Vol.65: e22210238, 2022
https://doi.org/10.1590/1678-4324-2022210238
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Effects of Pressure and Magnetic Field on Glutathione Production by *Saccharomyces cerevisiae*

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Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Alexandre Rasi Aoki

Received: 14-Apr-2021; Accepted: 11-Feb-2022.

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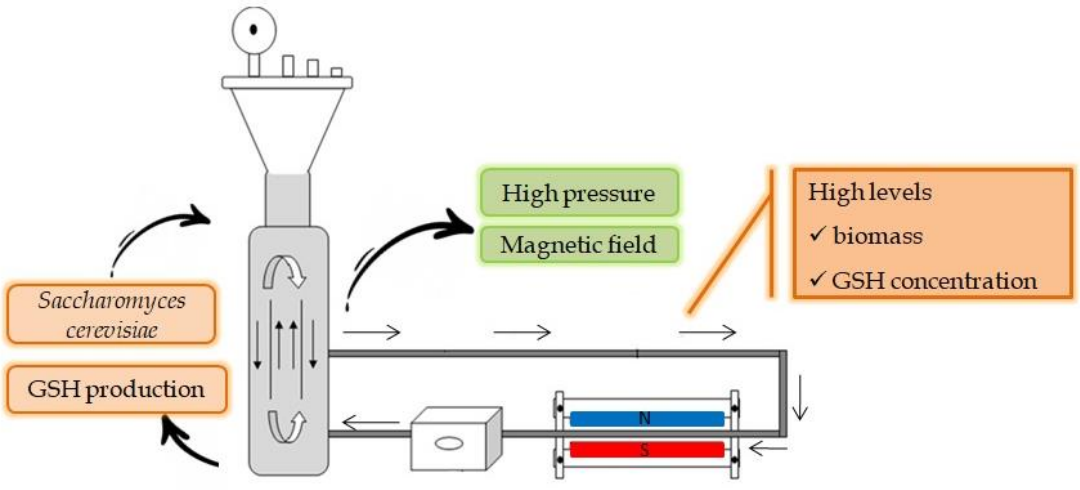
HIGHLIGHTS

- GSH may be produced by *Saccharomyces cerevisiae* in an airlift bioreactor.
- The higher the pressure, the higher GSH and biomass concentrations.
- The highest level of GSH concentration was 178.72 mg L^{-1} at 0.05 MPa after 96 h.

Abstract: *Saccharomyces cerevisiae* (*S. cerevisiae*) has been studied as a model microorganism to understand the mechanism of glutathione (GSH) production. Furthermore, magnetic field (MF) application is a novel approach to stimulate microorganism metabolism to increase growth and produce more biomolecules. This study aimed to evaluate whether GSH production by *S. cerevisiae* ATCC 7754 would be affected by different pressure levels and MF application in an airlift bioreactor. Influence of pressure (0.0 - 0.15 MPa), medium recycle velocity (3.0 and 15.0 cm s^{-1}) and MF application (0.0 and 3.0 mT) was evaluated. Biomass and GSH concentrations at high pressure and medium recycle velocity were higher than the ones in the control, whereas MF decreased biomass and GSH concentrations. The best results of biomass concentration and GSH production were 17.79 g L^{-1} and 162.92 mg L^{-1}, respectively. at 0.05 MPa, 15.0 cm s^{-1} and without any MF application. Results of the pressured airlift bioreactor showed that this kind of reactor may be used for producing GSH and for reducing energy costs.

Keywords: yeast; airlift reactor; magnets.

GRAPHICAL ABSTRACT



INTRODUCTION

Glutathione (GSH) is the most abundant nonprotein thiol peptide found in almost all living organisms [1]. It consists of L-glutamate, L-cysteine, and glycine and is very important to cells because it is a natural antioxidant [2]. GSH mitigates damaging effects of reactive oxygen species, either by direct scavenging free radicals or by indirect action of antioxidant enzymes, such as glutathione peroxidase [1]. Trehalose and GSH are examples of protective molecules produced by *S. cerevisiae* under stress conditions, such as salt shock, nutrient depletion, osmotic shock and heat increase [2]. GSH synthesis based on oxidative stress and energy metabolism in *S. cerevisiae* may be used as a strategy to increase its content [3].

