Effect of moderated pressure on the activity and termostability of three microbial enzymes: catalase, β-galactosidase and alcohol dehydrogenase

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Abstract

The effect of moderate gas pressure on the activity and termostability of three microbial enzymes: catalase from *Aspergillus niger*, β-galactosidase from *Escherichia coli* and alcohol dehydrogenase from *Saccharomyces cerevisiae* was study. Batch assays were carried out in a hyperbaric bioreactor at increased pressure up to 9 bar using the activity at atmospheric pressure as pattern. Interactions between the effects of pressure and temperature on the activity of β-galactosidase and alcohol dehydrogenase were also studied. Experiments with air, N_2 and CO_2 were performed with catalase.

It was observed that the increase of air pressure didn't affect the activity of catalase. When the pressuring gas was CO_2 at 3 bar or higher, a large loss of activity was observed. However, an increase of catalase activity between 3 bar to 6 bar of pure N_2 was found.

Among all the three enzymes, β-galactosidase was the most sensitive to pressure rise from 1 bar to 9 bar, since its activity was reduced by long periods of pressure exposure and it was the only enzyme that showed termostability reduction by pressure increase. Studies with β-galactosidase and alcohol dehydrogenase didn't show any interaction between air pressure effects and temperature in the reaction velocity.

1. Introduction

For many years, pressure was disregarded by biochemists and microbiologists, due to the idea of incompability of life in pressure environments different from atmospheric pressure. Nowadays, there is a growing interest on the part of researchers of biosciences and biotechnology, to introduce pressure as a variable acting on biosystems. This is partly due to the discovery of microbial life on high pressure environments, such as the deep sea (Masson 2002).

A little increase on the total pressure on a reactor can improve biotechnological process, mainly if a gas component is involved. Some authors have already reported the behaviour of microorganisms in hyperbaric conditions (Yang 1989; Wendlant 1993; Belo 2003).The increase of total air pressure in aerobic cultures improved the production of biomass since cell growth limitation due to high demand of oxygen by the cell was prevented (Yang 1989; Wendlant 1993; Pinheiro 1996; Belo 1999; Belo 2003). Nevertheless, there is an inhibition of cell grow if the pressure gets too high, therefore there is a limit to the pressure increase (Belo 2003). The oxidative stress caused by the increase of oxygen partial pressure is the main reason of this effect, essentially because cell anti-oxidant systems are not enough efficient (Moradas-Ferreira 1996). The metabolic changes showed by some microorganisms when grown under pressure suggest different degrees of inhibition on several enzymes that are involved in their metabolic system (Belo 2003). On the other hand, *Pinheiro et al.* (Pinheiro 2003) observed an increase of metabolic activity *in vivo* of the enzyme β-galactosidase from *Kluyveromyces marxianus* by increasing the total air pressure. Increasing the total air pressure to improve the oxygen

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transfer rate in microorganisms cultures with high oxygen demand showed to be a very efficient way to optimise the expression of recombinant proteins in *Escherichia coli* cultures (Belo 1998).

In this work, an attempt to follow the microbial enzyme activities under hyperbaric pressure *in vitro* was made, to compare with previous work on the effects of hyperbaric pressure on *in vivo* enzymes (Pinheiro 1996). On the other hand, it was intended to find potential pressure conditions for the optimization of the enzyme reactions studied.

2. Materials and methods

2.1 Enzymes and chemicals

Catalase (EC 1.11.1.6) from *Aspergillus niger* (Sigma C-3515) was used. The determination of catalase activity was performed by Beers and Sizer method (Beers 1952), being a catalase unit defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute at 25ºC and pH=7.

β-galactosidase (EC 3.2.1.23) from *Escherichia coli* (FLUKA 48274) was used. A catalytic unit of this enzyme is the amount of enzyme that hydrolyses 1μmol of pNPG per minute at 25 ºC and pH=7.8 (Domingues 1996). A wavelength of 405 nm was used to measure the pNP produced with a milimolar extinction factor of 6.8 mM^{-1} cm^{-1} .

