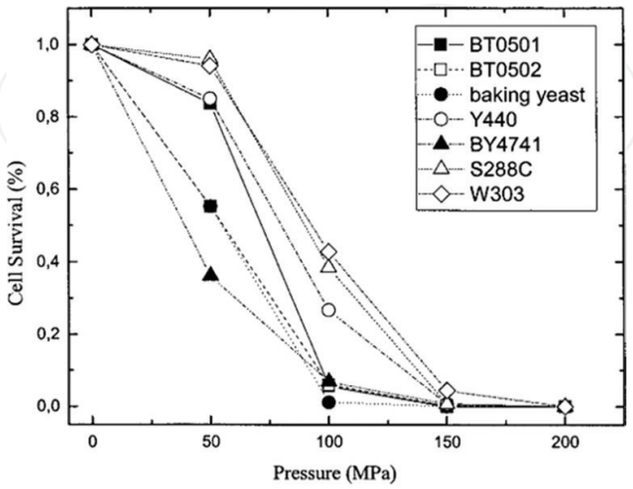

Figure 5. Effect of HHP on different wild-type yeast cells. *Saccharomyces cerevisiae* survival, expressed as percentage of viable cells, was measured on yeast cells at logarithmic phase submitted to various hydrostatic pressures for 30 min [43].

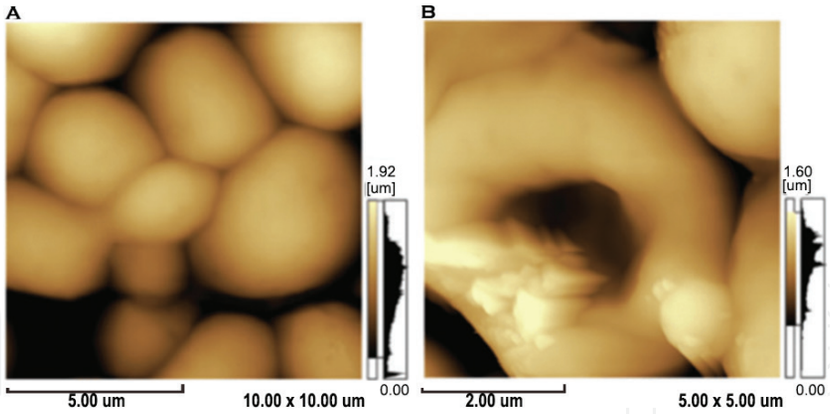

Figure 6. Atomic force micrograph of *Saccharomyces cerevisiae* wild-type cells Y440. (A) Yeast cells at atmospheric pressure. (B) Detail of a yeast cell after hydrostatic pressure treatment of 250 MPa for 30 min [43].

in a lower quantity by chitin. It presents a thickness around 100–200 nm. In nonstressed yeast cells, chitin can be seen in the neck and scars by using the fluorescence of calcofluor. Cells treated with HHP present abnormal distribution of the calcofluor fluorescence in the cell wall. Transmission electron microscopy images suggest that HHP induces alterations in the cell wall and cytoskeleton affecting the cell membrane and the dynamic of cell organelles (**Figure 7**) [41].

Another effect that pressure has is the upregulation of the gene HPS12 [45], which codifies a hydrophilic protein of 12 kDa that increases flexibility in the cell wall and the cell membrane [46, 47]. The suppression of HSP12 induces changes in the size of cells submitted to hypo and hyperosmotic stress and an increase in sensitivity to rapid pressure variations [48]. This characteristic of the HSP12 protein (HSP12p) can be observed using a model with agarose, which is a carbohydrate polymer, to represent the glucan found in the cell wall of yeasts. It was seen that adding known upregulating solutes of HSP12 to the agarose gel decreased its flexibility, but adding the HSP12p increased it. Atomic force microscopy studies suggest that HSP12p interrupts the hydrogen bond and ionic interactions between polysaccharide polymers found in the cell wall enabling more flexibility to the structure [48]. These findings suggest that high hydrostatic stress and osmotic stress affect the cell wall directly interfering with its flexibility and the cell responds by increasing the production of HSP12p.