In living tissues, GSH plays a fundamental role in bioreduction, protection against oxidative stress, detoxification of xenobiotics and endogenous toxic metabolites, enzymatic activity, besides sulfur and nitrogen metabolism [4, 5]. GSH may be used as a food additive to increase shelf life of meat and fruit, stabilize pigments, participate in the formulation of chemical and biological reagents and act as a parameter in environmental contamination and in food supplement capsules.

Previous studies have focused mainly on how to optimize fermentation conditions and, consequently, improve GSH productivity [6, 3]. GSH is an intracellular product in yeast, thus, its production may be enhanced in two ways: either by increasing cell biomass or by highly increasing GSH content in yeast. It is easier to increase cell mass by fermentation technology than increasing intracellular GSH content. After about 40 years of research on microbial GSH production, this topic is still very important to research and industry, since GSH and yeast enriched with GSH may be widely applied to food and pharmaceuticals [6].

Biological effects of microorganisms and macromolecules are usually affected by different pressure levels, especially high pressure which accelerates reactions that involve the volume change at the molecular level [7]. On the other hand, environmental stress induces changes in cell metabolism and accumulation of some protective molecules [4, 8]. Magnetic fields (MF) have been applied to the growth of different species of microorganisms with different results [9, 10], such as changes in their photosynthetic efficiency [11, 12], increase in pigments, such as chlorophyll and total carotenoids, besides antioxidants [13, 14], biomass composition [15], growth stimulation [16,17] and antioxidant defense system [18, 19].

MF application has shown differences in cellular behavior when cells are submitted to magnetic treatment [10]. Its effect may be either negative or positive and may include growth acceleration and changes in metabolism [20]. MF treatments may affect microorganism metabolism, such as their photosynthetic efficiency [11], photosystem II [12], carbohydrate, lipid and pigment synthesis [21], protein, lipid and carbohydrate contents [22, 23, 16], microalga growth [17], antioxidant defense system [18,19] and chlorophyll content [24]. MF affect growth and reproduction of microorganisms, cause changes in DNA synthesis and in the orientation of biomolecules and biomembranes and alter the flow of ions across membranes, thus, generating changes in the rate of cell reproduction [25, 26].

Researchers have developed several theories and explanations about how MF affect microorganisms. Studies of the influence of MF on biotechnological processes have been carried out while research on the yeast *S. cerevisiae* has been scarce. Thus, this study aimed to evaluate whether GSH production by

Saccharomyces cerevisiae ATCC 7754 would be affected by different pressure levels, medium recycle velocity and MF application in an airlift bioreactor.

MATERIAL AND METHODS

Inoculum preparation and culture media for GSH production

The yeast *Saccharomyces cerevisiae* ATCC 7754 was kept at 4°C in YM agar that contained (g L^{-1}): glucose (10.0), peptone (5.0), yeast extract (3.0), malt extract (3.0) and agar (2.0). Slants were transferred to shake-flasks (250 mL) with 75 mL YM broth to prepare the inoculum and incubated at 30°C, 150 rpm for 24 h in a rotary shaker (Certomat, BS-1, Germany).

To produce GSH, the medium contained glucose (54 g L^{-1}), yeast extract (50 g L^{-1}) and magnesium sulfate (12 g L^{-1}) [27]. Glucose was sterilized separately and added to the medium at the beginning of each experiment. Amino acids were added in two steps: firstly, 2 mM cysteine solution was added after 6 h. Then, a solution with 3.35 mM cysteine, 10 mM glutamic acid and 18 mM glycine was added after 28 h incubation [28].

Cultures at different pressures

Cultures were carried out in an airlift bioreactor (3.5 L) with concentric stainless-steel tubes. Compressed air was used for pressurizing the system for 96 h. Valves and a manometer were used for measuring pressure.

Pressure levels under evaluation were 0.0, 0.025, 0.05, 0.075, 0.1, 0.125 and 0.15 MPa. Temperature of cultures was set at 20°C by a thermostatized bath (Tecnal-TE 2005, Brazil) and total volume of the culture medium was 1.5 L. Prior to fermentation, glucose was mixed with other components, the $pH_{initial}$ was adjusted to 5.0 and 5% (v v^{-1}) inoculum was added.

