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Review

Adaptation of *Saccharomyces cerevisiae* to high pressure (15, 25 and 35 MPa) to enhance the production of bioethanol

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ABSTRACT

Saccharomyces cerevisiae is a yeast of great importance in many industries and it has been frequently used to produce food products and beverages. More recently, other uses have also been described for this microorganism, such as the production of bioethanol, as a clean, renewable and sustainable alternative fuel.

High pressure processing (HPP) is a technology that has attracted a lot of interest and is increasingly being used in the food industry as a non-thermal method of food processing. However, other applications of high pressure (HP) are being studied with this technology in different areas, for example, for fermentation processes, because microbial cells can resist to pressure sub-lethal levels, due to the development of different adaptation mechanisms.

The present work intended to study the adaptation of *S. cerevisiae* to high pressure, using consecutive cycles of fermentation under pressure (at sub-lethal levels), in an attempt to enhance the production of bioethanol. In this context, three pressure levels (15, 25 and 35 MPa) were tested, with each of them showing different effects on *S. cerevisiae* fermentation behavior. 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, the ethanol production was higher under pressure than at atmospheric pressure (0.1 MPa) (8.75 $g.L^{-1}$ and 10.69 $g.L^{-1}$ at 15 and 25 MPa, respectively, compared to 8.02 $g.L^{-1}$ at atmospheric pressure). However, when the pressure was increased to 35 MPa, cell growth and bioethanol production decreased, with minimal production after the 4 consecutive fermentation cycles.

In general, the results of this work suggest that consecutive cycles of fermentation under sub-lethal pressure conditions (15 and 25 MPa) can stimulate adaptation to pressure and improve the bioethanol production capacity by *S. cerevisiae*; hence, this technology can be used to increase rates, yields and productivities of alcoholic fermentation.

1. Introduction

Yeasts are eukaryotic, unicellular microorganisms classified as members of the kingdom Fungi. These microorganisms, and particularly, *Saccharomyces cerevisiae,* are of major economic, social and health significance in human culture (Pompon, 1999). They have been used to produce alcoholic beverages (such as beer and wine) and ferment bread dough for millennia. Nowadays, other uses have been described for *S. cerevisiae*, including the production of bioethanol as a fuel, by alcoholic fermentation processes (Atkinson & Sherwood, 2014; Fox, Bellini, & Pellegrini, 2014; Pompon, 1999). Bioethanol is a clean, renewable and sustainable alternative fuel that has been increasingly produced in order to support the need of new and cleaner energies (Bajpai, 2013; Deesuth, Laopaiboon, Klanrit, & Laopaiboon, 2015; Deesuth, Laopaiboon, & Laopaiboon, 2016).

High pressure processing (HPP) is a technology that has attracted a lot of interest in the food industry, since it is being increasingly used as a non-thermal method for food processing (up to 600 MPa). However, other applications of high pressure (HP) at mucg lower pressure (up to 100 MPa) are being studied in different fields as, for example, the use of sub-lethal pressure levels during fermentation processes. In this case, cells are able to resist to these sub-lethal pressures due to the development of different mechanisms of adaptation, such as production of heat shock proteins (HSP), modification of fatty acids and modulation

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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 increase of tolerance when they are subsequently submitted to higher levels of the same stress or even other stresses, like cross-protection and general stress response (GSR) (Malone, Shellhammer, & Courtney, 2002).

Mesophilic microorganisms, such as *S. cerevisiae,* cannot normally grow under pressure due to its sensitivity to pressure. 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 (organisms whose survival and reproduction are optimized to high pressures (Ichiye, 2018)), but with lower efficiency (Oger & Jebbar, 2010). High pressure 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. For instance, Iwahashi and Kaul (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 use of cryoprotectants (like dimethylsulfoxide or 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). For instance, *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). Miura, Minegishi, Usami, and Abe (2006) demonstrated that upregulated genes are not always responsible for the piezotolerance, which shows the great complexity of this subject (Miura et al., 2006). When submitted to 200 MPa for 30 min, *S. cerevisiae* cells revealed a HP-stress response pattern by adjusting its genomic expression pattern accordingly (Fernandes, Domitrovic, Kao, & Kurtenbach, 2004). Furthermore, Picard, Daniel, Montagnac, and Oger (2007) noticed that the use of lower levels of pressure, 5 and 10 MPa, improved the production of ethanol (3–4%) and the rate of fermentation was higher when compared to atmospheric pressure (Picard et al., 2007). These results suggest that sub-lethal pressure conditions can promote the development of specific genetic, physiologic and metabolic stress responses, opening the possibility to obtain fermentation products and processes with different characteristics. In some cases these modifications can represent significant improvements, such as increased yields, productivities, and fermentation rates, lower accumulation of by-products (a secondary product derived from a chemical reaction or a metabolic process such as some organic acids) and/or production of different compounds.