Alcohol dehydrogenase (EC 1.1.1.1) from *Saccharomyces cerevisiae* (Sigma A-7011) was used. This enzyme was stored at -20ºC in the solid form or in concentrated solution of 1 mg/mL. From this stock solution, and immediately before the assays, dilutions were prepared using cold sodium phosphate buffer 10 mM, pH=7.5 in order to obtain the required concentration. Reaction velocity was determined by Kägi e Vallee method (Kägi 1960), measuring the reduction rate of NAD^+ to NADH by the increasing absorbance at 340 nm. An enzyme activity unit is described as the amount of enzyme that reduces 1 μmol of NAD^+ per minute at 25 ºC and pH=8.8. A milimolar extinction factor of 5.47 mM^{-1} cm^{-1} of NADH was used for the unit's determination.

2.2 Reactor

A cylindrical stainless steel reactor (Whithey 304-HDF4) of 300 mL of capacity was used to run the hyperbaric assays. The reactor was horizontally installed in a shaker bath (EX-600-Neslab) that allows agitation and temperature control. Each gas, from a gas container, was fed to the reactor through two valves. The pressure of the inlet gas regulates pressure. The pressure transducer (HD 9220, Delta OHM) measures the total pressure inside the reactor and overpressure is released by another valve. The last valve was used to charge the reactor with the reactants and also to collect the liquid samples. The introduction of the reactant mixture was performed with a peristaltic pump (MS REGLO-Ismatec).

2.3 Enzyme reactions and assays

Before any assay, the reactor was washed with distilled water and, afterwards the reactant mixture was introduced (30 mL). 2 mL samples were collected by gently open the charge/discharge valve along reaction time intervals. Tube purge was made between samples.

2.3.1 Catalase

The reaction studied was the degradation of hydrogen peroxide to oxygen and water and was evaluated by measuring the disappearance of hydrogen peroxide, spectrophotometrically at 240 nm. Diluted solutions were prepared from the initial stock catalase solution, using 50 mM phosphate buffer, pH=7. Since oxygen is a hydrogen peroxide decomposition product, not only air has been used to perform the assays, but also carbon dioxide and nitrogen. By this way, it was possible to evaluate the effect of the presence of one of reaction products in the pressuring gas. After sample collection, the enzymatic reaction was immediately stopped by adding KCN to a concentration of 8 mM. All the assays performed in order to evaluate the effect of pressure on catalase activity were made at 25ºC, the optimum temperature for this enzyme.

2.3.2 β-galactosidase

The studied reaction was the hydrolysis of lactose to glucose and galactose and the reaction velocity was evaluated by measuring the amount of glucose produced. Enzyme and lactose solutions were prepared with phosphate buffer, pH=7.8. The temperature was stabilized at 37 ºC, optimum temperature. Only air was used to pressurize the reactor since there was no gas components involved on the studied reaction. After sample collection, the inactivation of the enzyme in samples was

performed by thermal shock (2 min, 100 °C). Quantification of glucose was performed with an enzymatic kit (Spinreact 1001190).

2.3.3 Alcohol dehydrogenase

The enzyme alcohol dehydrogenase (ADH) catalyses the ethanol oxidation to acetaldehyde, with the parallel reduction of NAD^+ to NADH. Solutions of enzyme and ethanol were prepared with phosphate buffer pH=8.8. Reaction rate was evaluated by measuring at 304 nm the amount of formed NADH. The assays were run at 25 °C, optimum temperature for this enzyme. 2,2,2- trifluoroetanol was added to stop the reaction, immediately after the collection of samples.

2.4 Effect of pressure on enzyme thermostability

In order to study the effect of pressure on enzyme thermostability, experiments were performed at tree different temperatures: 25°C, 50 °C and 75 °C. Two values of air pressure were applied: 1 bar and 9 bar to catalase and β-galactosidase, and 1 bar to 6 bar to alcohol dehydrogenase. On these assays, the reactor was filled with the solution of each enzyme, prepared in phosphate buffer, then pressure was established at the required value and, at defined times, samples were collected to determine the remaining enzyme activity.