Cultures at different pressures and MF application

Different conditions of pressure (0.05 and 0.15 MPa), medium recycle velocity (3 and 15 cm s^{-1}) and MF application (0.0 and 3.0 mT) were evaluated (Table 1). Two pairs of ferrite magnets were used for exposing yeast to 3.0 mT. Figure 1 shows the layout of experimental apparatus used in fermentations with MF application.

Magnetic intensity was measured by a teslameter. The airlift bioreactor was coupled with a peristaltic pump (Millan- BP-600, Brazil) so that the culture medium could circulate between the bioreactor and ferrite magnets. Control fermentations were carried out in each condition of pressure and medium recycle velocity under study (Assays 1_C, 2_C, 3_C and 4_C– Table 1).

Table 1. Conditions of assays under the influence of pressure, medium recycle velocity (MRV) and magnetic fields (MF) in a pressurized airlift bioreactor.

Assay*	Pressure (MPa)	MRV (cm s^{-1})	MF (mT)
1_C	0.05	3.0	0.0
1_{MF}	0.05	3.0	3.0
2_C	0.05	15.0	0.0
2_{MF}	0.05	15.0	3.0
3_C	0.15	3.0	0.0
3_{MF}	0.15	3.0	3.0
4_C	0.15	15.0	0.0
4_{MF}	0.15	15.0	3.0

* C – control fermentation; MF – fermentation with MF application

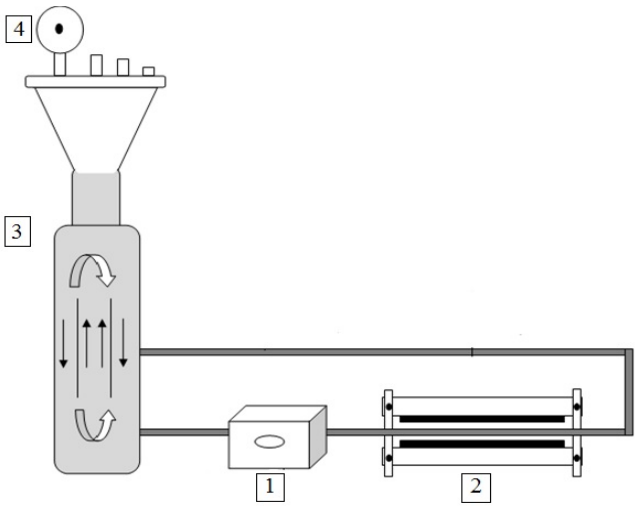


Figure 1. A schematic diagram of the experimental apparatus with magnetic field application. (Caption: 1- peristaltic pump, 2- magnet, 3- airlift bioreactor, 4- manometer).

Analytical methods

Biomass concentration was determined by optical density measurements at 600 nm by a UV–vis spectrophotometer (Tecnal, BEL photonics 1105, Brazil) and related to the optical density by the standard curve in agreement with Santos and coauthors [27].

Intracellular GSH was extracted from biomass with 40% ethanol (at 30°C for 2h). Concentration was determined by the method described by Owens and Belcher [29] and Santos and coauthors [27]. Biomass was collected by centrifugation (3,400 x g for 20 min). Colorimetric reaction was done with 0.5 mL supernatant, 1.5 mL 0.5 M phosphate buffer (pH 8) and 0.03 ml 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) and was incubated for 3 min. Ethanol solution 40% was used as blank. GSH concentration (mg L^{-1}) was found by reading absorbance at 412 nm with the use of a previously determined standard curve.

Specific GSH yield ($mg_{GSH}/g_{biomass}$) was calculated by the equation $\rho = C_G/C_B$, where C_G is GSH concentration (mg L^{-1}) and C_B is biomass concentration (g L^{-1}). Resulting data are means of triplicate samples.

RESULTS

Effects of pressure on *S. cerevisiae* growth and GSH production

Biomass concentration (g L^{-1}) and GSH production (mg L^{-1}) at different pressures are shown in Figure 2 (A and B, respectively). Biomass concentrations were higher in pressurized cultures than in assays carried out at atmospheric pressure (P_{atm}). The highest biomass concentrations were found at 0.15 MPa, after 72 h (17.7 g L^{-1}) and 96 h (17.3 g L^{-1}). Pressurized cultures increased biomass concentration after 48 h.

