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Physiological behaviour of *Saccharomyces cerevisiae* under increased air and oxygen pressures

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Saccharomyces cerevisiae, in a pressure batch reactor, coped with higher air (1.2–3 bar) pressures better than with pure oxygen pressures (1.2–3 bar) for an equivalent dissolved oxygen concentration. However, pure oxygen pressure enhanced ethanol production. Both pressures did not influence the type of metabolism followed by the organism which was always oxidoreductive. Growth was inhibited with the increase of air and pure oxygen pressure and almost completely inhibited with 8 bar of pure oxygen. Above 3 bar activities of mitochondrial superoxide dismutase and glutathione reductase increased with air pressure, but cytosolic superoxide dismutase and catalase increased activity only in pure oxygen pressure.

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Introduction

Processes with microbial cultures are generally carried out at controlled and defined conditions. In batch processes several parameters change with time, i.e., concentrations of cells, substrates, and products. Spatial gradients of these environmental parameters may be avoided in laboratory reactors by efficient mixing. However, with an increase in reactor size, substrate and product concentrations, and temperatures gradient must be taken into account and, especially for dissolved gases, it is not possible to attain spatially constant concentrations in large bioreactors. The total pressure is a function of liquid height. As a consequence local differences of gas solubility, e.g., for O_2 and CO_2, occur in industrial bioreactors. In some industrial bioreactors local differences in pressure up to the order of 10 bars may occur (Onken and Liefke, 1990; Sweere *et al.*, 1988). Obviously for a more detailed analysis of pressure effects O_2 and CO_2 partial pressures must be considered. From these, O_2 partial pressure deserves more interest since it is an indispensable nutrient in aerobic processes and can be the limiting factor for growth or product formation (Onken, 1990; Sweere *et al.*, 1988).

O_2 may have toxic effects on aerobic microorganisms at partial pressures not much higher than in air at 1 bar. This seems surprising at first sight, because aerobes need O_2 for their metabolism (Onken, 1990). It is generally accepted that O_2 toxicity is initiated by univalent reduction of O_2 which finally leads to damage in some way or other to enzymes, nucleic acids or lipids. As a safeguard against O_2 radicals, aerobic organisms have developed enzymes which are able to transform these reactive species (superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$)) into non-reactive ones (Onken and Liefke, 1990; Gille and Sigler, 1995). Both prokaryotic and eukaryotic cells acquire adaptive advantages by a response to oxidative stress (Mager and De Kruijff, 1995). The more important ones are the cytosolic and mitochondrial superoxide dismutase, glutathione reductase and catalase (Onken and Liefke, 1990; Gille and Sigler, 1995; Clarkson *et al.*, 1991).

Clearly, over-provision of O_2 in the pre-fermentation aeration stage of brewing may have an adverse effect on yeast cells. This paper reports an investigation into the effects of the air and pure oxygen pressures on the efficiency of the fermentation and on the enzymatic levels in the baker's yeast, *Saccharomyces cerevisiae*. This study may be helpful to learn more about the physiological behaviour of this yeast as an eukaryotic model (Rose, 1981; Sweere *et al.*, 1988).

Material and methods

Microorganism

Commercial baker's yeast was used at 2 g biomass/l initial concentration.

Operation conditions

Batch cultivation was carried out using a cylindrical pressure reactor with a total volume of 300 ml. The working volume was of 150 ml. The mineral medium (5 g KH_2PO_4/l, 1.2 g $(NH_4)SO_4$/l, 0.4 g $MgSO_4 \cdot 7H_2O$/l and 1 g yeast extract/l) contained

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4 g glucose/l. The temperature was controlled at 30°C and agitation at 150 rpm. The air feeding rate was of 150 ml/min, 1 vvm. Three different air pressures: 1.2, 3 and 6 bar, and 5 different O_2 total pressures: 1.2, 2, 3, 4 and 8 bar were studied.