3. Results and discussion

3.1 Catalase

The effect of pressure on catalase activity was assessed by the determination of the first order kinetic constant, k, of the hydrogen peroxide degradation under different values of pressure and gas composition. According with the results obtained and shown on Figure 1, an influence of the nature of the gas on pressure effects was observed. (ANOVA, P <0.01).
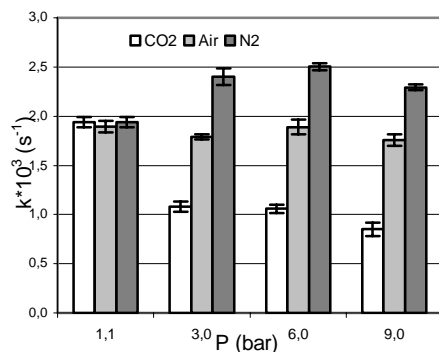

Figure 1:

Effect of gas pressure on the kinetic constant, k, of the degradation of hydrogen peroxide at T=25 °C and pH=7, [catalase] = 1.5 U/mL, $[H_2O_2]_{initial}$ = 23 mM. Data are mean±standard deviation of three independent experiments.

When air were used to pressurize the system, no alteration on the first order constant , was observed, in fact, with 95% confidence level (*t*-student test), that air pressure has no effect on catalase activity. Previous studies performed by Vasudevan and Takur (Vasudevan 1994), using bovine liver catalase on a semi-batch reactor, at a temperature of 25 °C and pH=7.0, with an initial concentration of hydrogen peroxide of 4.9 M and air pressure between 1.5 bar and 4 bar, showed that the maximum velocity for this reaction was observed when air pressure was 3.1 bar. It is important to remark that the assays performed by these authors were based on the stationary state study and not on the study of initial velocities, as in the herein reported work.

Since air pressure does not affect the first order constant, similar assays were performed using an inert gas to pressurize the system to discriminate the effects of pressure from the effects of oxygen.

When N_2 were used to pressurize the system, it was observed a maximum of activity at pressure values between 3 bar and 6 bar. In fact, the increase of k by N_2 pressure increase from 1.1 bar to 6 bar is significative at a 95% confidence level. Nevertheless, an increase of N_2 pressure from 6 bar to 9 bar has a negative effect on k.

Carbon dioxide is well known as a gas useful to microorganisms inactivation (Nguyen 2002) when used in high pressure values. In this work, it was intended to evaluate the effect of CO_2, at moderated pressure, on the activity of catalase. Thus catalase runs were repeated with CO_2 to pressurize the reactor. Assays were run using two different concentrations of enzyme: [catalase] =0.15 U/mL and [catalase] =1.5 U/mL. The CO_2 pressure was raised from 1 bar to 9 bar. Evaluating the ratio k/k_0, where k_0 is the value of k, the first order constant, at 1 bar, as the pressure of CO_2 increases, it was

possible to observe that when [catalase] =0.15 U/mL, there was a decrease of 77% in the ratio k/k_0 and when [catalase] =1.5 U/mL the decrease was only about 56%; this results confirm the knowledge that enzyme solutions are more stable in the concentrated form.

Previous assays preformed in a pressurized reactor equipped with a pH sensor showed that an increase of P_{CO2} from 1 bar to 2 bar induced reduction of buffer pH from 7 to 6. This variation on pH values can be explained by the fact that the reaction between CO_2 and water produces carbonic acid which decomposes into hydrogen carbonate ions that cause a decrease in pH. Thus, the reduction of the enzyme catalase when exposed to CO_2 pressure could be attributed to the reduction of buffer pH to values different from the optimal pH for the enzyme catalase.

