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

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Nossa/Our Refª. Vossa/Your Refª. Data/Date
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Dear Dr. Ching T. Hou, Editor-in-Chief of Biocatalysis and Agricultural Biotechnology,

Please find enclosed the revised manuscript (ref. BAB_2020_146) entitled The use of different fermentative approaches on *Paracoccus denitrificans*: Effect of high pressure and air availability on growth and metabolism, authored by Maria J. Mota, Rita P. Lopes, Carlos A. Pinto, Sérgio Sousa, Ana M. Gomes, Ivonne Delgadillo, Jorge A. Saraiva. I would like to state that all the aforementioned authors contributed for the manuscript, as follows:

- Maria J. Mota and Rita P. Lopes (PhD) performed the experiments and wrote several sections of the manuscript;
- Carlos A. Pinto, (PhD student), assisted Maria J. Mota and Rita P. Lopes with the experiments and wrote some sections of the manuscript;
- Sérgio Sousa (PhD student), performed the organic acid analyses and data interpretation by HPLC;
- Ana M. Gomes (Full Professor) and Jorge A. Saraiva (Full professor) coordinated and designed all the experimental work and reviewed the manuscript.

Yours sincerely,

Dr. Jorge M. A. Saraiva, Ph.D.
(Full Professor)

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

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Abstract

The performance of fermentation under sub-lethal high pressure (HP) is a strategy for stimulation of microbial growth and/or improvement of fermentation titers, rates and yields. The present work intended to study the possibility of applying HP to *Paracoccus denitrificans* glycerol fermentation, considering that HP-fermentation usually involves some process constrains, such as limited air volumes. Consequently, the work was divided in two main goals: *i)* study the effects of air availability on *P. denitrificans*; *ii)* assess if the strain is able to grow and maintain metabolic activity under HP (10 – 35 MPa).

Paracoccus denitrificans growth and metabolism were highly affected by air availability. Samples under higher air availability showed considerable cell growth, but no production of ethanol or organic acids. On the other hand, samples without air had lower cell growth, but active metabolic activity (with the production of ethanol and organic acids). Regarding the HP experiments, *P. denitrificans* was able to grow at 10, 25 and 35 MPa, but to a lower extent compared to atmospheric pressure. Application of HP promoted modifications in the production of ethanol, acetate and succinate, and the fermentative profile varied according to the pressure level. Overall, the present work demonstrated new metabolic features of *P. denitrificans* at atmospheric pressure and HP conditions. It also opened the way for further studies regarding *P. denitrificans* fermentation under HP, as well as utilization of this technology for other glycerol fermentations, in particular in the case of high requirements of air availability.

Keywords: Fermentation, glycerol, stress, high pressure.

1. Introduction

Biodiesel is a fuel originated from biomass, produced from vegetable oils and animal fats, and represents one of the most promising alternatives to fossil fuels (da Silva et al., 2009). The increasing biodiesel production is raising constrains that may compromise the sustainability of the process, and one of the major problems corresponds to the formation of crude glycerol as a reaction by-product (da Silva et al., 2009; Kolesárová et al., 2011). In order to maintain the viability of biofuel economy, it became necessary to develop new and sustainable applications for glycerol, such as the use as substrate for microbial fermentation processes, resulting in the production of different value-added products, such as organic acids, alcohols, polymers, among others (Mattam et al., 2013). For example, the industrial viability of glycerol fermentation for ethanol production was assessed by Yazdani and Gonzalez, (2007), who also compared the fermentative approach with the traditional ethanol production pathway (from corn) and observed that the use of glycerol was the most cost-effective approach, as both feedstock and operational costs were lower. Another example is the production of 1,3-propanediol, a very interesting compound in synthetic chemical reactions, namely for the production polytrimethylene terephthalate (Varrone et al., 2017). Nevertheless, the use of fermentative approaches for glycerol valorization remains a challenge due to the limit number of strains able to use it as substrate, as usually other chemicals are present in crude glycerol that will inhibit microbial metabolism.

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

The interest in exposing microbial cells to HP is related to growth stimulation and/or improvement of fermentation (Mota et al., 2018). This approach involves the use of sub-lethal HP levels that affect cell growth and metabolism, but without compromising cell viability. In some cases, these modifications can represent considerable improvements, such as increased yields, productivities and fermentation rates, lower accumulation of by-products and/or production of different compounds. For instance, Picard et al. (2007) accelerated alcoholic fermentation and increased ethanol yields in *Saccharomyces cerevisiae* by performing fermentation at 5 and 10 MPa. Later on, Bravim et al. (2012) observed that pre-treatment of *S. cerevisiae* with HP led to an increase in ethanol content upon fermentation. A global transcriptional analysis revealed the over-expression of several genes related to cell recovery and stress tolerance induced by HP. The most relevant case was the gene SYM1, which was related to enhancement of ethanol production and increase of stress tolerance upon fermentation.