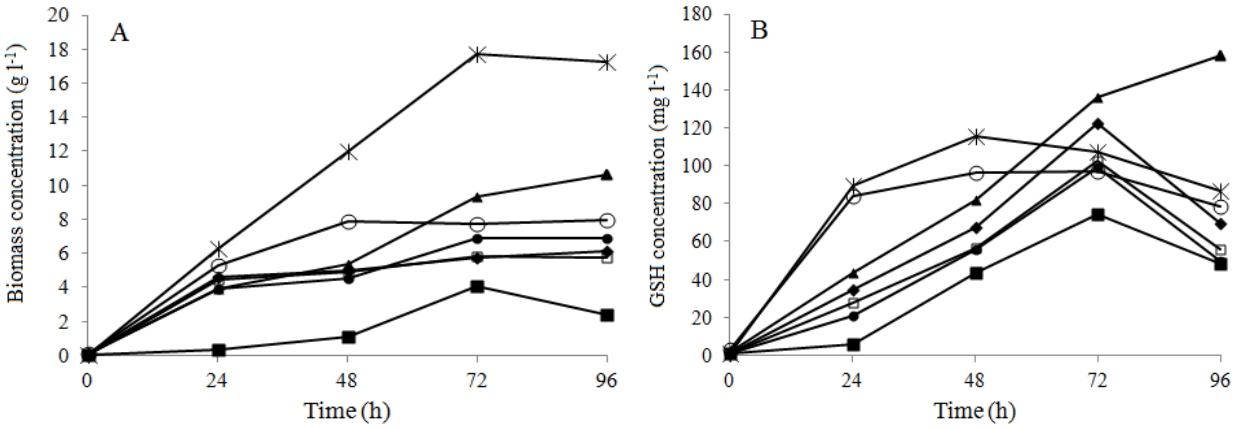


Figure 2. (A) Biomass concentration (g L^{-1}) of *S. cerevisiae* at different pressures. (■ 0.0; ◆ 0.025; ▲ 0.05; ☐ 0.075; ● 0.1; ○ 0.125 and ✳ 0.15 MPa). (B) GSH concentration (mg L^{-1}) at different pressures. (■ 0.0; ◆ 0.025; ▲ 0.05; ☐ 0.075; ● 0.1; ○ 0.125 and ✳ 0.15 MPa). Standard deviation brackets (>= 0.01) are not visible due to the scale used in this figure.

The highest level of GSH concentration was 178.72 mg L^{-1} at 0.05 MPa after 96 h of cultivation, this result was 272% higher than the one of the control (Fig. 2-B).

Effects of MF application

MF of 3 mT enhanced biomass concentration in 48 h (Assay 1_{MF}), 8% higher than in the control. In the other assays, MF was not sufficient to increase biomass concentration and values were lower than those of the control (without MF) (Table 2).

GSH concentration was higher in Assay 2_C (162.92 mg L^{-1}) after 96 h at 0.05 MPa, 15.0 cm s^{-1} (medium recycle velocity) and without any MF application (Table 2). MF increased GSH production in the first 24 h and compared 1_{MF} to 1_C, demonstrating that depending on the exposure time, MF may increase GSH production when there is low biomass production.

Table 2. Biomass and GSH concentrations for fermentation.