Analytical methods

Growth was measured by optical density at 620 nm, and converted to g cell dry/l (or g biomass/l). Viability was determined by epifluorescence technique with acridine orange (orange–live cells, green–dead cells). Glucose was determined using the 3,5-dinitrosalicilic acid (DNS) method. Ethanol, acetaldehyde, 2,3-butanediol, propanol and ethyl acetate were determined by GC and glycerol by HPLC. ATP was measured by bioluminescence according to Siro *et al.* (1982). Protein was measured using coomassie blue method.

Enzymatic assays

Superoxide dismutase (SOD) was assayed by the method of McCord and Fridovich (1969), mitochondrial superoxide dismutase (MnSOD) by the same method but in the presence of 0.6 M KCN, and cytosolic superoxide dismutase (CuZnSOD) by difference between the two rates. Catalase was assayed using the method described by Beers and Sizer (1952) and glutathione reductase was assayed by the method of Smith *et al.* (1988).

Results

Air effects on growth and production

Three different air pressures were investigated: 1.2, 3 and 6 bar. Pressures were selected considering the existence or not of enough O_2 transfer to provide an optimum oxidative metabolism. The O_2 transfer rate required for growth with maximum oxidative capacity of the cell is 40 mmol O_2/l.h with 5 g biomass/l (Sonnleitner and Kappeli, 1986), the maximum specific O_2 consumption rate corresponding to 8 mmol O_2/g biomass.h. In this case the O_2 transfer rate, with an initial concentration of cells of 2 g/l, is 512 mg O_2/l.h. Table 1 shows that above 3 bar there is enough O_2 for the cellular growth.

Typical batch growth curves for the experiments with 1.2, 3 and 6 bar air pressure are shown in Figure 1. With 3 and 6 bar air pressure, yeast cultures showed a diauxic growth. During the first phase cells consumed glucose, which is metabolized oxidoreductively, producing biomass and ethanol. During the second phase, ethanol is metabolized oxidatively by the cells. At 3 bar pressure, ethanol is metabolized more rapidly than in 1.2 bar air pressure, although the biomass yield decreases.

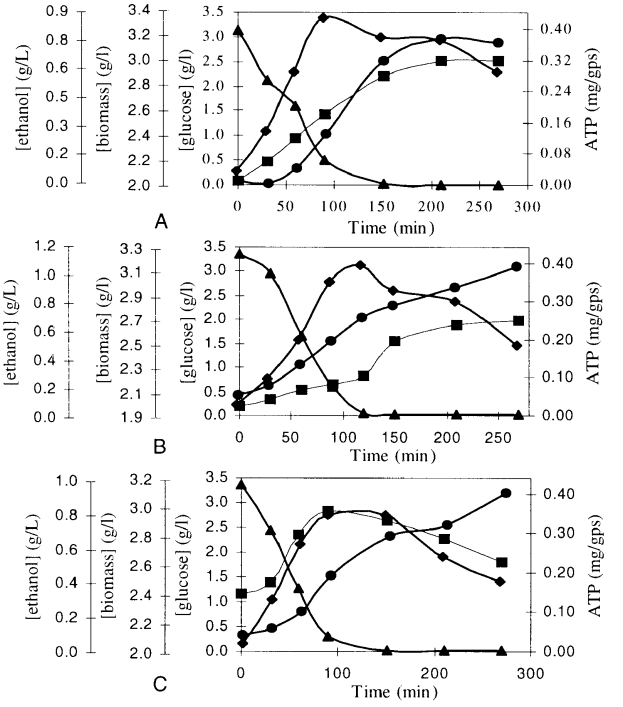

Figure 1 Batch growth of *Saccharomyces cerevisiae* at different air pressures: A) 1.2 bar, B) 3 bar and C) 6 bar (● biomass; ◆ ethanol; ■ ATP; ▲ glucose).