Therefore, such strategies are not only relevant for food fermentations (e.g. for the production of dairy products, alcoholic beverages, and others), but may also 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) (Ananta & Knorr, 2004; Marietou, Nguyen, Allen, & Bartlett, 2014; Mota, Lopes, Delgadillo, & Saraiva, 2013, 2015; Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017; Serrazanetti, Guerzoni, Corsetti, & Vogel, 2009). In addition, several studies showed the ability of mesophilic microorganisms to adapt to HP, such as in the case of *S. cerevisiae*. Since HP seems to have a positive effect on alcoholic fermentation by this microorganism, the production of bioethanol might be further enhanced in pressure-adapted cells. Therefore, the main goal of this work was to study the adaptation of *S. cerevisiae* to HP and to understand how this parameter affected cell viability, growth and fermentation, using consecutive cycles of fermentation under sub-lethal pressures, between 5 and 100 MPa.

2. Material and methods

2.1. Microorganism

Saccharomyces 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, sub-cultured on Yeast Malt agar plates, and subsequently incubated at 30 °C for 48 h.

2.2. 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 $g.L^{-1}$ of peptic digest of animal tissue (peptone), 3.00 $g.L^{-1}$ of yeast extract, 3.00 $g.L^{-1}$ of malt extract and 10.00 $g.L^{-1}$ of dextrose. The culture was incubated at 30 °C and 150 rpm for 18 h. The inoculum was ready to use when the optical density (at 600 nm) of the culture medium was 0.8.

2.3. Fermentation under high pressure

The work was divided in two different parts: i) in the first phase, sub-lethal pressures (between 15 and 50 MPa) were applied during only one-cycle fermentation process, to determine the most suitable pressures to use in the consecutive fermentation cycle; ii) in the second phase, *S. cerevisiae* cultures underwent fermentation under the same pressures over four consecutive fermentation cycles, in an attempt to trigger pressure adaptation mechanisms. In both cases, fermentation was performed 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 anaerobic process. As such, the samples to be used were sealed with the minimum level of oxygen possible, promoting fermentation over aerobic respiration.

The inoculated medium (100 mL) was homogenized and then portions of 5 mL were transferred to low permeability polyamide-polyethylene bags (11 cm × 3.5 cm) (PA/PE-90, Albipack – Packaging Solutions, Portugal), previously sterilized with UV radiation (BioSafety CabinetTelstar Bio II Advance, Terrassa, Spain), being manually heat sealed to minimise the amount of air inside the bags. All the steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination.

2.3.1. One-cycle fermentation under high pressure

In this first part of the work, with only one cycle, fermentations were performed at 0.1, 15, 25, 35 and 50 MPa, at 30 °C, for 24 h or 48 h. Fermentation was also performed at 0.1 MPa (atmospheric pressure), keeping all conditions equal to those applied for fermentation under HP. Samples were collected throughout the fermentation period, and each experiment was run in duplicate. The HP experiments were conducted using two HP equipments, depending on the availability, one with a vessel capacity of 2 L (vessel internal length/diameter: 250/100 mm) and with pressure come up-time of about 20 s for 50 MPa, and the other a High Pressure System U33, Unipress Equipment, Poland, with a capacity of 100 mL (vessel internal length/diameter: 100/35 mm) and with pressure come up-time of about 15 s for 50 MPa. A mixture of propylene glycol and water (40:60) was used as pressurizing fluid and to control the temperature in the external jackets.

2.3.2. Consecutive cycles of fermentation under high pressure

In the second phase, fermentations were performed during four consecutive HP cycles (Fig. 1). After each fermentative cycle, new fresh culture medium was used (in order to maintain the initial conditions in

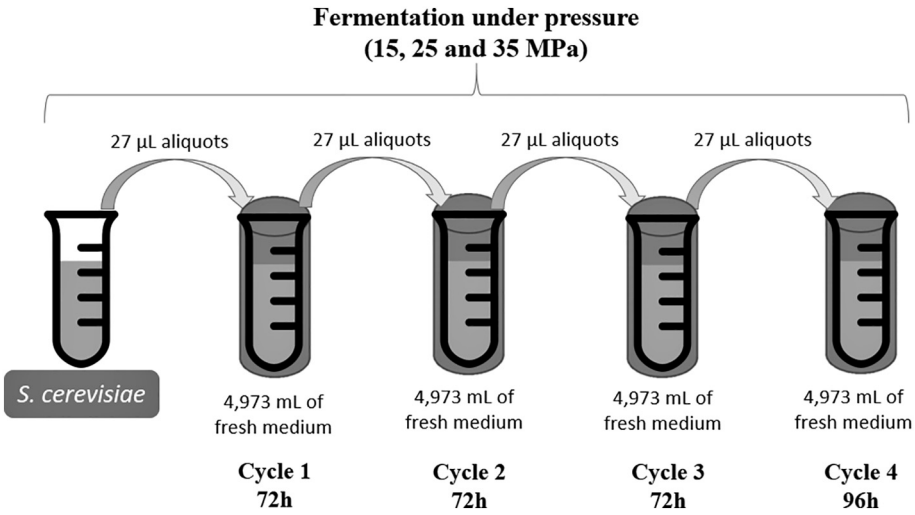

Fig. 1. Representation of the four consecutive cycles of fermentation under pressure.

each cycle) and aliquots of the previously fermentative cycle were used as inoculum to ascertain for the possible adaptation of *Saccharomyces cerevisiae* to pressure.