	Biomass concentration (g L^{-1})				
	0	24 h	48 h	72 h	96 h
1_{MF}	0.3 ± 0.01	0.30 ± 0.01	5.88 ≤ 0.01	6.38 ≤ 0.01	6.94 ≤ 0.01
1_C	0.12 ± 0.01	2.22 ± 0.02	5.36 ± 0.01	10.32 ≤ 0.01	10.06 ± 0.01
2_{MF}	0.04 ≤ 0.01	1.44 ≤ 0.01	3.72 ± 0.01	2.73 ± 0.08	3.88 ± 0.01
2_C	0.06 ≤ 0.01	3.23 ≤ 0.01	5.86 ≤ 0.01	10.34 ≤ 0.01	9.77 ≤ 0.01
3_{MF}	0.08 ± 0.01	1.92 ± 0.01	3.74 ≤ 0.01	7.16 ≤ 0.01	6.32 ≤ 0.01
3_C	0.07 ± 0.01	4.35 ± 0.01	10.78 ≤ 0.01	15.22 ≤ 0.01	14.00 ± 0.01
4_{MF}	0.18 ± 0.01	2.19 ± 0.01	1.57 ± 0.01	7.26 ± 0.01	8.80 ± 0.01
4_C	0.03 ± 0.01	5.18 ≤ 0.01	12.34 ≤ 0.01	15.78 ± 0.01	15.09 ± 0.01
	GSH production (mg L^{-1})				
	0	24 h	48 h	72 h	96 h
1_{MF}	0.11 ≤ 0.01	45.30 ± 0.01	30.75 ≤ 0.01	31.74 ± 0.01	31.45 ± 0.01
1_C	0.09 ≤ 0.01	32.55 ≤ 0.01	72.55 ≤ 0.01	135.77 ± 0.01	155.21 ± 0.01
2_{MF}	0.17 ≤ 0.01	9.45 ≤ 0.01	29.22 ± 0.01	67.24 ± 0.01	64.07 ≤ 0.01
2_C	0.09 ≤ 0.01	44.59 ≤ 0.01	83.67 ≤ 0.01	155.54 ± 0.01	162.92 ± 0.01
3_{MF}	0.94 ≤ 0.01	27.23 ≤ 0.01	40.90 ± 0.01	63.66 ± 0.01	38.67 ± 0.01
3_C	1.86 ± 0.01	34.84 ± 0.01	110.65 ± 0.01	135.56 ± 0.01	70.55 ≤ 0.01
4_{MF}	0.71 ≤ 0.01	25.17 ≤ 0.01	31.04 ≤ 0.01	42.72 ≤ 0.01	30.40 ≤ 0.01
4_C	0.26 ≤ 0.01	34.66 ≤ 0.01	89.66 ± 0.01	113.77 ≤ 0.01	112.88 ± 0.01

C – control fermentation (0 mT); MF – fermentation with MF application (3.0 mT)
Assay 1 (0.05 MPa, 3.0 cm s^{-1}), Assay 2 (0.05 MPa, 15 cm s^{-1}), Assay 3 (0.15 MPa, 3.0 cm s^{-1}), Assay 4 (0.15 MPa, 15.0 cm s^{-1}).

This study improved GSH production by a bioprocess in *S. cerevisiae*, which is the microorganism that has been used for industrial GSH production in a bioreactor. Table 3 shows that results of specific GSH yield (ρ), after 48, 72 and 96 h of cultivation, were satisfactory because, even with low biomass production (2_{MF} after 72 h and 4_{MF} after 48 h), they showed higher yield than the ones of the control. These results indicated that exposure time to MF varies during cultivation, a fact that reflects in the response under evaluated.

In pressurized culture, the highest biomass concentrations were reached under the following conditions: at 0.15 MPa, after 72 h (17.7 g L^{-1}) and 96 h (17.3 g L^{-1}). It shows that pressurized fermentations may increase biomass concentration after 48 h. The highest level of GSH concentration was 162.92 mg L^{-1} at 0.05 MPa after 96 h of fermentation, i. e., 272% higher than the one of the control. In assays with MF application, the highest value was 67.24 mg L^{-1}.

Table 3. Specific GSH yield (ρ) ($mg_{GSH}.g_{biomass}^{1}$) in the culture of *Saccharomyces cerevisiae* ATCC 7754 after 48, 72 and 96 h.

	Specific GSH yield ($mg_{GSH}.g_{biomass}^{1}$)		
Condition	48 h	72 h	96 h
$\mathbf{1_{MF}}$	5.23 ± 0.01	4.97 ± 0.01	4.53 ± 0.01
$\mathbf{1_{C}}$	13.54 ± 0.01	13.16 ± 0.01	15.43 ± 0.01
$\mathbf{2_{MF}}$	7.85 ± 0.01	24.63 ± 0.01	16.51 ± 0.01
$\mathbf{2_{C}}$	14.28 ± 0.01	15.04 ± 0.01	16.68 ± 0.01
$\mathbf{3_{MF}}$	10.94 ± 0.01	8.89 ± 0.01	6.12 ± 0.01
$\mathbf{3_{C}}$	10.26 ± 0.01	8.91± 0.01	5.04 ± 0.01
$\mathbf{4_{MF}}$	19.77± 0.01	5.88 ± 0.01	3.45 ± 0.01
$\mathbf{4_{C}}$	12.38 ± 0.01	13.13 ± 0.01	15.89 ± 0.01