HP was also tested to change the metabolic selectivity of fermentative strains: application of pressures of 7 and 17 MPa during fermentation by *Clostridium thermocellum* redirected the metabolism from the production of by-products (such as acetic acid) to ethanol, compared to fermentation at atmospheric pressure (Bothun et al., 2004). Another example is the use of HP to modify the properties of biopolymers produced during fermentation. Production of bacterial cellulose by *Gluconacetobacter xylinus* under HP (30, 60 and 100 MPa) showed profound differences in morphological properties of the polymer depending on the applied pressure conditions. The cellulose produced under HP had a significantly higher density compared with the cellulose produced at atmospheric pressure (Kato et al., 2007). Regarding polymer production, Follonier et al. (2012) applied a low pressure level (0.7 MPa) to *Pseudomonas putida* KT2440 and enhanced productivity of medium-chain-length polyhydroxyalkanoate production, although with a significant decrease in specific growth rates. The effects of HP have also been evaluated in the context of food fermentation: on lactic acid fermentation, for production of probiotic yogurt (Mota et al., 2015); and in the beginning of malolactic fermentation by *Oenococcus oeni* (Neto et al., 2016). In the first case, HP was found to reduce the fermentation rate, but it was still possible to produce yogurt under pressure by extension of the fermentation time (Mota et al., 2015). The probiotic yogurt produced at 5 MPa showed different biochemical composition (unpublished results), and possibly different organoleptic properties. In the study with *O. oeni* (microorganism used by the wine industry to perform malolactic fermentation), the strain was able to perform fermentation during and after HP-stresses of 50 and 100 MPa, with some metabolic changes. For instance, the HP-stress of 100 MPa stimulated the production of the D-lactic acid isomer, relative to the L-isomer. In addition to HP-assisted fermentation processes, these are also suitable to be assisted by pulsed electric fields (Al Daccache et al., 2020a, 2020c) and by ultrasounds (Al Daccache et al., 2020b).

There is still a great potential to explore in this field, with the studies conducted so far showing promising results, not only regarding food fermentations, but also for biotechnological processes. *Paracoccus denitrificans* ATCC 17741 was selected to perform this study due to its metabolic versatility, as well as high biotechnological potential and applicability. There are some interesting but preliminary results regarding the behavior of this microorganism in response to HP stress (Deguchi et al., 2011), indicating ability to grow at 30 MPa, but complete inhibition at 40 MPa. Considering that HP can be a useful tool for improving the glycerol-based fermentation processes, and since there is limited information about the effects of pressure on *P. denitrificans* cells, we intended to perform a preliminary study on the subject. The aim of this work was to obtain general information about the effects of HP and air availability on *P. denitrificans* growth, and how the combination of both factors could be applied to re-direct cell metabolism. As a result, the present work was divided in two main goals: *i)* to evaluate how different conditions of air availability could affect *P. denitrificans* growth and metabolism; *ii)* to assess if *P. denitrificans* is able to grow and maintain metabolic activity under HP (10, 25 and 35 MPa), even with limitations in terms of volume and air supply.

2. Material and methods

2.1. Microorganism and culture media

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

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

2.2. Inoculum preparation and inoculation

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

2.3. Fermentation experiments: Effect of air availability

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

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

2.4. Fermentation experiments: Effect of high pressure

Fermentation was carried out in polyethylene bags, with controlled V_{medium}:V_{air} ratio (1.0:2.2), at 35 ºC under different HP conditions (10, 25, and 35 MPa), for 72 h. The experiments were conducted in a Hydrostatic press (FPG7100, Stanstead Fluid Power, Stanstead, United Kingdom), with a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature, using a mixture of propylene glycol and water (40:60 v/v) as pressurizing fluid. In parallel, a control sample (at atmospheric pressure, 0.1 MPa) was also performed, maintaining the exact same conditions of the HP-samples. Fermentation samples were collected over time in duplicate and all the analyses were also performed in duplicate.

2.5. Analytical methods

2.5.1. Biomass concentration

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

2.5.2. Glycerol concentration

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

2.5.3. Characterization of the extracellular medium

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

2.5.4. Statistical analysis

The results obtained for the previously indicated parameters were tested at a 0.05 level of significance and the effect of pressure was tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons' test (Tukey HSD) to identify the differences between samples.

3. Results

3.1. Effect of air availability on *P. denitrificans* growth and fermentation at atmospheric pressure

The results for variation of biomass and glycerol concentrations are shown in **Figure 1**. In samples "without air", cell growth (**Figure 1a**) was nearly inexistent and substrate consumption was low, with glycerol concentrations (**Figure 1b**) varying from 18.95 g L^{-1} in the beginning of fermentation to 17.90 g L^{-1} after 72 h. This indicates that *P. denitrificans* was inhibited by the absence of aeration/agitation and confirms that oxygen was required for cell growth, at least when using this culture medium and conditions. In the present work, the selected culture medium and conditions did not seem suitable for growth under low oxygen environments. In fact, higher air availability and agitation conditions were more suitable for *P. denitrificans* growth, since the samples "with air" showed pronounced cell growth and substrate consumption over time. In the "24 h with air + 48 h without air" samples, cell growth and glycerol consumption were similar to samples "with air" during the first 24 h, as expected, since the fermentation was performed at the same conditions during that period. Afterwards, when