The first three pressure cycles were performed for 72 h and the last one was 24 h longer (96 h). Furthermore, the experiments were performed at different pressure conditions (15, 25 and 35 MPa) using the same equipments described in section 2.3.1 and at the controlled temperature of 30 °C. As control, fermentation was also performed at 0.1 MPa (atmospheric pressure), keeping all conditions equal to those for fermentation under HP.

2.4. Determination of biomass concentration

For the determination of biomass concentration, the optical density (OD) of the samples from the first part experiments was measured at 600 nm and the cell dry weight was determined after 24, 48 and 72 h fermentation, by freeze-drying. A calibration curve correlating these two parameters was determined and utilized in the second part experiments to determine sample biomass concentration through the measurement of OD, at 600 nm.

2.5. Viable cell enumeration

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

2.6. Sugar and organic acids quantification

Fermented samples were centrifuged at 10,000 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, formic, 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 × 7.8 mm) (Bio-Rad Laboratories Pty Ltd., Hercules, CA, USA). The mobile phase was 13 mM H_2SO_4, delivered at a flow rate of 0.6 mL.min^{-1} and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards.

2.7. Kinetic calculations

Different kinetic parameters were determined in order to allow a better comparison between the results. The parameters were glucose consumption (%) and yields (Y) of bioethanol, and biomass using the formulas below. Furthermore, the productivity (Q ($g.L^{-1}.h^{-1}$)) and specific productivity (q ($g.g^{-1}.h^{-1}$)) of bioethanol were also calculated.

$$Glucose\ consumption\ (\%) = \frac{[Glucose]_{final} - [Glucose]_{initial}}{[Glucose]_{final}}$$

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

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

$$Produtivity\ of\ bioethanol\ (Q) = \frac{[Bioethanol]_{final} - [Bioethanol]_{initial}}{time_{final} - time_{initial}}$$

$$Specific\ produtivity\ of\ bioethanol\ (q) = \frac{Q}{[Biomass]_{final}}$$

3. Results and discussion

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

As previously mentioned, the main goal of the present work was to study the adaptation of *S. cerevisiae* cultures to HP, by performing consecutive cycles of alcoholic fermentation under pressure conditions. However, in order to determine the most suitable HP conditions to use in these experiments, a first study of *S. cerevisiae* fermentation was performed under different pressure levels (0.1, 15, 25, 35 and 50 MPa) at 30 °C for 72 h. The results of these preliminary tests indicated that the increase in pressure level considerably affected the fermentation rate, decreasing the consumption of glucose and the production of ethanol.

For that reason, the variation of the concentration of glucose and ethanol was monitored during this preliminary study and the results obtained are shown in Fig. 2. A decrease in glucose concentration over time was observed in all cases (Fig. 2a), even at the highest pressures (35 and 50 MPa), but with a slower consumption rate in these cases. At atmospheric pressure (0.1 MPa), a more accentuated consumption in glucose was noticed during the first 48 h of fermentation, when the

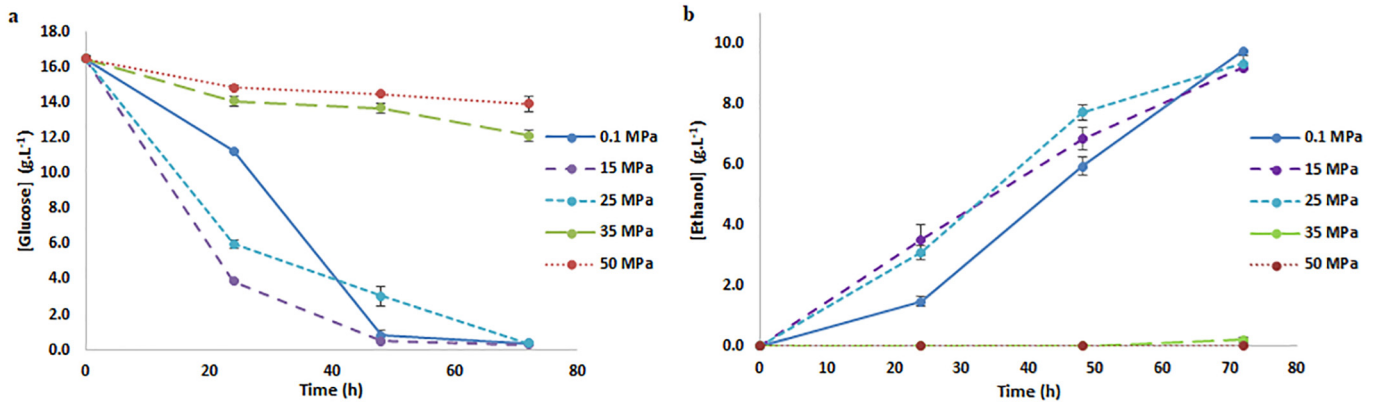

Fig. 2. Glucose consumption (a) and ethanol production (b) over time, for fermentation by *S. cerevisiae* under different pressure conditions and at 30 °C.

concentration of glucose reached 0.813 g.L^{-1}. Thereafter, the glucose consumption rate stabilized gradually, 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.