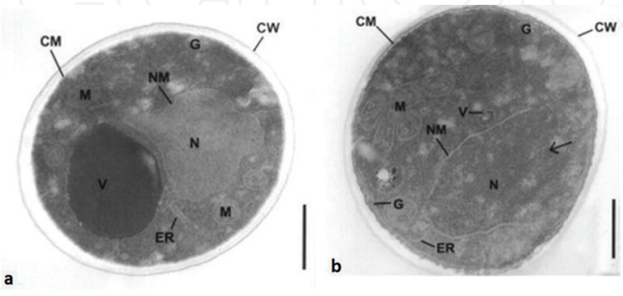

Figure 7. Transmission electron micrographs of a thin section through *Saccharomyces cerevisiae* Y440 wild-type cells. (a) Typical *S. cerevisiae* cell at atmospheric pressure. (b) Cell submitted to 200 MPa for 30 min. CM, cell membrane; CW, cell wall; NM, nuclear membrane; N, nucleus; V, vacuoles; M, mitochondria; G, Golgi apparatus; ER, endoplasmic reticulum. The bar in panel a represents 0.8 μm; the bars in panel b represent 0.5 μm [41].

http://dx.doi.org/10.5772/intechopen.78712

HHP also interferes with the structure of the cell membrane by increasing the level of arrangement of the lipids (**Figure 8**), especially in surrounding molecules as a consequence of volume reduction. This characteristic induces a decrease in the cell membrane fluidity followed by an increase in thickness [29]. The effect of pressure over the cell membrane is explained by the fact that lipids are more compressible than proteins, reason why they are more sensible to pressure [49]. To compensate pressure, there is an increase in the unsaturation of fatty acids so there is more flexibility in the membrane, and it can stay in its functional liquid-crystalline phase as unsaturated membranes have a less ordered structure than saturated bilayers. This mechanism is used by organisms that live in the bottom of the sea to adapt to the increase in pressure [29, 50]. Fatty acid composition content also might play a role in the protection of the cell membrane from oxidative damage produced by HHP. A desaturase-deficient *Saccharomyces cerevisiae* mutant strain (OLE1 gene deletion) grown in media supplemented with fatty acids differing in size and number of unsaturations and submitted to pressure up to 200 MPa for 30 min shows different responses after the stress. Desaturase-deficient yeast supplemented with palmitoleic acid demonstrated increased sensitivity to pressure compared to cells supplemented with oleic acid or a proportionate mixture of both acids. In contrast, yeast cells grown with linoleic and linolenic acids were more piezoresistant than cells treated with oleic acid. Furthermore, growth with palmitoleic acid led to higher levels of lipid peroxidation [51].

Wild-type yeast cells submitted to 200 MPa for 30 min were observed using transmission electron microscopy and showed that they maintained their external shape, but the cell membrane presented an increase in ondulation, invaginations and evidences of a diluted nuclear membrane [41].

Scanning electron microscopy (SEM) analysis revealed that *S. cerevisiae* submitted to 300 MPa does not show apparent consequences in the cell surface, but at 500 MPa, there is a visible damage and disruption in the cell wall [52]. After pressures above 200 MPa, the nucleus and other organelles are no longer differentiated and membranous fragments can be detected [41]. There are no major visible external changes in the cell under pressure of 80–150 MPa, which might be related to the rigidity of the cell wall [53] The cell mortality as a result of a HHP treatment might be related to the mass transfer through the cell membrane, which causes a change in the permeability of the membrane leading to the intracellular solutes leakage.