C – control fermentation (0 mT); MF – fermentation with MF application (3.0 mT)
Assay 1 (0.05 MPa, 3.0 cm s^{-1}), Assay 2 (0.05 MPa, 15 cm s^{-1}), Assay 3 (0.15 MPa, 3.0 cm s^{-1}), Assay 4 (0.15 MPa, 15.0 cm s^{-1})

DISCUSSION

Effects of pressure on *S. cerevisiae* growth and GSH production

Pressure accelerates chemical reactions and leads to volume changes at the molecular level, which are the key to understand biological effects on macromolecules and microorganisms [7]. According to Cheftel [30], high pressures cause morphological, biochemical and genetic changes, mainly in membranes and intracellular parts of microorganisms. In addition, they cause changes in their operation and reproduction. It made 0.15 MPa increase biomass concentration. Some pressure values may affect yeast growth positively or negatively.

S. cerevisiae showed resistance to pressure variation in the stationary phase, by comparison with other growth phases. It impacted directly on cell physiology and metabolism correlated to GSH production and cell defense mechanism (Dong and coauthors [31]).

Specifically, Dong and coauthors [31] evaluated the effect of high pressures on accumulation of GSH and trehalose in *S. cerevisiae.* The highest GSH concentration (103 mg_{GSH}/g_{cel}) was found when 1.0 MPa was used, i. e., 68.8% higher than the result found only with P_{atm}. The use of a pressurized reactor system to produce molecules responsible for cell protection, trehalose and GSH led to yeast growth, by comparison with fermentation under P_{atm}.

The configuration of the airlift bioreactor allows good mass and heat transfer, thus, favoring quick and efficient conversion of the substrate into GSH. Biomass concentration remained constant after 72 h in cultures at pressures above 0.05 MPa. Pressurized cultivation with *S. cerevisiae* promoted stress condition in the cell, a fact that may increase yeast energy consumption and change metabolism. Therefore, there is an accumulation of substances related to the formation of singlet (reactive) oxygen in aerobic condition, responsible for cell protection.

The highest level of GSH concentration obtained in this study was higher than that found by Santos and coauthors [27], who found 154 mg L^{-1} GSH after 72h of cultivation in the same culture conditions, but in shake flasks without any pressurization. This fact corroborates results found by this study, i. e., high pressures accelerate chemical reactions, thus raising GSH concentration. Anschau and coauthors [32] evaluated the effect of the carbon source on GSH production by *S. cerevisiae* ATCC 7754 for 96 h, at 20ºC, pH 5, 300 rpm. The highest production of GSH was 119 mg L^{-1}, which was lower than GSH concentration found by this study in pressurized cultivation.

Microorganisms, such as yeasts, are known to respond to extreme conditions, such as high temperatures, high concentrations of heavy metals and high osmotic pressure. GSH was an example of an intracellular molecule that is produced when some of these conditions occurs. However, the effect of high pressure has been shown to be an extreme condition in GSH production by *S. cerevisiae.* The use of high-pressure levels to culture microorganism has been increasing in the search for the key mechanism that alters the metabolism and synthesis of compounds responsible for cell protection. Results of this study were

satisfactory to demonstrate that some pressures under investigation increased biomass and GSH concentration.

Effects of MF application

Santos and coauthors [19] reached 16.26 g L^{-1} of biomass concentration of *S. cerevisiae* after 72 h of cultivation with application of 25 mT in the first 16 h of fermentation. Besides, these authors found 16.11 g L^{-1} when exposure time was 8 h -25 mT. Ruiz-Gómez and coauthors [33] reported growth effects induced by static and sinusoidal 50 Hz MF in *S. cerevisiae* WS8105-1. Resulting data showed that MF induction of 0.35 and 2.45 mT did not induce alterations in yeast growth as occurred in this study (3 mT); *S. cerevisiae* is stimulated with intensities above than those values. Novák and coauthors [34] found inhibitory effects on *S. cerevisiae* CCY 21-4-59 growth after its exposure to MF (≤10mT and ≤60 min). Therefore, it may be inhibited since the first moments of MF application.

MF generate different effects on microorganisms, such as either increase or decrease in their growth [16,17, 24], biomass productivity [35] and specific GSH yield [19]. In this study, MF application decreased GSH production. The inhibitory effect may have occurred because the yeast was exposed to MF, different pressures and external circulation through peristaltic pump, thus changing mechanisms responsible for the development of cells and/or changes in the cytoplasmatic membrane.