Table 1 also shows yields variation with pressure. As pressure increases biomass and ethanol yields decrease. Although biomass yield has not a big decrease with pressure, even having sufficient O_2, the specific growth rate decreases a lot. Like biomass yield, ATP yield also decreases with pressure. The time course of ATP concentration (Figure 1) followed growth behaviour on its growth phases for all air pressures studied. The final viability is similar for all the experiments (Table 1). The viability was maximum at the end of the first logarithmic phase (on glucose) and remained constant in the stationary phase (data not shown).

While for other authors glycerol concentration varied between 2 and 5 g/l, in this work the glycerol content did not change with pressure. There was no accumulation in by-products, like propanol, ethyl acetate, 2,3-butanediol and its concentrations were very low (Kuriyama and Kobayashi, 1993). No toxic effects could therefore be caused by these metabolites.

Air effects on the antioxidant enzymes

Four different enzymes were analysed. As Figure 2 shows, catalase activity decreased during the three different growth phases, meaning that there was no

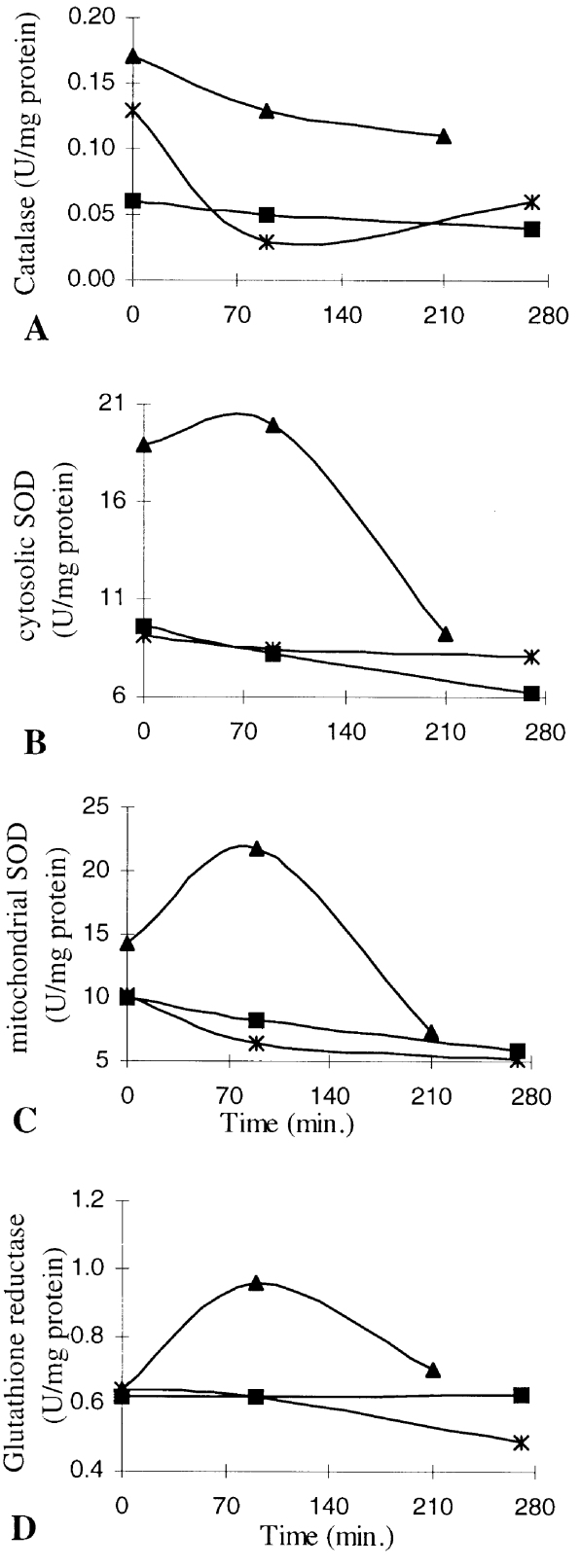

Figure 2 Enzymatic activity for different air pressures: A) Catalase; B) cytosolic superoxide dismutase (CuZnSOD); C) mitochondrial superoxide dismutase (MnSOD); D) Glutathione reductase. The points represented correspond to the growth phases: lag, logarithmic and stationary (■ 1.2 bar; ▲ 3 bar; ★ 6 bar).