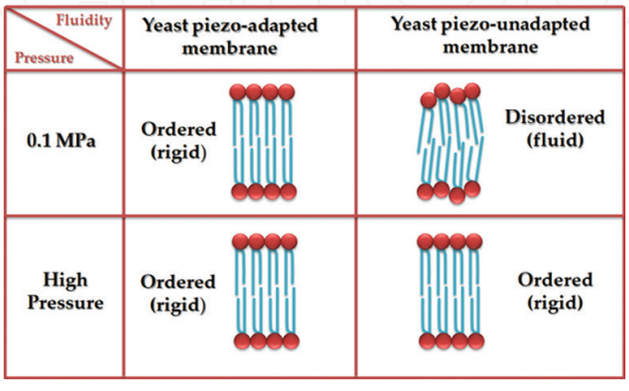

Figure 8. The effects of high pressure in yeast membrane cell (arrangement of lipids).

It seems that trehalose is also involved in cellular protection when HHP is applied. It was shown that trehalose acts inside of the cell as its effect was only observed when applied intracellularly. Actually, cells with a mutation on the trehalose-6-phosphate synthase gene present more sensitivity to high pressure compared to the parental strain [41, 51]. During stress caused by HHP, there is a compression of lipids and increase in ROS [54, 55]. Therefore, it is possible that trehalose acts in the internal bilayers protecting the cell from free radicals and inhibiting lipid peroxidation [51].

2.4.2.2. HHP influence on yeast physiology

HHP affects various structures and cellular functions [36]. Depending on its extent, cytokinetic and mitotic activities are delayed or inhibited, the reactivity of enzymes and other proteins are affected and cell viability decreases with the increase of pressure. This effect is more effective in pressures over 100 MPa and wild-type strains do not survive over 220 MPa. A pressure of 50 MPa is not high enough to kill the cell or modify its cellular morphology, but changes in gene expression and physiology can be observed. Yeast cells in stationary phase have various alterations in morphology and physiology and are more resistant to pressure than proliferative cells [38].

When S. cerevisiae is submitted to 50 MPa for 30 min, it presents an arrest in the log phase of the cellular cycle. If these cells are incubated at ambient pressure after the stress, they show a diminution in the formation of buds up to 45 min after pressurization. Cell recovery starts around 60 min after the stress and achieves full recovery after 2 h [6].

Studies showed that the sensibility of the strain to HHP is related to its genotypic background. The comparison of critical pressure for survival was studied with strains isolated from Brazilian distilleries and laboratory strains (Y440, BY4741, W303 e S228C), and it was seen that industrial strains were more sensitive to HHP (**Figure 5**). The critical pressure for the strains varied between 50 and 100 MPa. It was also observed that nonetheless of the variation in survival, all the strains share a universal mechanism for survival after HHP, which is related to cellular volume [36].

It was found that the cells have a higher tolerance to HHP during stationary phase and are capable of acquiring higher tolerance after a heat shock [36, 41]. A HHP treatment at 50 MPa for 30 min increases the production of ROS in yeast cells, dropping 15 min after the cells are taken out of the HHP and grown in ambient pressure. This showed that oxidative defense mechanisms are induced during cellular recovery after HHP to prevent the accumulation of ROS [55].

2.4.2.3. Alteration on gene expression upon HHP treatment

Gene expression profile in *S. cerevisiae* after 50 MPa HHP treatment (sublethal stress) [7], and 200 MPa treatment (lethal stress) [45] was assessed by microarray analysis. After the piezotreatment with 200 MPa for 30 min at room temperature, 5% of the 6200 known or predicted genes of *S. cerevisiae* are affected. From the 274 genes that shows more than twofold change in the expression, 131 are upregulated, while 143 are downregulated. The most upregulated

genes code small HSPs, HSP30 and HSP12 [45]. HHP, as well as other stresses, promotes cytoplasmic acidification in yeast cells increasing the activity of the H^+-ATPase [9]. HSP30 is important for ATPase activity regulation allowing preservation of the cell energy during stress [56].

Genes related to stress defense and carbohydrate metabolism are also upregulated after 200 MPa, while several genes related to cellular transcription, cell cycle regulation and protein synthesis and target are downregulated. Other response seen after the treatment with 200 MPa was that some gene categories related with transport, cellular organization control, and translation exhibited the same amount of upregulated and downregulated genes. However, other categories show a strict upregulated or downregulated profile. The amount of genes downregulated with strong inhibition were involved with protein regulation and destination, cell cycle progression [45], and this response justifies the cell cycle arrest displayed in cells after HHP treatment.