The effect of CO_2 pressure on the catalase activity, given by kinetic constant, k, for the studied pressure range can be described by equation 1.

$$k = k_0 \times P_{CO_2}^{\alpha} \qquad\qquad (1)$$

Fitting the data to equation 1, k_0 and α were obtained for the two assays conditions: [catalase] =0.15 U/mL and [catalase] =1.5 U/mL. The obtained value of k0 increased from 0.0003 when the enzyme was in more diluted solution to a value of 0.0019 in assays performed with the more concentrated enzyme solution. The α parameter suffered a variation from a value of -0.85 to a value of -0.37, also by increasing the concentration of enzyme solution from 0.15 U/mL to 1.5 U/mL.

The difference of the absolute value of α for the two enzyme concentrations shows that the inhibition effect of CO_2 pressure is dependant of enzyme concentration: when the enzyme was in a diluted solution it showed more sensibility to CO_2 pressure than in concentrated solution. This statement was expected by the knowledge that, in general, enzyme stability decreases as its solutions become more diluted.

3.2 β-Galactosidase

The effect of pressure on β-galactosidase activity was studied using the lactose decomposition as reaction system. Previous assays, performed at atmospheric pressure, showed that glucose formation rate was constant until values of 50 % to 60 % of lactose conversion.

Figure 2 resumes the results obtained for lactose conversion evolution trough time under different conditions of air pressure.
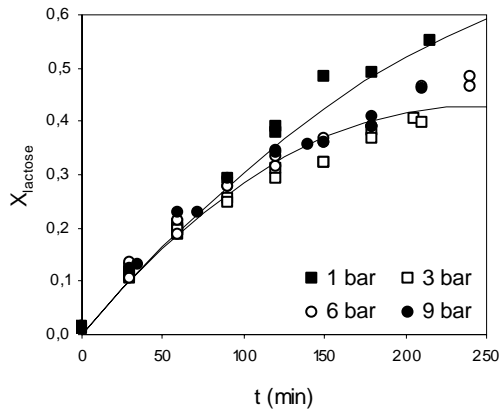

Figure 2:
Effect of air pressure on activity of β-galactosidase, in terms of converted lactose ($X_{lactose}$). Temperature= 37 ºC, pH=7.8 [lactose]$_0$=50 g/L and [β-galactosidase]=1200 U/mL.

It is possible to observe that, in the first hour of reaction, there is no difference between the lactose conversion obtained at atmospheric pressure and that at higher pressure values. Thus, air pressure does not affect the initial rate of the reaction. Nevertheless, as reaction time goes on, a decrease on lactose hydrolysis velocity was observed, which was more pronounced in the assays performed at air pressure higher than atmospheric value. In fact, the increase on air pressure causes a decrease of about 20% to 30% of the final conversion. Those results suggest that long exposition time of β-galactosidase to air pressure of 3 bar, or higher, cause enzyme deactivation.

3.3 Alcohol dehydrogenase

In order to study the effect of air pressure on alcohol dehydrogenase activity, the ethanol oxidation to acetaldehyde, with the parallel reduction of NAD^+ to NADH was followed. The range of air pressure values studied was 1.1 bar to 6.8 bar. Activity studies were based on determination of initial velocity
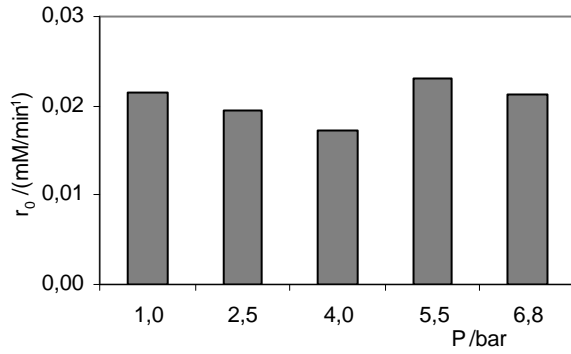
of NADH formation.

Figure 3:
Effect of air pressure on the initial velocity (r_0) of reduction of NAD^+ to NADH, by alcohol dehydrogenase at T=25 °C and pH=8.8, [alcohol dehydrogenase] = 0.025 U/mL. Values are means of three independent experiments.