The application of HP up to 25 MPa affected glucose consumption positively ($p < .05$). With an increasing pressure, it was observed a higher glucose consumption variation during the first 24 h for both 15 and 25 MPa. Thereafter, glucose concentration at 15 and 25 MPa was lower than that reported at 0.1 MPa, indicating a higher and faster glucose consumption under pressure. However, after 48 h of fermentation, 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 glucose consumption was similar (approximately 0.350 g.L^{-1}) for all pressures 0.1, 15 and 25 MPa. At 35 and 50 MPa, glucose consumption was considerably slowed down, as almost no variation was observed in glucose concentration over the 72 h fermentation period.

The production of bioethanol during fermentation under pressure is represented by an increase in the bioethanol production at lower pressures (15 and 25 MPa), and no production at higher pressures (pressure higher than 25 MPa). At 0.1 MPa, 15 MPa and 25 MPa, the concentration of bioethanol reached values close to 10 g.L^{-1}. Furthermore, for 15 and 25 MPa, the production of bioethanol was faster, 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. Albeit the almost absence of bioethanol production, the slight variation observed in glucose concentration in these conditions, may indicate that sugars were being 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 (Bravo, Camacho, Sánchez, & Jaén, 1993) and/or adaption to HP (Mota et al., 2013).

Regarding these preliminary results, it may be concluded that at 35 and 50 MPa the fermentation process is very slow, which probably indicates that *S. cerevisiae* was inhibited or even 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 their viability at 100 MPa (Bartlett, 2002Picard et al., 2007). For example, certain levels of HP affect relevant cellular processes, mostly due to changes in DNA structure. The DNA hydrogen bonds are stabilized and, as a consequence, the replication/transcription/translation processes may become more difficult (Macgregor, 2002). Furthermore, pressure increases compression of bacterial membranes causing a reduction in the intermolecular distance between acyl chains and membrane lipids. This process can cause leaks 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). High pressure may also have effects on the secondary, tertiary and quaternary structures of proteins, affecting protein unfolding and eventually causing their denaturation (Moreirinha, Almeida, Saraiva, & Delgadillo, 2016).

3.2. Consecutive cycles of fermentation under high pressure at 30 °C

Taking into account the results of the first phase described in the previous section, the most suitable conditions for *S. cerevisiae* adaptation to pressure were selected (15 and 25 MPa). Furthermore, 35 MPa was also included, despite de results of Fig. 2 that suggest that almost no bioethanol was produced, because the slight decrease observed in glucose concentration at this pressure (and also for 50 MPa) indicates that sugars were used, possibly in metabolic pathways involved in cellular viability maintenance (Bravo et al., 1993) and/or adaption to HP (Mota et al., 2013) that could lead to pressure adaptation and ethanol production in further fermentation cycles. Therefore, four consecutive fermentation cycles were performed at 15, 25 and 35 MPa: the first three pressure cycles were carried out with a fermentation time of 72 h, and the fourth was 24 h longer, i.e. 96 h.

The results obtained for glucose concentration, indicated in Table 1, show higher glucose consumption at 0.1 MPa and at lower pressure levels (15 and 25 MPa), compared to fermentation at 35 MPa. Since the glucose concentrations in Fig. 3a cannot be properly analysed and discussed for the lower pressure conditions, due to the highest concentrations observed, a more zoomed version of the figure is presented in Fig. 3b. A similar behaviour in the four fermentation cycles at 0.1 MPa was observed, with similar final glucose concentrations achieved in all cases (below 0.50 g.L^{-1}). At this pressure, glucose was almost entirely consumed ($\approx$ 99%) in all cycles. Similarly, high glucose consumptions were observed at 15 and 25 MPa during the fermentation cycles. At 15 MPa, a decrease in glucose concentration was observed after each fermentation cycle: from 0.56 g.L^{-1} at the end of the first cycle, to 0.26 g.L^{-1} at the end of the last one. This resulted in an

Table 1
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 °C.