The main theories which attempt to explain biological effects of MF are based on effects on the permeability of membrane ion channels. It may affect ion transport into cells and may result in biological changes in organisms. However, each microorganism reacts differently to magnetic action. According to Fojt and coauthors [36], other effects are free radical formation due to exposure to MF. Nie and coauthors [37] reported that GSH synthesis was essential for cell growth under stress conditions. Blank [38] reported that MF may be interpreted as a stressful event for cells and that the system responds to neutralize such events. Thus, if the yeast is subject to such conditions, GSH production is likely to increase.

According to Fologea and coauthors [39], biological response is non-linear, thus, only frequency or amplitude values in a so-called 'window' significant response may be recorded. Mehedintu and Berg [40] cultivated *S. cerevisiae* H192, with 0.2 mT (50 Hz) and 0.5 mT (50 Hz), for 10 h of fermentation, and got 16% inhibition at the end of the process. Novák and coauthors [34] evaluated the effect of 10 mT on *S. cerevisiae* CCY 21-4-59 after 24 min of exposure. MF decreased the number of yeasts and slowed down their growth. These results are different from those found by Santos and coauthors [19, 41] who described that only MF (without any pressure and aeration) increased *S. cerevisiae* ATCC 7754 growth from 8.7% to 43.1%, depending on the variable, *i. e*., MF induction and exposure time.

The mechanism that helps microorganism growth is directly related to the stress resulting from the external environment, such as high temperature, osmotic stress, high salt concentration, heavy metals and high pressure. In this case, pressure was the stressful situation that promoted GSH production in the first assays (without any MF). In the second case (with pressure, MF application and external circulation of medium), variables may change metabolic pathways that stimulate the synthesis of the three GS gene families (GSI, GSII, and GSIII), which are responsible for GSH synthesis.

Studies of GSH production with MF application are very scarce in the literature. Some authors have studied GSH production with the use of yeast and bacterium strains. Their investigations focused on the study of culture conditions: culture medium, bacterial strains, type of bioreactor and batch or fed-batch cultures. However, apparently, only Santos and coauthors [27, 41] have addressed MF application to GSH production with the use of yeast.

Santos and coauthors [41] observed increase in GSH production and biomass concentration by *S. cerevisiae,* but these authors used different application times, a fact that enhanced their results. In 72 h of fermentation, three "electromagnetic windows" were found positive for GSH production: 25 mT for 8 h; 25 mT for 16 h and 34.3 mT for 16 h.

Stress increased energy consumption in yeast, which induces changes in the metabolism and accumulation of some protective molecules. Trehalose and GSH are examples of these protective molecules produced by *S. cerevisiae* under stress conditions, such as nutrient reduction, osmotic shock and temperature rise [31].

Novák and coauthors [34] studied three bacterial strains and the yeast *S. cerevisiae* exposed to MF, and concluded that bacteria were more sensitive to exposure to MF than the yeast. Differences between the bacteria and the yeast may be due to the type of cell (eukaryotic or prokaryotic). Santos and coauthors [19] showed that when MF were applied to cellular suspensions of *S. cerevisiae*, the combination of exposure times and MF intensities may enhance GSH yield and biomass concentration in the fermentation process. Therefore, high GSH yields may be correlated to MF application as described above. It may help to simulate

processes that aim to increase cell growth and metabolic activities to reach higher amounts of GSH produced by *S. cerevisae* strains (Lopes and Borzani [42]; Hristov and Perez [43]; and Santos and coauthors [19]).

CONCLUSION

The pressurized airlift bioreactor may be used as a potential bioreactor for GSH production. The medium recycle system may be used to scale up biomass and GSH production with no major changes in the reactor. High pressure positively affected GSH production and biomass concentration, but when it was combined with MF application, the effect decreased. Even though MF application to fermentation processes is quite unexplored, results are promising, especially in cultures with yeasts.

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, the Foundation Support Research of Rio de Janeiro (FAPERJ) of Brazil and by the Coordination for the National Council for Scientific and Technological Development (CNPq).
Conflicts of Interest: The authors declare no conflict of interest.
Acknowledgments: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, National Council for Scientific and Technological Development (CNPq) and Foundation Support Research of Rio de Janeiro (FAPERJ) of Brazil.

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