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

The effects of air availability were also evaluated in terms of production of extracellular compounds by *P. denitrificans* during fermentation. This is a rather unusual approach, since typically only *P. denitrificans* intracellular products are analyzed, in attempt to find biopolymers (Kalaiyezhini and Ramachandran, 2015; Mothes et al., 2007). However, since that was not the purpose of the present work, only the extracellular products were analyzed, in particular alcohols and organic acids. As a result, ethanol, acetic and succinic acids were identified for each fermentative batch. The variation of these compounds over fermentation time is represented in **Figure 2**, while the respective yields (Y, g g^{-1}) are indicated in **Table 1**. The formation of the extracellular products was profoundly affected by air availability. It is interesting to point out that the conditions "without air", which highly inhibited cell growth and glycerol uptake, were the ones that promoted the formation of extracellular products. While all these compounds were formed in samples fermenting "without air", none of them was detected in samples with high air availability. In the mixed samples, ethanol and acetic acid (**Figure 2a, b**) were both produced, but only during the period of fermentation without air (24-72 h). It would be expected that the higher cell density accumulated in mixed samples during the first 24 h would result in increased production of extracellular compounds, compared to samples "without air". However, acetic acid concentration was lower in mixed samples, and succinic acid (**Figure 2c**) was not produced, which suggests that both samples developed different mechanisms to survive under low oxygen availability conditions. In addition, the absence of succinic acid may indicate that this compound was produced by different metabolic pathways, which were differently affected by air availability, relative to ethanol and acetic acid. In addition, in the present work, there was high ethanol production during fermentation with low aeration (**Figure 2** and **Table 1**), which resulted in a concentration of 1.00 g L^{-1} and a yield of 0.95 g g^{-1}, both considerably higher than the values obtained for acetic acid (0.15 g L^{-1} and 0.14 g g^{-1}) after the same time.

Glucose concentration showed low variation over time, from 1.07 g L^{-1} at 0 h, to 1.03 - 1.66 g L^{-1} after 72 h. In contrast, maltose initially present in the medium (0.26 g L^{-1}) was entirely consumed after 48 h of fermentation. These results indicate that ethanol, acetic and succinic acids may also be produced from maltose, and not exclusively from glycerol. To understand which substrate is used for the production of each compound, and which are the metabolic pathways used for that purpose, the metabolic profile of *P. denitrificans* should be studied in detail, using specific and suitable metabolomics tools.

In samples "without air", *P. denitrificans* showed metabolic activity, with the production of ethanol, acetic and succinic acids, but no cell growth over time. In contrast, samples "with air" showed considerable cell growth, but no production of ethanol, acetic or succinic acids.

Samples "24 h with air + 48 h without air" were able to accumulate biomass during the first 24 h, and to produce ethanol and acetic acid during the period without air. However, it did not achieve the concentrations produced without air, and succinic acid was not even detected.

3.2. Effect of high pressure on *P. denitrificans* growth and fermentation

Similar cell growth profiles (**Figure 3a**) were observed at 10 and 25 MPa, with similar biomass concentrations (p>0.05) reached after 72 h of fermentation (2.52 and 2.42 g L^{-1}, respectively). In both cases, biomass concentration was significantly lower (p<0.05) compared to the obtained at 0.1 MPa (3.01 g L^{-1}). At 35 MPa, inhibition of cell growth was even more pronounced, showing only slight variation over time, which resulted in a final biomass concentration of only 1.14 g L^{-1}. In a study concerning microbial growth under hyperaccelerations (in centrifuges), Deguchi et al. (2011) tested the effects of HP (where the pressure was generated by hyperacceleration) on *P. denitrificans* proliferation, and observed that the strain was able to grow at 30 MPa, but was completely inhibited at 40 MPa (at 30 ºC, in LB agar). This negative effect of HP on cell growth was previously reported for other microorganisms such as *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis*, at 5 and 100 MPa (Mota et al., 2015); for *Gluconacetobacter xylinus* at 100 MPa (Kato et al., 2007) and for *Clostridium thermocellum* at 7 and 17.3 MPa (Bothun et al., 2004).

Glycerol consumption (**Figure 3b**) seemed to be less affected by HP, at least at 10 and 25 MPa: in those cases, glycerol consumption after 48 h was slightly but significantly lower (p<0.05) compared to 0.1 MPa, but reached similar concentrations (p>0.05) after 72 h (in the range of 9.04 and 9.26 g L^{-1}). In contrast, biomass concentration at these same pressure conditions was always lower (p<0.05) compared to 0.1 MPa. This discrepancy between the pressure effects on growth and substrate consumption may be related to the development of stress response mechanisms to ensure cell survival.

As indicated in the previous section, glucose and maltose can both be found in the culture medium, and their presence (and consumption) may have an impact on *P. denitrificans* metabolism. Therefore, the concentrations of these sugars were analyzed throughout the fermentation time, at the end of each fermentation condition. In all cases, maltose showed the same behavior, with 0.31 g L^{-1} of maltose, initially present in the medium, being completely consumed, regardless of the pressure applied (data not shown). On the other hand, glucose consumption was highly affected by pressure, as indicated in **Figure 4**. After 48 h, glucose concentrations were similar (p>0.05) at 0.1, 10 and 25 MPa (≈ 1.38 g L^{-1}), but changed considerably at 72 h: *i)* at 0.1 MPa, the concentration remained as 1.38 g L^{-1}; *ii)* at 10 MPa, it decreased to 0.76 g L^{-1}; and *iii)* at 25 MPa, it decreased to 0.97 g L^{-1}. This suggests that HP stimulates glucose consumption during fermentation, possibly due to the higher need of

substrate and energy to ensure cell survival, with development of general and/or specific stress responses. Interestingly, glucose concentrations at 35 MPa were significantly lower (p<0.05) that for all other conditions, after 48 and 72 h (0.83 and 0.20 g L^{-1}, respectively), showing an opposite behavior relative to glycerol consumption. Therefore, fermentation at 35 MPa seems to stimulate glucose consumption, while the same effect was not observed for glycerol. These results suggest specific metabolic changes at this pressure level, which affected differently *P. denitrificans* growth and fermentation compared to the lower pressure levels.