Table 1 Changes in biomass, ethanol and ATP yields, specific growth rate, productivity and metabolite residual concentrations with air pressure in batch experiments

Air pressure (bar)	*1.2*	*3.0*	*6.0*
OTR (mg O_2/l.h)	*310*	*626*	*1152*
$Y_{X/S}$ (g biomass/g glucose) (%)	35.5	32.1	29.4
$Y_{E/S}$ (g ethanol/g glucose) (%)	16.7	12.5	10.8
Y_{ATP} (mg ATP/g biomass)	0.31	0.22	0.08
μ (h^{-1})	0.2	0.13	0.09
P (g biomass/l.h)	0.25	0.24	0.22
Glycerol (g/l)	0.07	0.09	0.08
Acetaldehyde (g/l)	0.023	0.019	0.019
2,3-Butanediol (g/l)	< 0.027	< 0.027	< 0.027
Ethyl acetate (g/l)	< 0.002	< 0.002	< 0.002
Propanol (g/l)	< 0.005	< 0.005	< 0.005
Final viability (%)	60	63	57

sufficient hydrogen peroxide to induce this enzyme. Clarkson *et al.* (1991) found that catalase is not directly involved in the response to O_2 toxicity. On the contrary, cytosolic and mitochondrial superoxide dismutase increased for the experiment with 3 bar air pressure, decreasing in the final stage.

There was no change in the activity for the rest of the experiments. This behaviour was similar to the glutathione reductase, which increased for the logarithmic phase and then decreased in the last phase, for a 3 bar of air pressure. Presumably, glutathione reductase and SOD have an important role as antioxidant enzymes in the hydrogen peroxide detoxification in the case of air pressure.

O_2 effects on growth and production

The influence of total pressure on the cell growth may be caused either by the effect of pressure or by partial pressures of CO_2 or O_2, which increase with increasing total pressure (Onken, 1990). To distinguish between these possible causes, experiments were carried out in which the cultures were aerated at different pure O_2 pressures. It is also very interesting to observe the differences between air and O_2 effects.

Typical batch growth curves for the experiments with 1.2, 2, 3, 4 and 8 bar pressure are shown in Figure 3