Some specific pathways are induced after the 200 MPa treatment as lipidic, fatty acid and carbohydrate metabolism, glycolysis, gluconeogenesis, respiration, while amino acid and nucleotide metabolism are repressed. Actually, contrasting with other stresses, the metabolism of trehalose does not show modulation in its expression after HH, even though trehalose plays an important role in the response to this stress. The gene ERG25, associated to ergosterol synthesis, and OLE1, that codes a Δ9-desaturase, which increases the unsaturation of fatty acids in the lipidic membranes, are both induced [45, 57].

The overall microarray analysis of *S. cerevisiae* exposed to HHP of 50 MPa also reveals transcriptional changes in a wide range of genes. Among 6200 known or predicted genes in yeast, mRNA levels for approximately 2.7% of genes were altered more than twofold after 30 min of pressurization when compared to untreated cells. From these 167 genes, 123 were induced and 47 were repressed. Gene expression after 15 min of incubation at atmospheric pressure (0.1 MPa) after 50 MPa treatment showed alteration in 12.9% of the genes, with 408 genes being over-regulated and 392 genes were downregulated more than twofold. This temporal profile of gene transcription presented by cells after HHP suggests that gene regulation follows a priority line. First, genes corresponding to repair and membrane modifications, mitochondria, vacuoles, as well as genes related to aggregation protection are regulated. Then, along the recovery period, other groups of genes, such as the ones encoding membrane proteins and chaperone proteins, genes related to cellular respiration and spore formation are regulated [58].

Transcriptional profile analysis indicates an activation of the general stress response, for instance cell cycle arrest and energy metabolism that is maintained after 15 min of recovery at ambient pressure. The comparison between the groups of genes altered immediately after the pressure and after 15 min of recovery demonstrates that the promoters of genes up or downregulated in response to HHP harbor different motifs governing transcriptional control. Analysis of gene expression and gene ontology made after 5 and 10 min postpressurization showed an effect in categories involved in the regulation of sulfur metabolism. After 15 min of incubation at atmospheric pressure, the affected categories are those related to amine transporter activity and cell cycle. Of the three motifs known to regulate gene expression, all are

identified within 15 min after piezotreatment. On the other hand, only one motif is found after 10 min and another one after 15 min [58].

Interesting enough, genes related to oxidative damage are also induced after HHP treatment [7, 45]. In addition, studies that submit yeast cells to HHP in the presence of glutathione exhibit piezoresistance. This confirms the importance of an oxidative defense mechanism to reduce the damage caused by hydrostatic pressure [6].

Moreover, genes associated with ATP synthesis through glycolysis were modified after pressure. HXK1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism, were upregulated after 50 MPa for 30 min, increasing after 15 min of recuperation. Genes related to high affinity glucose transportation, HXT6 and HXT7, also were highly regulated after treatment with pressure. An increase in the expression of the ADH1 gene was observed 15 min after the treatment with hydrostatic pressure. This gene is responsible for coding the alcohol dehydrogenase enzyme that is required for the reduction of acetaldehyde to ethanol in the last step of the glycolytic pathway [*Saccharomyces* Genome Database (SGD)] [58]. Those results prove the interference of HHP in fermentation.

2.5. Improvement on ethanol production by HHP

Ethanol production may be based on direct access to sugar found in fruit extract (first-generation production) or access to sugar present in lignocellulosic biomass (second-generation production). Applying HHP in *S. cerevisiae* cells is a strategy to optimize both first-generation and second-generation ethanol production. However, for second-generation ethanol, HHP can also be used in previous steps, to treat the lignocellulosic biomass and obtain higher sugar concentrations.

The effects of high pressure in microorganisms and lignocellulosic biomass for bioethanol production differ according to the pressure value and duration of treatment. Therefore, the process used is case-specific, being differentiated when used for pretreatment, continuous pressure during the fermentation processes or applied in lignocellulosic biomass and enzymes.