Application of HP on *P. denitrificans* was also evaluated in terms of ethanol, acetic and succinic acids production (**Figure 5**). Different pressure levels showed different effects on the formation of these compounds. Ethanol production (**Figure 5a**) was observed for all pressure conditions, with a general increasing trend for the first 48 h of fermentation, and decreasing thereafter. The highest ethanol concentration was reached after 48 h at 0.1 MPa (1.92 g L^{-1}), but this compound was not detected after 72 h at this pressure. Similarly, ethanol concentrations of 1.34 and 1.50 g L^{-1} were observed after 48 h at 10 and 25 MPa, respectively, but these values decreased to 0.43 and 0.40 g L^{-1} at 72 h of fermentation.

Succinic acid production (**Figure 5b**) was also detected during fermentation at 0.1, 10 and 25 MPa, but not at 35 MPa. After 72 h of fermentation, succinic acid concentrations were significantly different (p<0.05) for all pressures tested: the highest succinic acid concentration was achieved for samples at 10 MPa (0.28 g L^{-1}), followed by those at 0.1 MPa (0.21 g L^{-1}) and, finally, at 25 MPa (0.13 g L^{-1}). Stimulation of succinic acid production at 10 MPa could be an interesting outcome of *P. denitrificans* fermentation under HP, since this compound is widely used as a precursor of many industrially important compounds in food, chemical, and pharmaceutical industries (Jiang et al., 2017). However, the concentrations produced by *P. denitrificans* are considerably low compared to other microorganisms typically used for that purpose, such as *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, or *Anaerobiospirillum succiniciproducens*. For these microbial strains, succinic acid concentrations in the range of ≈ 10 – 83 g L^{-1} are usually reported in literature (Jiang et al., 2017). However, further optimization is highly likely to be possible, and could increase the succinic acid production by *P. denitrificans*, possibly resulting in titers and yields more similar to the ones reported for other microorganisms.

As observed for other results in this section, fermentation at 35 MPa exhibited a different metabolic profile compared to other pressure conditions, with low ethanol production (max. 0.17 g L^{-1}), and no detected production of acetic and succinic acids. This suggests that HP is inhibiting the formation of these compounds, an effect that was also observed at lower extent for fermentation at 10 and 25 MPa. To clarify this inhibitory effect of pressure, the yields (Y, g g^{-1}) of biomass, ethanol, acetic acid, and succinic acid on glycerol were estimated at the end of fermentation (72 h) and are indicated in **Table 2**. In the cases of biomass and acetate, the yields

followed a decreasing trend with the increase of pressure, suggesting a negative impact on these features. Ethanol yields were only estimated for fermentation at 10 and 25 MPa, as this compound was not detected after 72 h at the other conditions. Due to the high variation of ethanol concentrations over time, the yields at the end of fermentation did not allow a pertinent evaluation of the HP effects on ethanol formation. In contrast, succinic acid yields reflected the behavior observed for concentrations over time, with the yield at 10 MPa (0.29 g g^{-1}) being slightly higher than at 0.1 MPa (0.22 g g^{-1}).

4. Discussion

4.1. Effect of air availability on P. denitrificans growth and fermentation at atmospheric pressure

In the first part of this work, *P. denitrificans* metabolism was evaluated under different conditions of air availability and agitation, in order to understand how they affected growth and fermentation. For that purpose, three different approaches were tested: (1) in the absence of air (or, at least, minimizing the air availability) and without agitation, i.e. samples "without air"; (2) in the presence of air (V_{medium}:V_{air} ratio of 1:5) and with agitation, i.e. samples "with air"; and (3) in a mixed process, which corresponded to fermentation with air in the first 24 h, and without air in the remaining time, i.e. samples "24 h with air + 48 h without air" (without adding fresh culture medium). We intended to assess if *P. denitrificans* was able to maintain growth and activity under all these conditions, and to analyze potential metabolic differences between them.

In literature, *P. denitrificans* strains are reported to grow both aerobically and anaerobically (Beijerinck and Minkman, 1910), but with specific requirements of culture medium composition and culture conditions at each one of these environments (Hahnke et al., 2014; Nokhal and Schlegel, 1983).

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

The high value obtained for ethanol yield on glycerol raised the possibility of production of this compound (as well as other extracellular compounds) from carbon sources other than glycerol. In fact, the experiments were performed using complex culture medium, since some ingredients had unspecified chemical composition, e.g. yeast or meat extracts. Therefore, it would be possible to have different carbon sources present in the media. In order to address this issue, the presence of alternative carbon sources was evaluated, and glucose and maltose were detected in the initial samples (0 h), as indicated in **Table S1** (supplementary material).