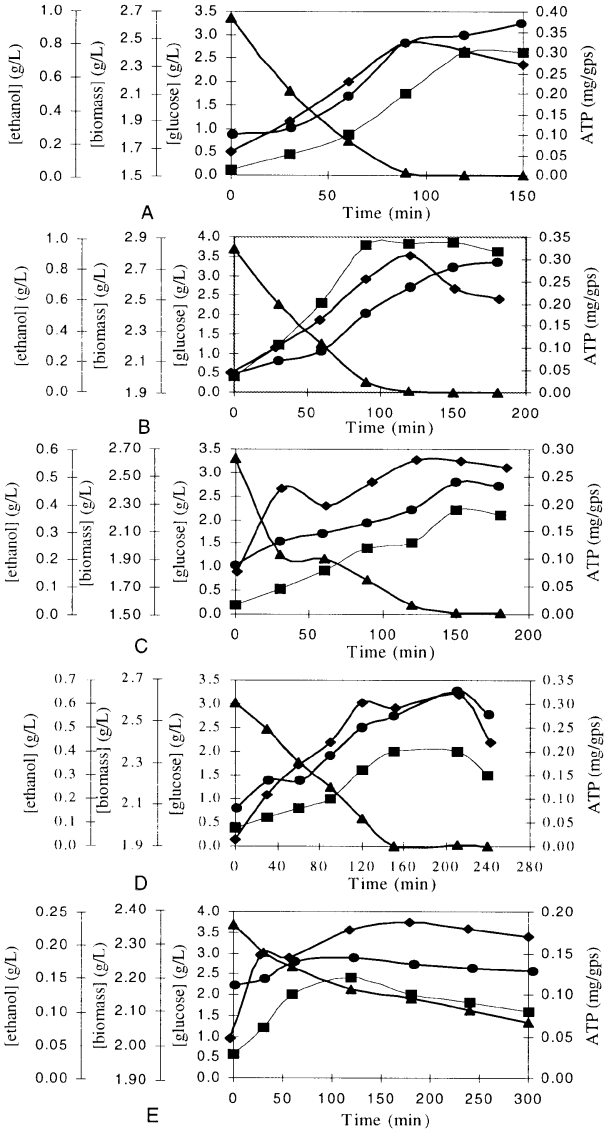

Figure 3 Batch growth of *Saccharomyces cerevisiae* at different pure O_2 pressures: A) 1.2 bar, B) 2 bar, C) 3 bar, D) 4 bar and E) 8 bar (● biomass; ◆ ethanol; ■ ATP; ▲ glucose).

A), B), C), D) and E) respectively. In the first two experiments, 1.2 and 2 bar, yeast cultures show diauxic growth and ethanol is metabolized oxidatively in a second phase. Some differences can be observed in the remaining experiments, the ethanol produced is not subsequently oxidised by the cells, then cellular growth stabilises and starts to decrease for the final O_2 pres-