2.5.1. Use of HHP on first-generation ethanol production

2.5.1.1. HHP as pretreatment

Positive effects on HHP treated *S. cerevisiae* cells can be observed during the fermentation process. The use of HHP can induce cross protection to other stresses in the fermentative vats. Therefore, HHP in mild conditions can be used to increase stress tolerance to high temperature, high pressure, and ultra-cold shock. The acquisition of stress tolerance by applying HHP occurs after the cells are incubated for 15 min in ambient pressure, but it is lost after 1 h [28, 38].

Yeast cells pretreated with 50 MPa already begins to produce ethanol after 4 h of being inoculated in the fermentation vat, reaching 0.3% of ethanol. After 10 h of fermentation, those cells produced up to 0.8% of ethanol, while nontreated cells produced 0.6% (**Figure 9**) [7].

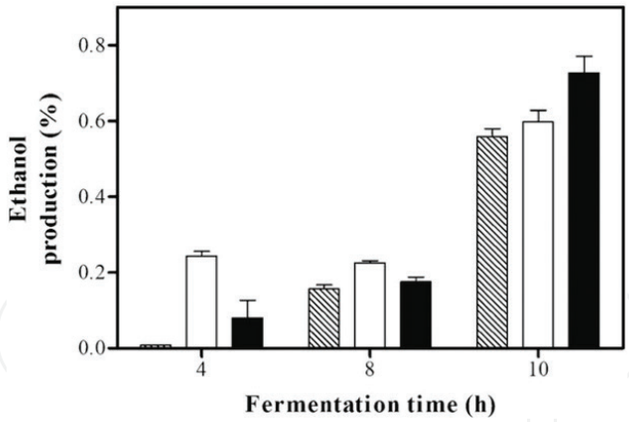

Figure 9. Ethanol production (in percent) after pressure treatment. Hydrostatic pressure of 50 MPa for 30 min (empty bars) and 50 MPa for 30 min and then incubated at room pressure (0.1 MPa) for 15 min (filled bars), and after that, the fermentative efficiency of this strain was evaluated. A nonpressurised sample was used as a control (striped bars) [5].

Other techniques described to induce piezotolerance in wild yeast strains used UV light and HHP. Two methods were compared trying to produce a tolerant strain. The first method treated the wild *S. cerevisiae* with UV light to induce a mutation and subsequently to test them in HHP at 200 MPa for 240 s. These cells proved to be piezotolerant. The other method consisted in using UV light on the strain and then putting them under pressure of 250 MPa for 240 s followed by 48 h of recovery with agitation. These medium was analyzed to determine the cells survival. Then, the medium with less viable cells was submitted to pressure (250 MPa for 240 s) until the survival rate stayed constant. These cells were grown in solid medium and the distinct colonies were put under pressure three more times to produce piezotolerant strains. One of these strains also presented tolerance to high pressure but showed a growth delay, which evidences piezotolerance and piezosensitivity. This delay was also seen in the wild strain but not in the cells treated with the first method. The use of pressure to obtain this delay and mutations proved to be effective to induce piezotolerance and piezosensibility improving the metabolism including ethanol production [59].

2.5.2. Continuous pressure for first-generation ethanol

When used during fermentation, the positive effects are not only described in literature relating to gene expression or cell morphology, but also relating to the increase of glucose flow for the production of ethanol and its relationship with the efficiency/deficiency of some enzymes. *S. cerevisiae* at room temperature produces about 90–95% of the maximum theoretical ethanol yield, since some nutrients are also used for cell maintenance biomolecules synthesis. The kinetic reactions for ethanol production are characterized as firstorder: [42].

$$[Eth] = 2 \times [Glc] \times (1 - e^{-kt}) \quad (2)$$

with [Eth] as the ethanol concentration expressed in mol.L^{-1}, [Glc] as the glucose concentration used to produce ethanol, expressed in mol.L^{-1} (the factor 2 comes from the general equation of fermentation, 1 mole of glucose giving 2 moles of ethanol), k as the reaction constant in h^{-1} and t the time in h [42].