Overall, the results in this section showed that *P. denitrificans* growth and metabolism were highly affected by air availability. It is also important to consider that the low cell growth under low air availability can represent a serious limitation to achieve reasonable concentrations of fermentation products, due to the reduced number of cells. Therefore, the use of moderate air availability conditions would possibly favor the process, balancing the formation of biomass and the fermentative activity. In addition, these would be the most suitable conditions to perform fermentation under HP, due to volume limitations of the HP vessel used at the present work, which does not easily allow high air volumes or agitation. Considering these constrains, two different V_{medium}:V_{air} ratios (1.0:1.8 and 1.0:2.2) were selected and tested for *P. denitrificans* growth and fermentation during 24 h. As indicated in **Table 3**, specific growth rates (μ, h^{-1}), final biomass concentrations and glycerol consumption percentages were all slightly higher for the 1.0:2.2 ratio, correspondent to higher air availability. It is certain that high air availability and high specific growth rates do not only always translate in higher product formation (de Almeida et al., 2010; Kalaiyezhini and Ramachandran, 2015). However, since the study under HP would be limited by other factors, such as the lack of agitation, and the stress induced by the pressure itself, the highest ratio (1.0:2.2) was selected to proceed the studies with *P. denitrificans* at HP conditions.

4.2. Effect of high pressure on *P. denitrificans* growth and fermentation

The previously discussed experiments provided relevant information about the fermentation process and how it depends on air availability. In the second stage of the work, HP (at 10, 25 and 35 MPa) was applied during the 72 h at 35 ºC of *P. denitrificans* fermentation. Fermentation was also tested at atmospheric pressure (0.1 MPa), to use as control. The pressure effects on cell growth and glycerol consumption (**Figure 3**) showed a clear inhibitory effect, which was more accentuated with the increase of pressure level.

The inhibitory effect of pressure on cell growth may result from a wide variety of damaging effects. Generally, low pressure levels, such as the ones used in this work, may be enough to impair several cellular processes, such as motility, cell division, nutrient uptake or membrane protein function. A pressure of 50 MPa can inhibit protein synthesis and reduce the number of functional ribosomes (due to subunit dissociation), while 100 MPa can induce partial

protein denaturation (Abe, 2007; Huang et al., 2014). However, it is important to take into account that pressure effects on microbial growth are highly variable depending on several factors, such as the organisms' degree of piezotolerance, the growth stage, the extent and duration of pressure treatment, as well as other environmental parameters (such as temperature, pH, and medium composition) (Mota et al., 2013).

With the increasing pressure, relevant cell structures and functions are successively compromised until it turns impossible to withstand the stress and survive at these hostile conditions (Mota et al., 2013). In terms of cell structure, different organelles show different sensitivity to HP. For instance, lipid membranes are particularly pressure sensitive, because of its high compressible potential. Thus, changes in membrane composition and fluidity are observed under HP, as well as weakening of important protein-lipid interactions (Winter and Jeworrek, 2009). HP treatments may also affect the structure of DNA, ribosomes and proteins (Abe, 2007; Macgregor, 2002; Niven et al., 1999) possibly leading to inhibition of cell processes (such as replication, transcription and translation) and metabolic reactions essential for cell maintenance. Additional information regarding genetic regulation for pressure-induced stress can be found in Mota et al. (2013).

Under stress conditions, cell growth is usually disregarded, in order to favor other processes more relevant to their survival, i.e. cell maintenance processes. Cell maintenance refers to the fraction of substrate consumed to generate energy for functions other than the production of new cell material (Pirt, 1965). These functions include energy costs of osmoregulation, cell motility, turnover of macromolecular compounds, as well as defense mechanisms (Van Bodegom, 2007). In short, when the energy is used for these maintenance processes, bacterial growth is reduced, even if substrate consumption is maintained. Therefore, it is expectable that under stressful conditions, such as HP, biomass production will be more affected than substrate consumption. However, this effect was not observed at 35 MPa, which showed low glycerol consumption during the entire process, possibly indicating metabolic activity inhibition at this pressure.

The results of the present work for the formation of extracellular products suggest that ethanol is produced during the first hours of fermentation, and was then converted into other metabolic products. An option for the ethanol degradation pathway is the oxidation into acetaldehyde, which can be followed by oxidation into acetate. In fact, Felux et al. (2013) indicated that *P. denitrificans* Pd1222 (a derivative of DSM 413) has the genetic machinery to perform these metabolic reactions: a gene that encodes an alcohol dehydrogenase (locus tag Pden_2367) able to convert ethanol into acetaldehyde; and a gene that encodes an NAD^+-dependent aldehyde dehydrogenase (locus tag Pden_2366) that oxidizes acetaldehyde to acetate. This is also supported by the production of acetic acid (**Figure 5c**) during the period between 48 and 72 h, with the highest concentration at 0.1 MPa (0.69 g L^{-1}), followed by significantly lower

concentrations (p<0.05) at 10 and 25 MPa (0.16 and 0.17 g L^{-1}, respectively). However, these acetic acid concentrations are too low relative to the concentration of ethanol consumed. If ethanol was entirely converted into acetic acid, ≈ 2.5 g L^{-1} would be obtained at 0.1 MPa, which is quite higher than the concentration actually detected (0.69 g L^{-1}). There may be two possible explanations for this discrepancy: *i)* acetaldehyde (obtained from ethanol) was not entirely converted into acetic acid and accumulated in the cell, which is unlikely due to the high toxicity of this compound; *ii)* acetate obtained from this pathway was further converted into acetyl-CoA by an acetyl-CoA synthetase, possibly entering in a wide variety of metabolic pathways, such as the tricarboxylic acid cycle (TCA cycle) or fatty acid synthesis.