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

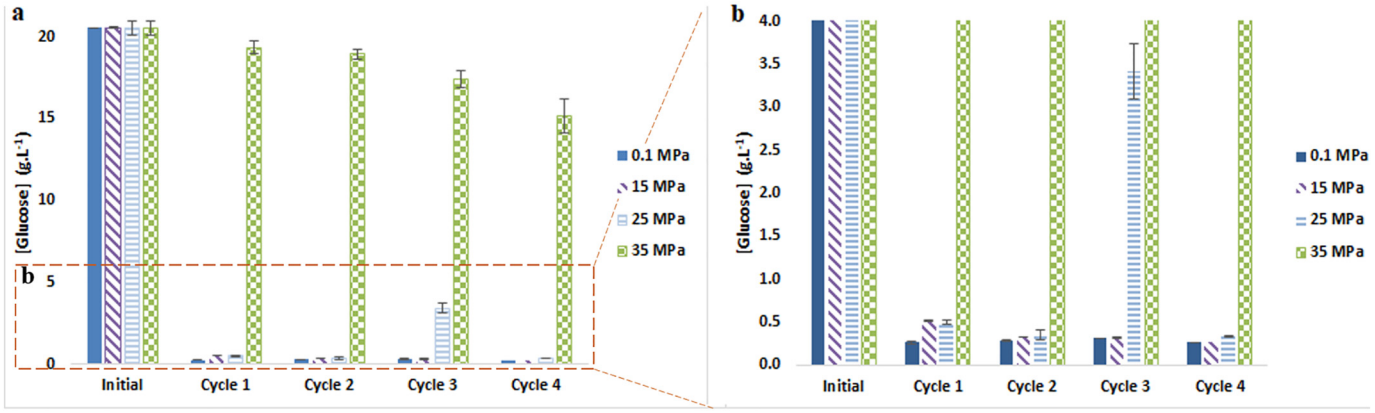

Fig. 3. Final concentration of glucose after each of the four cycles under different pressure conditions (0.1, 15, 25 and 35 MPa) and the four cycles at 0.1 MPa, at 30 °C (a), zoomed image of the final glucose concentration of the four fermentative cycles of pressure (b).

increase of glucose consumption from 85% to 98.7%. Similar results were obtained for the fermentation cycles at 25 MPa, with a general decrease in glucose concentration from 0.50 $g.L^{-1}$ at the end of the first cycle, to 0.33 $g.L^{-1}$ at the end of the fourth one. At this pressure, the percentage of glucose consumed was more variable throughout the cycles, showing a decrease in consumption in the third cycle that could be a result of an adaptation process. In the other three cycles, the percentage of glucose consumed was similar to the one obtained for 0.1 MPa. Glucose consumption at 25 MPa varied from 97.6% in the first cycle, to 98.4% in the fourth one. The increase in glucose consumption over the consecutive fermentation cycles under pressure (15 and 25 MPa) may indicate cell adaptation to these pressure conditions, possibly resulting in improved fermentation rates (and improved bioethanol production) after each fermentation cycle, which was also analysed in the course of this work. Glucose consumption at 35 MPa was found to be lower than at the other pressures tested but, even so, tended to increase over the consecutive cycles. In this case, only ≈ 6% of the total glucose available was consumed after the first cycle, contrasting with the 25% consumed at the last cycle. Therefore, these results may also indicate adaptation of *S. cerevisiae* cells to pressure, even if to a lower extent when compared to 15 or 25 MPa.

Fig. 4 indicates the production of bioethanol throughout the four cycles of fermentation, at 0.1, 15, 25 and 35 MPa. At 0.1 MPa, the concentration of bioethanol produced at the end of the fermentation process was enhanced from the first to the last cycle, which suggests the occurrence of an adaptive process that stimulated the formation of this compound. In this case, adaptation may be related to some stress factors that the yeast is naturally exposed to during fermentation, such as osmotic pressure, pH reduction, ethanol stress, and others. Interestingly,

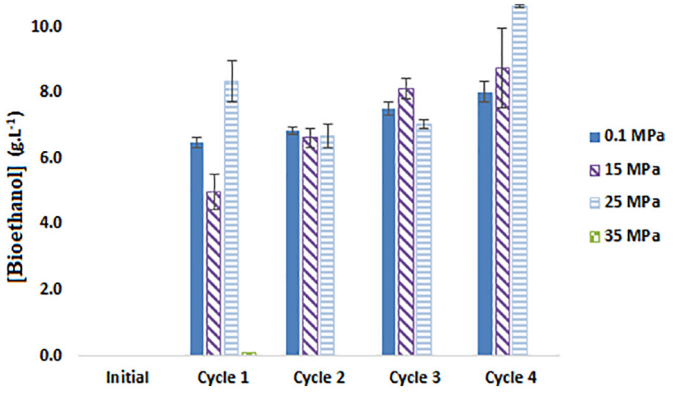

Fig. 4. Final concentration of bioethanol after each of the four cycles under different pressure conditions (0.1, 15, 25 and 35 MPa) and the four cycles at 0.1 MPa, at 30 °C.

at 15 MPa, the production of bioethanol in the first cycle (5.0 $g.L^{-1}$) was lower than that reports at 0.1 MPa (6.5 $g.L^{-1}$), but increased in the next three fermentation cycles and, in the latter two, the amount produced at 15 MPa was even higher than at 0.1 MPa. At 25 MPa, bioethanol production after each fermentation cycle did not follow a clear pattern throughout the cycles. The final ethanol concentration decreased from the first to the second cycle, but, thereafter, the concentration was enhanced over the cycles, with the maximum bioethanol concentration achieved at the fourth cycle (10.30 $g.L^{-1}$). In fact, this concentration was even higher than that 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 bioethanol concentration was minimal (0.11 $g.L^{-1}$) 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 *S. cerevisiae* cells were not able to adapt and survive to consecutive cycles under 35 MPa; indeed viable cell numbers decreased ca. 4 log cycles during treatment as discussed below.

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. In terms of bioethanol production, an increased concentration was observed at 15 MPa and 25 MPa, when compared to the final concentration of bioethanol at 0.1 MPa. This enhancement in bioethanol production was 2–3 fold higher than that obtained by Picard et al. (2007) for *S. cerevisiae* at 10 MPa for 24 h (3–4%). Even though the fermentation times used in the present work were longer, the results suggest that the use of consecutive cycles of pressure can stimulate the adaptation of *S. cerevisiae* to sub-lethal levels of pressure, and thus lead to changes in metabolic capacity, namely improve its ability to produce bioethanol.