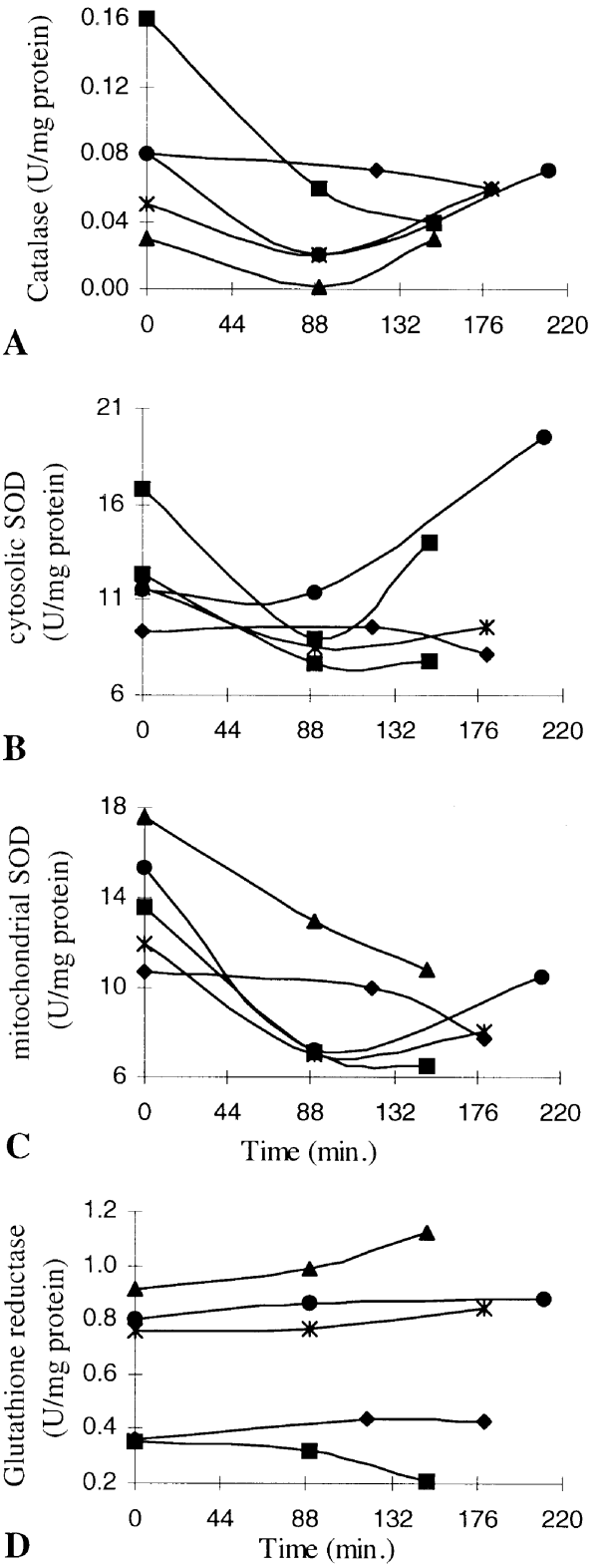

Figure 4 Enzymatic activity for different pure O_2 pressures. A) Catalase; B) cytosolic superoxide dismutase (CuZnSOD); C) mitochondrial superoxide dismutase (MnSOD); D) glutathione reductase. The points represented correspond to the growth phases: lag, logarithmic and stationary (■ 1.2 bar; ◆ 2 bar; ▲ 3 bar; ● 4 bar; ★ 8 bar).

Table 2 Changes in biomass, ethanol and ATP yields, specific growth rate, productivity and metabolite residual concentrations with O_2 pressure in batch experiments

O_2 pressure (bar)	*1.2*	*2*	*3*	*4*	*8*
OTR (mg O_2/l h)	*2400*	*3400*	*12200*	*26220*	*69400*
$Y_{X/S}$ (g biomass/g glucose) (%)	23.8	19.4	17.4	14.7	1.7
$Y_{E/S}$ (g ethanol/g glucose) (%)	15.6	13.2	11.5	13.5	6.50
Y_{ATP} (mg ATP/g biomass)	0.29	0.21	0.16	0.11	0.05
μ (h^{-1})	0.29	0.15	0.09	0.07	0.02
P (g biomass/l.h)	0.32	0.24	0.19	0.11	0.02
Glycerol (g/l)	0.16	0.18	0.07	0.13	0.22
Acetaldehyde (g/l)	0.024	0.022	0.024	0.029	0.024
2,3-Butanediol (g/l)	0.16	0.33	0.20	0.26	0.24
Ethyl acetate (g/l)	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
Propanol (g/l)	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Viability (%)	77	62	67	63	46

sure, 8 bar. For all experiments ATP curves followed growth behaviour during the different growth phases. Maximum ATP concentration was reached in 2 bar pure O_2 pressure and earlier than for the other pressures.

Table 2 shows pure O_2 pressure effects on the physiological behaviour of *Saccharomyces cerevisiae*. As happened with air pressure, pure O_2 pressure influenced biomass as well as ethanol yield. It is clear that pure O_2 has an inhibitory effect on growth and production. A 8 bar pure O_2 pressure leads to nearly complete inhibition of yeast growth. The specific growth rate is about 90% lower than the value obtained with 1.2 bar. Similar results for *Pseudomonas fluorescens* were found in the work of Onken (1990). The viability did not increase during fermentation time, it was kept constant during the experiments and decreased with O_2 pressure. At 8 bar, the viability decreased to a value 40% lower than its initial value.

The increase in death rate was also confirmed by the decrease in ATP yield. Kuriyama and Kobayashi (1993) say that when there is formation and accumulation of some toxic products such as acetaldehyde, membrane activity is reduced, increasing ATP consumption for maintenance, reducing the amount of ATP available for cell growth. However, Table 2 shows no change in by-product formation like acetaldehyde, propanol, 2,3-butanediol, ethyl acetate. As for glycerol, its production was higher with increasing pressures. The results for the productivity show that the efficiency of the process decreased with pure O_2 pressure.

O_2 effects on the antioxidant enzymes

Figure 4 shows the activity of antioxidant enzymes throughout growth for all the experiments made with pure O_2. Glutathione reductase activity remained constant in all the experiments. Catalase response to the O_2 pressures was different. In the two first experiments, 1.2 and 2 bar, catalase activity decreased, but when the pressure reached 3 bar the activity had a different behaviour, decreasing in logarithmic phase and then increasing in stationary phase. Likewise, superoxide dismutase activity decreased for the 1.2 and 2 bar of O_2 pressure.