Overall, HP was found to affect *P. denitrificans* cell growth and metabolism, with different effects on substrate consumption, as well as on production of ethanol, acetic and succinic acids. These effects varied according to the pressure level, with the lower pressures (10 and 25 MPa) showing a behavior approximate to 0.1 MPa, while the highest pressure (35 MPa) presented a more extensive impact on *P. denitrificans* metabolism. As previously reported for other microorganisms (Mota et al., 2018), the application of pressure stresses resulted in particular and interesting effects on *P. denitrificans* growth and metabolism. These results open the way for application of HP to other glycerol fermentation processes, in particular to the ones with high requirements of air availability. Most information in literature concerning cell growth and fermentation under pressure is regarding anaerobic or facultative anaerobic microbial strains (thus with low oxygen requirements), since these processes are easier and simpler to perform with most of the HP equipment. It is certain that, in aerobic processes, there are more limitations that demand a more complex development and optimization, but the results obtained so far for *P. denitrificans* confirm that it is possible to perform aerobic fermentation under these lower air availability conditions.

In this preliminary study, the experiments were performed in a lab-scale HP equipment, designed for pasteurization and food technology purposes; such equipment can also be used for a broader range of applications, including extraction, hyperbaric storage or microbial growth, but with inherent limitations. In the case of microbial growth and fermentation processes under pressure, the main constrains are related to volume limitations, absence of agitation mechanisms, as well as unpractical oxygen supply. Therefore, it may be worth to perform equipment and process optimization, in order to perform further studies in more suitable and tailor-made systems, able to meet the specifications of these microbial processes. In fact, such type of pressure equipment is now becoming more widely available, making it possible to evaluate the full potential of fermentation under pressure. It should be highlighted that, in this context, this technology is highly versatile, since it can be applied intermittently, as pressure stresses, but can also be maintained during the whole fermentation time, without serious cell loss and no heating effect. Since there is no refrigeration requirement (because the continuous

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

5. Conclusions

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

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

further optimize the fermentation process at these conditions, and to improve the titers and yields. As future work, it would also be relevant to study the pressure effects on the production of different compounds, such as the intracellular biopolymers polyhydroxyalkanoates. It would also be important to evaluate the physiologic, genetic and metabolic effects of HP on *P. denitrificans* cells, using genomics and proteomics tools, as well as cell imaging techniques that allow the analysis of cell structure - with particular interest for the cell membrane.

Overall, the implications of the pressure-promoted changes in *P. denitrificans* growth and fermentation process are still not completely understood, but the results obtained in this work unveil new metabolic features of this bacterial strain, and provide useful information for further studies regarding *P. denitrificans* under pressure. Although there is no immediate and direct practical application of the study to the industry, we consider that these results show great relevance in the field, since they also open the way for application of this technology to other glycerol fermentation processes, in particular to the ones with high requirements of air availability.

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure captions:

Figure 1. Concentrations of biomass (a) and glycerol (b) throughout fermentation, for different air availability conditions: without air during the entire process (●); with air during the first 24 h, and without air during the remaining 48 h (▲); or with air during the entire process (■).

Figure 2. Concentrations of ethanol (a), acetic (b) and succinic acids (c) throughout fermentation, for different air availability conditions: without air during the entire process (●); with air during the first 24 h, and without air during the remaining 48 h (▲); or with air during the entire process (■).

Figure 3. Concentrations of biomass (a) and glycerol (b) throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

Figure 4. Glucose concentrations throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

Figure 5. Concentrations of ethanol (a), acetic (b) and succinic acids (c) throughout fermentation at different HP conditions: 10 MPa (▲), 25 MPa (●), or 35 MPa (■). Control samples (0.1 MPa) are also represented (*).

Table 1. Yields of biomass ($Y_{X/S}$), ethanol ($Y_{EtOH/S}$), acetic ($Y_{Acet/S}$), and succinic acids ($Y_{Succ/S}$) on glycerol, for fermentation under different air availability conditions.

Samples	$Y_{X/S}$ **(g g^{-1})**	$Y_{EtOH/S}$ **(g g^{-1})**	$Y_{Acet/S}$ **(g g^{-1})**	$Y_{Succ/S}$ **(g g^{-1})**
Without air	0.061 ± 0.019	0.945 ± 0.005	0.140 ± 0.089	0.106 ± 0.018
24h with air + 48h without air	0.492 ± 0.005	0.095 ± 0.002	0.028 ± 0.001	n.d.
With air	0.221 ± 0.008	n.d.	n.d.	n.d.

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

Table 2. Yields of biomass ($Y_{X/S}$), ethanol ($Y_{EtOH/S}$), acetic acid ($Y_{Acet/S}$), and succinic acid ($Y_{Succ/Gly}$) on glycerol, for fermentation under different pressure conditions.