Microbial growth was evaluated by the determination of biomass concentration and viable cell numbers, and both results are represented in Fig. 5. It is well established that in a fermentation process, the concentration of microorganisms is, in general, related to substrate consumption rate and to product generation rate. Regarding biomass concentration (Fig. 5a), the highest values were observed at 0.1 MPa, and were maintained almost constant throughout the four 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 enhancement of biomass concentration was higher at 15 and 25 MPa than at 35 MPa (from an initial 1.79 $g.L^{-1}$ to ≈ 8.0 $g.L^{-1}$ at 15 and 25 MPa, and to ≈ 5.0 $g.L^{-1}$ at 35 MPa). From these results it seemed that despite the low glucose

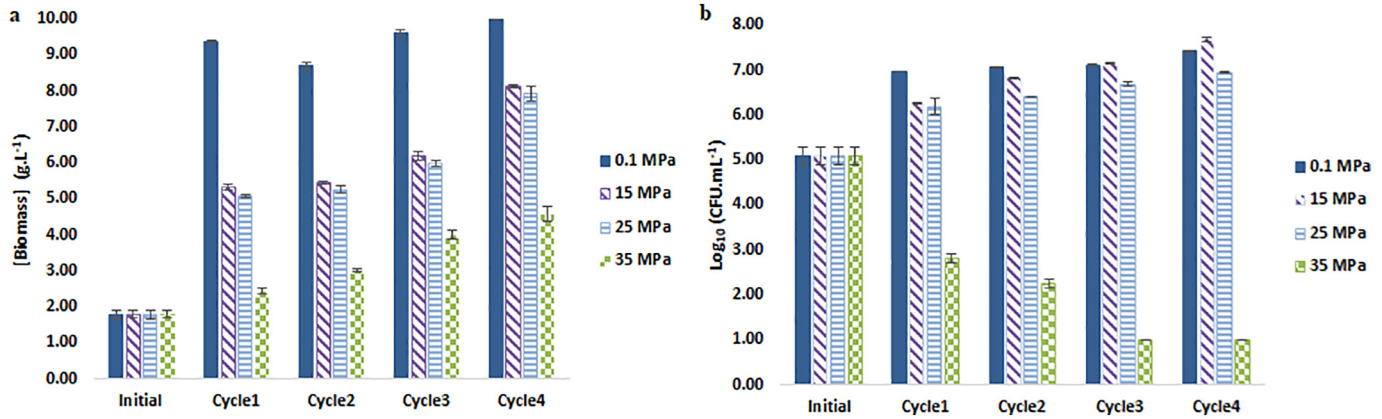

Fig. 5. Final biomass concentration (a) and viable cell numbers (log CFU mL^{-1}) (b) after each of the four cycles under different pressure conditions (0.1, 15, 25 and 35 MPa) and the four cycles at 0.1 MPa, at 30 °C.

consumption and bioethanol production at 35 MPa, biomass concentration slightly increased throughout the fermentative cycles, suggesting that *S. cerevisiae* cells could still grow at this pressure, with slow but steady adaptation over the cycles.

However, biomass concentration does not only provide the number of viable cells in the culture medium but also the number of non-viable cells since the total number of cells present in the samples are measured. Therefore, viable cell numbers were also determined (Fig. 5b). At 0.1 and 25 MPa the number of viable cells (maximum of 7.53 CFU.mL^{-1} and 7.43 CFU.mL^{-1}, respectively) showed almost no variation throughout the cycles. However, bioethanol production tended to increase after the first fermentation cycle, such as indicated above, suggesting a possible adaptation effect of *S. cerevisiae* to this pressure. Therefore, the enhancement in bioethanol production was not a consequence of an increase in viable cell numbers, and possibly indicates that the cells at that pressure were re-directing their metabolism and converting a higher amount of glucose into bioethanol.

In contrast, when pressure cycles were performed at 15 MPa, there was a clear adaptation of *S. cerevisiae*, and viable cell numbers increased almost 1.5-log cycles between the end of the first and the fourth cycles; overall, viable cell numbers were enhanced from the initial value of 4.95 CFU.mL^{-1} to 7.93 CFU.mL^{-1}. At this higher pressure, *S. cerevisiae* cells may have developed mechanisms to survive and adapt to such sublethal conditions, resulting in an increase in the number of viable cells. On the other hand, no adaptation of *S. cerevisiae* seemed to occur at 35 MPa, since a 4-fold reduction in the number of viable cells was observed throughout the four cycles as mentioned before. At this pressure it is possible that cells were still growing but, due to the higher level of stress, their viability was lost during the process. Furthermore, it is possible that some of the cells could not resist to the harsh conditions, which may compromise many important cell structures and functions (Heremans & Smeller, 1998; Mota et al., 2013). The ones that were able to survive, as discussed by Iwahashi, Odani, Ishidou, & Kitagawa, 2005, might have increased in size and complexity, in order to try to resist to this pressure level. 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).