For higher pressures the activity decreased and then the enzyme was induced for the last growth phase. Cytosolic superoxide dismutase had a higher increase than mitochondrial superoxide dismutase. With pure O_2 it is possible that catalase and cytosolic superoxide dismutase have a more important role in cell protection against reactive O_2 species.

Discussion

Growth and metabolite production in a mineral medium were inhibited with increased air and pure O_2 pressure. Growth was almost completely inhibited with a 8 bar pure O_2 pressure. Process efficiency also decreased as productivity decreased with air and pure O_2 pressure.

Different biomass and ethanol yields were found for the experiments with the same O_2 pressure: 1.2 bar of pure O_2 and 1.2 bar of partial O_2 in 6 bar air pressure. With 6 bar air pressure (p_{O_2} = 1.2 bar) biomass yield was 29.4% whereas at 1.2 bar pure O_2 pressure it was 23.8%. Ethanol yield was not enhanced with increasing air and O_2 pressure. As with biomass, ethanol yield for 1.2 bar pure O_2 pressure was different from 6 bar air pressure. Therefore ethanol production seems to be increased with pure O_2. At 1.2 bar pure O_2 pressure a 15.6% of ethanol yield was obtained and at 1.2 bar O_2

partial pressure (6 bar air pressure), the yield was 10.8%. We must not forget the presence of CO_2 in air as a possible explanation for this difference. As a matter of fact CO_2 partial pressure in 6 bar air was 0.18%, and in pure O_2 there was no CO_2 initially.

From this work it is obvious that O_2 injection had a negative effect on biomass and ethanol yield, but it was possible to observe that the ethanol oxidative metabolism was improved with a 3 bar air pressure and at 1.2 bar pure O_2 pressure gave better growth rates and productivity.

Studied pressures did not interfere with metabolism type, which was always oxidoredutive. There was simultaneously biomass and ethanol production from glucose, and ethanol yield could not be neglected.

Moderate pure O_2 pressures between 1 and 4 bar had no effect on the metabolism type, oxidoredutive, and on cellular viability. The loss in viability was observed with increased pressures, 8 bar. It seems reasonable to infer that the higher specific activity of superoxide dismutase and catalase, in pure O_2 pressure higher than 3 bar, may provide protection against O_2 radicals damage.

References

Beers, RF and Sizer, IW (1952). *J Biol Chem* **195**: 276–287.

Clarkson, S, Large, P, Boulton, C, Bamforth, C (1991). *Yeast* 7: 91–103.

Fridovich, I (1989). *J Biol Chem* **264**: 7761–7764.

Gille, G and Sigler, K (1995). *Folia Microbiol* **40**, 2: 131–152.

Kuriyama, H and Kobayashi, H (1993). *J Ferment Bioeng* **75**, 5: 364–367.

Mager, WH and Kruijff, AJJ (1995). *Microbiol Rev* **59**: 506–531.

McCord, JM and Fridovich, I (1969). *J Biol Chem* **244**: 6049–6050.

Onken, U (1990). *Biotechnol Bioeng* **35**: 983–989.

Onken, U and Liefke, E (1990). *Adv Biochem Eng Biotechnol* **40**: 137–169.

Rose, AH (1981) *Saccharomyces cerevisiae* as a model eucaryote. In: *Advances in Biotechnology: Current Developments in Yeast Research*, Stewart, GG, Russell, I, eds, Toronto: Pergamon Press.

Saez, C (1989) Etude des parametres de la fermentation alcoolique. Aplication a l'élaboration du petillant de raisin. These de Doctorat, INSA, Toulouse, France.

Siro, M-R Romar, H, Lovgren, T (1982). *Eur J App Microbiol Biotechnol* **15**: 258–264.

Smith, I, Viertheller, T, Thorne, C (1988). *Anal Biochem* **175**: 408–413.

Sonnleitner, B and Kappeli, O (1986). *Biotechnol Bioeng* **28**: 927–937.

Sweere, APJ, Mesters, JR, Janse, L, Luyben, KCAM (1988). *Biotechnol Bioeng* **31**: 567–578.

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