Pressure (MPa)	$Y_{X/S}$ **(g g^{-1})**	$Y_{EtOH/S}$ **(g g^{-1})**	$Y_{Acet/S}$ **(g g^{-1})**	$Y_{Succ/S}$ **(g g^{-1})**
0.1	0.221 ± 0.008	n.d.	0.070 ± 0.006	0.022 ± 0.003
10	0.170 ± 0.012	0.043 ± 0.007	0.016 ± 0.003	0.029 ± 0.005
25	0.163 ± 0.010	0.041 ± 0.011	0.017 ± 0.001	0.014 ± 0.005
35	0.121 ± 0.056	n.d.	n.d.	n.d.

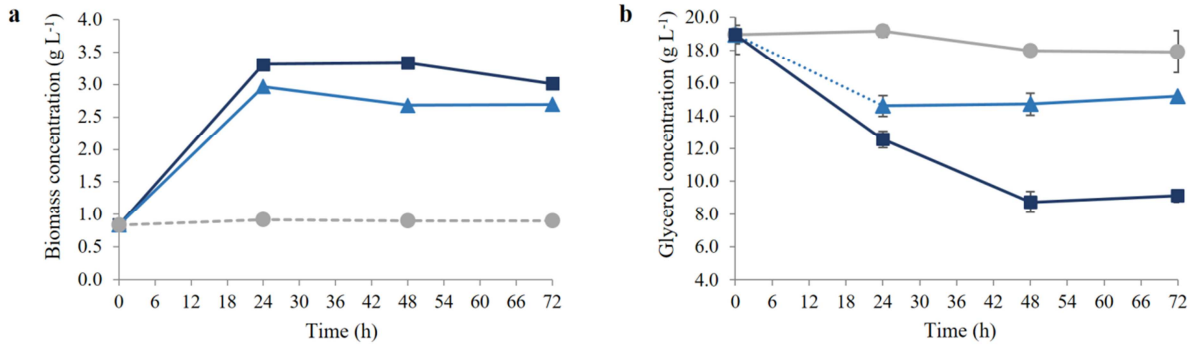
Yields were calculated from a single time-point corresponding to the end of the experiment (72 h). Values reported in the table represent the mean ± SD of two independent biological replicates, and analyzed in duplicated. N.d. indicates non-detected production of the compound.

Table 3. Specific growth rates (μ), final biomass concentrations and percentages of glycerol consumed, after 24 h of fermentation, for medium:air ratios of 1.0:1.8 and 1.0:2.2.

Samples		μ (h^{-1})	Final biomass concentration (g L^{-1})	Glycerol consumed (%)
0.1 MPa	1.0:1.8	0.098 ± 0.011	2.65 ± 0.14	28.34 ± 3.19
	1.0:2.2	0.104 ± 0.010	2.99 ± 0.16	33.54 ± 0.98

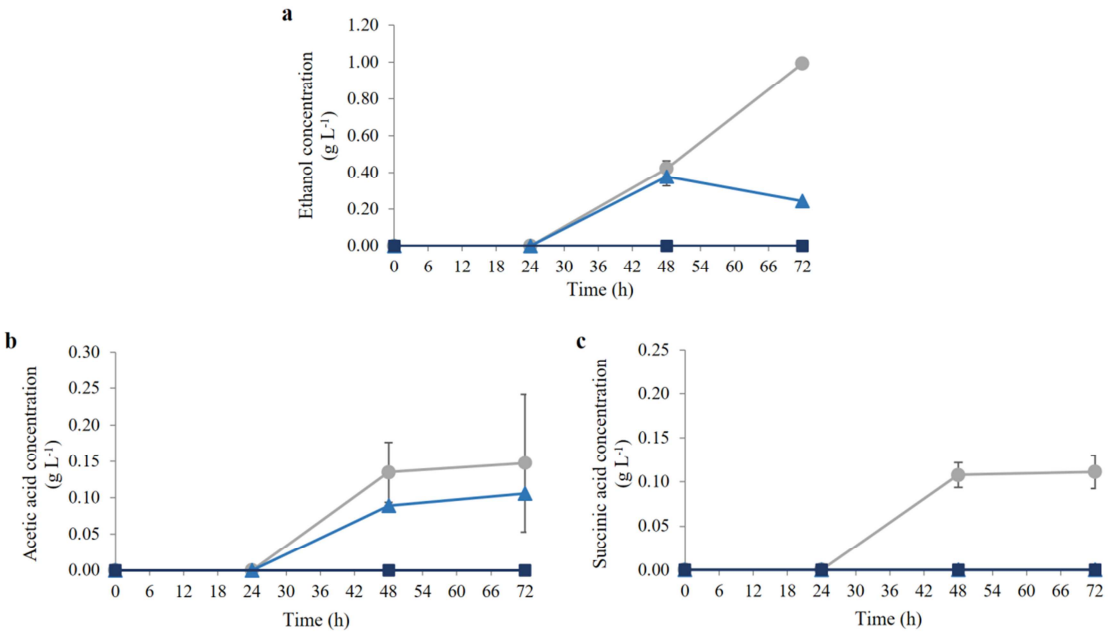
Values reported in the table represent the mean ± SD of two independent biological replicates, analyzed in duplicated.