In general, the results in this section revealed that it is possible to induce adaptation of *S. cerevisiae* (particularly, 15 and 25 MPa). This was verified not only for microbial growth and cellular viability, but also for the production of bioethanol, where an enhancement on the production of this fuel was also verified for the two lower pressures (15 and 25 MPa). For 15 MPa, in the last cycle, an increase of 8.5% and for 25 MPa 32.5% in the production of bioethanol was noticed. These considerable improvements can have a practical impact at the industrial level.

3.3. Kinetic analysis

Taking into account the results obtained in the previous section, some kinetic parameters were determined to characterize the pressure effect on substrate consumption and product formation over the fermentative cycles. For instance, bioethanol production yield was estimated for each fermentation cycle and each pressure level, the results being presented in Table 2. The other kinetic parameters analysed were the biomass production yield, the productivity and specific productivity of bioethanol, and the bioethanol:by-products ratio (with by-products meaning products different form the main product (ethanol), like organic acids).

For 35 MPa, the only cycle with bioethanol production was the first one and, because of that, only the first cycle shows values for the kinetic parameters. For example, 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.

Regarding bioethanol production yield, this parameter gives information about the production of bioethanol *per* molecule of glucose consumed during fermentation. For all conditions, bioethanol yield increased throughout the fermentation cycles. However, the highest variation between cycles was obtained at 15 MPa (from 0.171 g.g^{-1} to

Table 2
Yields of bioethanol and biomass production (per gram of glucose) by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 °C.

Bioethanol and Biomass yields of production (g.g^{-1})								
	0.1 MPa		15 MPa		25 MPa		35 MPa	
	$Y_{Bioethanol}$	$Y_{Biomass}$	$Y_{Bioethanol}$	$Y_{Biomass}$	$Y_{Bioethanol}$	$Y_{Biomass}$	$Y_{Bioethanol}$	$Y_{Biomass}$
Cycle 1	0.319	0.358	0.171	1.75	0.416	1.63	0.0941	0.319
Cycle 2	0.337	0.327	0.327	1.8	0.331	1.71	0	0.604
Cycle 3	0.371	0.369	0.401	2.17	0.411	2.06	0	1.09
Cycle 4	0.395	0.403	0.431	3.13	0.525	3.03	0	1.38

0.431 $g.g^{-1}$), with the last two cycles presenting a yield higher than the corresponding cycles at 0.1 MPa. Therefore, the efficiency of *S, cerevisiae* to produce bioethanol was considerably improved over the cycles at 15 MPa, producing a higher amount of bioethanol *per* glucose consumed. On the other hand, cycles at 25 MPa presented more variable results regarding these two kinetic parameters. However, bioethanol on glucose yields were generally higher after each cycle at 25 MPa than at 0.1 MPa. The maximum value for the yield of bioethanol on glucose was estimated for the fourth fermentation cycle at 25 MPa, corresponding to 0.525 $g.g^{-1}$. Thus, these results indicate that *S. cerevisiae* cells adapted throughout the cycles to these pressure levels, possibly through the development of stress responses that protect the cells against the harsh conditions and improve the efficiency to perform alcoholic fermentation, producing higher amounts of bioethanol.

In addition, the biomass yield on glucose consumption was also calculated, the results being also presented in Table 2. In general, an increase of the biomass production yield was observed at all cases, being that increase more accentuated for the cycles under pressure (15 and 25 MPa). While the biomass yield was very similar throughout the 4 cycles at 0.1 MPa (0.358 $g.g^{-1}$ and 0.403 $g.g^{-1}$ in the 1st and 4th cycles, respectively), the highest variations between cycles were observed at 15 and 25 MPa, from 1.85 $g.g^{-1}$ to 3.31 $g.g^{-1}$ and from 1.98 $g.g^{-1}$ to 3.56 $g.g^{-1}$, respectively. Interestingly, higher yields were observed for all the fermentative cycles under pressure, when compared to 0.1 MPa cycles. This possibly indicates that the biomass production was considerably improved over the cycles under pressure, with the cell producing a higher amount of biomass *per* glucose consumed. These results further uphold the theory that *S. cerevisiae* is adapting to pressure, possibly by developing HP stress responses, which may be promoting its ability to grow using glucose as an energy source.

Regarding bioethanol productivity (Table 3), similar results were observed throughout the cycles at 0.1 MPa and 15 MPa, increasing in the first three cycles and slightly decreasing in the 4th and last cycle, probably due to the increase in fermentation time. Nevertheless, at the end of the adapting process, bioethanol productivity was slightly higher at 15 MPa than at atmospheric pressure. In contrast, bioethanol productivity remained constant throughout the cycles at 25 MPa, with values slightly higher than values achieved for the other pressures tested. Taking into account the biomass produced, bioethanol specific productivity was also calculated, with the results showing a similar profile to bioethanol productivity. An exception was observed at 25 MPa, where the bioethanol specific productivity decreased throughout the cycles (from 0.0226 to 0.0141), instead of remaining constant as occurred in bioethanol productivity. However, higher values were also observed in this case, indicating that more bioethanol was produced at this pressure from biomass existing in the medium *per* hour. Therefore, the highest values for both parameters were achieved at the end of the adapting process at 15 and 25 MPa. In general, it was noticeable that for the 1st fermentative cycle the kinetic parameters were lower for the tests performed under pressure when compared to 0.1 MPa. However, throughout the fermentative cycles the kinetic parameters increased when lower pressures were applied becoming higher than the control group. Thus, these results showed that the application of consecutive fermentation cycles under pressure promoted the adaption of *S. cerevisiae* to sub-lethal levels of pressure and increased the bioethanol production. The enhancement in the yields and productivity of bioethanol were results of that adaption to the consecutive fermentation cycles.