Differing from the findings that high pressure is useful as a pretreatment for cells to later produce more ethanol at atmospheric pressure, it was seen that applying low hydrostatic pressure (up to 10 MPa) show better results when used during the whole fermentation due to the acceleration of ethanol production. The optimum value the obtaining of higher ethanol yield (relationship between the ethanol produced and to the amount of sugar present in the medium) is 5 MPa [42]. The *S. cerevisiae* mortality shows significance starting at 25 MPa as described in **Figure 5** [43]. It is also noticed that mortality varies between strains, reason why the effects of high pressure may undergo changes, so the applied pressure amount must be adjusted individually to each strain [30]. As the best results of ethanol production under continuous pressure are around 5–10 MPa, it can be concluded that does not affect cell mortality in a relevant manner.

Low pressure does not produce a delay in fermentation as it is not reported to induce protein synthesis. These results are related to the enzymatic kinetics, that show that pressure up to 10 MPa shifts the equilibrium to the state of lower volume, which is ethanol. Even though maximum ethanol yield was presented at 5 MPa (100% at 30 min), at 10 MPa the reaction rate is considerably higher, being more than two times the rate shown at atmospheric pressure, with a yield of 99% at 30 min [42].

There is a divergence in the reported pressure that is necessary to interrupt ethanol production that ranges from 50 to 87 MPa. This can be explained by experimental variations or the use of different yeast strains between studies. It is known that HHP interrupts fermentation in that pressure range because over 20 MPa there is a decrease in cytoplasmic pH, which disturbs a crucial stage of fermentation by negatively affecting the action of phosphofructokinase, an important enzyme for the glycolytic pathway [3–6]. From 20 to 87 MPa the ethanol yield is reduced and after that it comes to a halt (**Figure 10**).

$$\text{Yield decrease: } 2 \pm 0.1 \times 10^{-3} \times mo\, l^{-1} \times MPa^{-1} \tag{3}$$

The pressure is chosen depending on the strategy that wants to be used in a specific process as it has distinct effects. In the case of pretreating cells with pressure before fermentation, it acts on gene modulation, but when continuous pressure is applied during fermentation, the goal is only to shift the equilibrium of the reaction to ethanol. When the best pressure used for pretreatment (50 MPa) was used continuously for fermentation ethanol yield drops to 45% [7, 42].

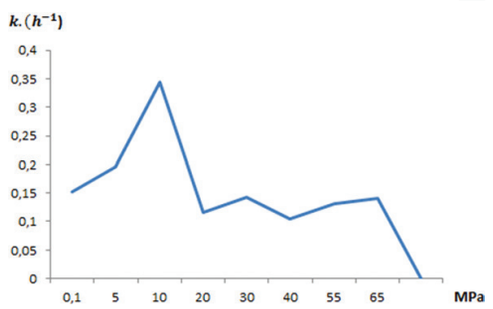

Figure 10. Relation between applied pressure and reaction constant in hours (modify from [42]).

2.5.3. Use of HHP for second-generation ethanol

Second-generation bioethanol characterizes for using lignocellulosic biomass, which are normally residues, so there is no competition with food production. The process to obtain fermentable sugars is more complex than for first-generation ethanol. First the biomass has to undergo a treatment to break the intricate structure and remove lignin. Then, cellulose and hemicellulose are degraded by enzymes to monosaccharides that can be used by the yeasts to produce ethanol.

One use of HHP for second-generation ethanol production is submitting the lignocellulosic biomass to pressure to open the structure, facilitating the access of digesting enzymes to complex sugars. Unlike the pressures reported for yeast cells, the pressure used in lignocellulosic biomass is much more aggressive, since it has the role of breaking cellulosic fibers facilitating the action enzymes. The values shown for lignocellulosic biomass use are higher than 300 MPa, showing a relevant efficiency in the breakage of these fibers. There is a release of fermentable sugars when the lignocellulosic biomass is treated with HHP even without enzymes. Also, an increase in phenols shown that the HHP mainly breaks lignin [60, 61].