From the results in Figure 3, it can be seen that air pressure at 25 ^{o}C, in the studied range, did not inhibit alcohol dehydrogenase activity, when temperature is settled to 25 ºC. Although Figure 6 shows a decrease of r_0 from 1.1 bar to 4.0 bar, that decrease is not confirmed at higher pressure values. In fact, for an air pressure of 6.8 bar, the enzyme activity is the same than the one observed at 1.1 bar. Moreover, for a 95% confidence level (*t*-student test), it can be stated that, r_0 values obtained in assays performed at 4 bar and 5.5 bar are not statistically different.

The insensibility of alcohol dehydrogenase to air pressure in the studied range (until about 1.43 bar pure O_2) could be explained by the presence of zinc in the active site of this enzyme, which protects it from oxygen inactivation, as it was proposed by Tamarit et al. (Tamarit 1997).

3.4 Termostability assays
In order to study the effect of air pressure, at 1 bar and 9 bar, on enzyme termostability, enzyme solutions were incubated at three different temperatures (25 ºC, 50 ºC and 75 ºC) and submitted to air pressure for defined time intervals. Samples were collected trough time to monitor residual activity.

The kinetics of enzyme activity decrease followed a first order kinetic. Data fitting to that model enabled the determination of the kinetic constant from which the half-life was assessed applying equation 2.

$$t_{1/2}= - (Ln (50/A_0))/ k \qquad\qquad (2)$$

Table 2 presents the results obtained for the enzymes studied, presenting the half-life values, in minutes.

Table 2: Effect of air pressure on enzyme termostability

Temperature → Pressure ↓	T = 25 ºC	T = 50 ºC	T = 75 ºC	Enzyme
P = 1 bar	6171	90	3.4	Catalase
P = 9 bar	5589	85	1.9	
P = 1 bar	3118	250	10.3	β- Galactosidase
P = 9 bar	203	25	10.2	
P = 1 bar	173	12	--	Alcohol dehydrogenase
P = 6 bar	178	9	--	

The half life time of catalase and alcohol dehydrogenase at 25ºC and 50ºC were not significantly affected by air pressure increase (1 bar to 9 bar to catalase and 1 bar to 6 bar to alcohol dehydrogenase). At 75ºC, catalase shows a 45 % half life time decrease at 9 bar, comparing to $t_{1/2}$ at 1 bar. However, as at this temperature the effect of temperature on enzyme deactivation was too strong to safely conclude on the effect of pressure.

The termostability of β-galactosidase was very affected by air pressure at 25ºC and at 50ºC, the half life time decreases 90% between 1 bar to 9 bar at bought temperatures. As the same away as it was observed for catalase, the effects of pressure at 75ºC are affected by significant experimental errors due to the high level of enzyme inactivation by the temperature. This effect was also observed with the ADH which activity was quickly inhibited at 75 ^{o}C.

4. Conclusion

The main goal of the present work was the study of the influence of moderated gas pressure in the activity of microbial enzymes *in vitro.*

In general it is possible to conclude that all studied enzymes were not affected by air pressure in the studied range (1 bar to 9 bar) at the optimal temperatures. It allows concluding that there is no advantage to use increased pressure to optimize the industrial process using those three enzymes. On the other hand, it can be stated that the three enzymes stand well increased air pressure.

Nevertheless, exceptions were observed, depending of the gas and the enzyme used. The maximums activity of catalase was observed between 3 bar to 6 bar of N_2 and the lowest activity was observed at 3 bar or higher of CO_2 pressure. On the assays using β-galactosidase and alcohol dehydrogenase, the initial velocity was not affected by the hyperbaric condition, but β-galactosidase showed a partial inhibition to do long exposition to raised air pressure.

The assays of termostability showed that the half-lives of catalase and alcohol dehydrogenase were not affected by pressure. Contrarily, β-galactosidase showed accentuated loss of stability by the simultaneously increase of temperature and air pressure. Among all of the enzymes studied,β-galactosidase is the most sensitive enzyme to the effect of air pressure increase.

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