Table 4
Ratio of bioethanol: by-products of fermentation after each of the four cycles of pressureand the four cycles at 0.1 MPa, at 30 °C.

Bioethanol:by-products ratio				
	0.1 MPa	15 MPa	25 MPa	35 MPa
Cycle1	2.96	4.34	4.61	0.331
Cycle2	3.36	3.36	4.68	–
Cycle3	3.91	4.33	4.88	–
Cycle4	5.85	5.99	7.23	–

3.4. Ratio of bioethanol: by-products of fermentation

In order to understand the effects of pressure on product selectivity, ratios between bioethanol and the fermentation by-products (formic and acetic acids) were estimated, after each of the four fermentation cycles at different pressure levels (Table 4). Citric, tartaric, malic, succinic acids were excluded of the by-products analysis because its concentration was near the detention capacity of the analytical apparatus.

The bioethanol:by-products ratios tended to increase over the cycles at 0.1, 15 and 25 MPa. Moreover, the ratios were always higher at 15 and 25 MPa than at 0.1 MPa (except in the second cycle at 15 MPa), which indicates that the proportion of ethanol produced increased under HP conditions, relatively to the production of the other compounds. However, the behaviour was highly variable over the cycles at 15 MPa and, consequently, the ratio in the last cycle was similar to 0.1 MPa, even if slightly higher (5.99 and 5.85, at 15 and 0.1 MPa, respectively). At 25 MPa, the ratios increased steadily over the cycles, with a considerable increase from the third to the fourth cycle. Thus, in the last cycle, the ratio at 25 MPa was much higher than those observed for 0.1 and 15 MPa. This indicates that, after four cycles at 25 MPa, the selectivity of the metabolism seems to be modified, leading to an increase in ethanol production in relation to the by-products. Since the goal of the work was to enhance the yield and productivities of bioethanol, the reduction of by-products formation corresponds to an improvement of the process. Furthermore, the reduction in by-products formation lowers the costs of purification and may enhance the quality of the final product (Atadashi, Aroua, & Aziz, 2011).

4. Conclusions

Overall, the results of the present study showed that some levels of pressure may be applied in consecutive fermentation cycles, to enhance the production of bioethanol by *S. cerevisiae*. For the first cycles at 15 MPa and 25 MPa, the fermentation rate was lower when compared to atmospheric pressure. However, after four fermentation cycles, the

Table 3
Bioethanol productivity and specific productivity by *S. cerevisiae* after each of the four cycles of pressure and the four cycles at 0.1 MPa, at 30 °C.

Bioethanol productivity, Q ($g.L^{-1}.h^{-1}$) and specific productivity, q ($g.g^{-1}.h^{-1}$)								
	0.1 MPa		15 MPa		25 MPa		35 MPa	
	Q	q	Q	q	Q	q	Q	q
Cycle 1	0.0899	0.0097	0.0691	0.013	0.116	0.0226	0.0015	0.0006
Cycle 2	0.0949	0.011	0.092	0.011	0.0929	0.0176	–	–
Cycle 3	0.104	0.0111	0.113	0.018	0.0978	0.0164	–	–
Cycle 4	0.0835	0.0084	0.0912	0.009	0.111	0.0141	–	–

fermentation rate reported at both pressures was higher than at 0.1 MPa and, in consequence, the consumption of glucose and the production of bioethanol were both higher. This effect may be a consequence of the development of stress adaptation mechanisms, which improve *S. cerevisiae* growth, as well as its energetic metabolism (that involves bioethanol production). At a higher pressure level, correspondent to 35 MPa, it became harder for *S. cerevisiae* to adapt and the alcoholic fermentation was inhibited. Therefore, lower levels of pressure (such as 15 and 25 MPa) enhanced the fermentative process and the production of bioethanol, while higher levels negatively affected alcoholic fermentation, probably due to the inhibition of *S. cerevisiae* cell growth and metabolic activity. Similarly, the fermentation kinetic parameters showed that, in general, the reactions involved in alcoholic fermentation were slowed down by higher pressures (35 MPa) and accelerated by lower pressures (15 and 25 MPa). Those results are supported by the increase in yield and productivities of bioethanol throughout the four fermentative cycles, at both 15 and 25 MPa. Therefore, in the last cycle, higher yield and productivity values were observed at these pressures, compared to atmospheric pressure: this indicated yield and productivity improvements of 1.30% and 3.68% at 15 MPa, respectively; and of 3.33% and 19.78% at 25 MPa, respectively. In addition, the production of by-products of fermentation (acetic and formic acids) was reduced at these levels of pressure, leading to higher bioethanol:by-products ratios. This may bring added value to the bioethanol production process and, in addition, this approach of fermentation cycles under HP can be applied to other processes for the production of different valuable products, with interest for different industries.

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