Figure 1:



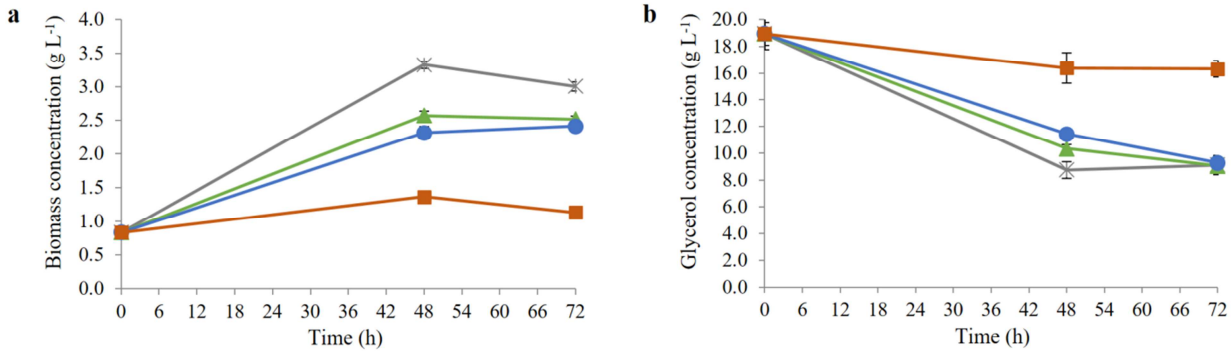
No air (●); With air on the first 24 h, followed by no air for 48 h (▲); With air (■).

Figure 2:



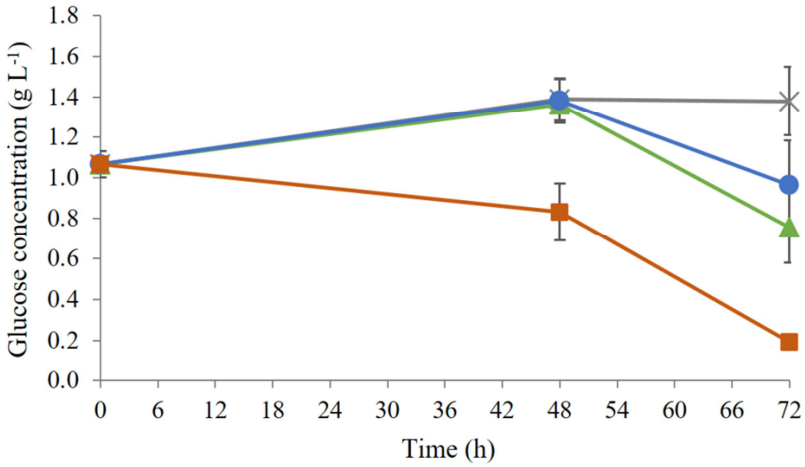
No air (●); With air on the first 24 h, followed by no air for 48 h (▲); With air (■).

Figure 3:



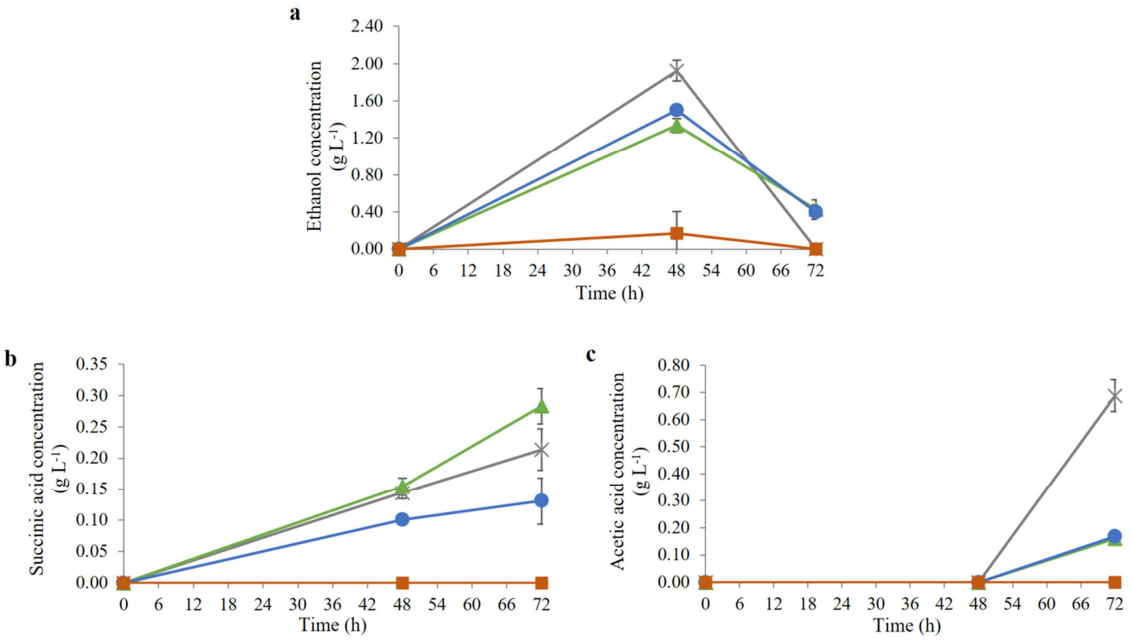
0.1 MPa (*) 10 MPa (▲), 25 MPa (●), or 35 MPa (■)

Figure 4:



0.1 MPa (*) 10 MPa (▲), 25 MPa (●), or 35 MPa (■)

Figure 5:



0.1 MPa (*) 10 MPa (▲), 25 MPa (●), or 35 MPa (■)