In addition to the effects on biomass fibers, HHP also affects the efficiency of the enzymes used for hydrolysis to obtain fermentable sugars from cellulose and hemicellulose to produce ethanol. These enzymes are known as cellulases, and they are composed of different kinds of enzymes with specific functions. They are normally used as cocktails that contain these different enzymes but the proportions and individual activities may vary, affecting the overall performance of the cocktail. In a study using coconut husk as a substrate, cellulases produced by fungi isolated from the husk and commercial cellulases were tested under pressure. First, the commercial enzymes were studied by submitting just the enzyme or the husk to 300 MPa for 30 min and then doing the hydrolysis in atmospheric pressure at 50°C or by introducing both the enzyme and the substrate to the pressure and performing the hydrolysis under pressure (300 MPa) at 22 and 50°C for 30 min. The highest hydrolysis rate was found when the hydrolysis was performed under pressure at 50°C, followed by doing the hydrolysis under pressure at 22°C. This shows that the effects induced by pressure are reversible. Then a comparison between the activity of the commercial enzymes and enzymes produced from isolated fungi was made. In these tests, the activity of different cellulases and the overall cellulase activity were measured at atmospheric pressure at 50°C, 300 MPa at 50°C and 300 MPa at 22°C. Best results were shown for cellulases produced by Penicillium variabile, which was isolated from the coconut husk, at 300 MPa at 50°C. As a general result, all the enzymes tested presented the highest activity at 300 MPa and 50°C. It was seen that the activity of the enzyme cellobiase was especially enhanced by HHP for enzymes isolated from the two fungi tested [60]. This effect was also seen in another study that showed that cellulase structural and functional stability are not negatively affected by HHP from 300 to 400 MPa and HHP gives enzyme stability hydrolysis in a larger range of temperatures [62].

These benefits can be used to obtain higher sugar concentration, which leads to higher ethanol concentration, with a variety of lignocellulosic substrates. This technique was also in used *Eucalyptus globulus*, which showed similar results while pressurizing cellulolytic enzymes. In this case, the pressure applied varied from 200 to 400 MPa using different treatment times from 15 to 15 min. It was seen that higher pressure or time did not have best results, demonstrating that optimum conditions must be found to optimize the process. In this case, the best

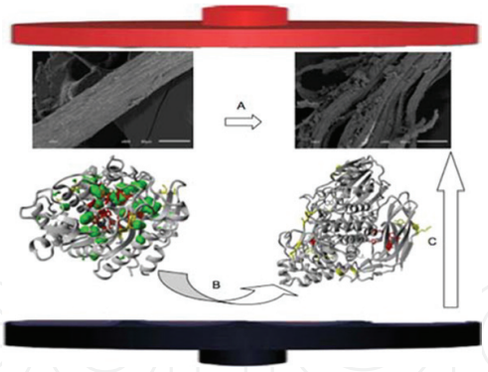

Figure 11. HHP effect on lignocellulosic matrix and enzyme.

results were found using 300 MPa for 45 min. This treatment promoted a better accessibility to xylan, and it was reflected in the sugar concentration after hydrolysis, which reached up to 35% with the HHP compared to the 10% obtained at atmospheric pressure.

The improvement in cellulase activity has been attributed to different factors that act at the same time. One is conformational change on enzyme by HHP, which leads to the exposition of hydrophobic amino acids that interact with the sugar through hydrophobicity creating new binding sites. Other is that, HHP causes a diminution in volume that brings the enzyme closer to the substrate (cellulose and hemicellulose). Finally, as mentioned earlier, HHP helps to break the lignocellulosic tight matrix, which facilitates its degradation by cellulases (**Figure 11**) [60].

These results demonstrate the importance of monitoring hydrostatic pressure, among other abiotic factors, so that ethanol production is maximized. Finally, it can be concluded that the use of high hydrostatic pressure can be used in different stages of fermentation processes and each stage will have its value and specific purpose.

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