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ORIGINAL PAPER

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Response of the thermophile *Thermus* sp. RQ-1 to hyperbaric air in batch and fed-batch cultivation

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Abstract The effects of increased air pressure in a culture of the thermophilic microorganism *Thermus* sp. RQ-1 were investigated. Cell growth dependence on oxygen supply was investigated in a fermenter at atmospheric pressure. Total oxygen depletion from the medium for low values of k_La was observed during the exponential growth phase. It was possible with this strain to enhance the oxygen transfer rate by increasing the air pressure. Cell productivity was improved by pressurisation up to 0.56 MPa for batch cultivation; and an induction of the antioxidant enzymes, superoxide dismutase and catalase, was observed with the rise in pressure. Cell pre-cultivation under pressurised conditions conferred to the cells more resistance to an exposure to hydrogen peroxide and more sensitivity to paraquat (methyl viologen). The usefulness of bioreactor pressurisation on the cultivation of *Thermus* sp. RQ-1 was demonstrated for fed-batch operation, with the attainment of higher cell densities. A two-fold increase in cell mass productivity was obtained by the use of hyperbaric air (0.5 MPa). With the pressurisation of the head-space in the reactor, it was also possible to eliminate the loss of liquid by evaporation, which amounted to more than 10% at 70 °C and atmospheric pressure.

Introduction

Thermophilic microorganisms have potential applications in industry due to high cell growth and enzymatic reaction rates, lower costs in cooling systems and lower susceptibility to contamination (Aiba et al. 1984; Krahe et al. 1996). Thermophiles are also important sources of thermostable enzymes (Hjörleifsdottir et al. 1997). Despite the adaptation of these microorganisms to low gas concentrations in their natural habitat due to high temperatures, significantly high values of specific oxygen up-take rate have been reported for some aerobic strains (Cometta et al. 1982). Thus, the industrial cultivation of thermophiles in high cell density systems can present oxygen limitations.

The oxygen transfer rate (OTR) from the gas phase into the broth does not change significantly from low to high temperatures, because OTR depends not only on oxygen solubility but also on the oxygen mass transfer coefficient (k_La; Equation 1),

$$\mathrm{OTR} = k_L a(C^* - C) \quad (1)$$

where C^* is the solubility of oxygen in the liquid and C is the actual oxygen concentration in the liquid. In fact, while the oxygen solubility decreases with temperature, k_La increases with temperature, due to a decrease in the viscosity of the medium, which consequently increases oxygen diffusivity (Krahe et al. 1996). Nevertheless, at atmospheric pressure k_La must often be enhanced by an increase in the stirring and aeration rates, in order to prevent oxygen depletion from the broth. This was observed by Pham et al. (1998) for *Bacillus* sp. I-1018 batch growth at 50 °C. High values of air flow rate at a high temperature can lead to increased loss of liquid and volatile nutrients due to evaporation, as well as to foam development. Also, increasing the stirring rates presents limitations, such as power consumption costs and cell sensitivity to shear stress (Toma et al. 1991). According to Eq. 1, increasing the driving force for oxygen transfer while keeping k_La low can also enhance OTR. This can be accomplished by pressurising the cultivation vessel, with the added advantage of preventing liquid evaporation. It is then crucial to study the effects of the air pressure increase on cell growth and physiology since it is well known that an increase in oxygen partial pressure can inhibit growth and damage the cells by oxidative stress (MacMichael 1988). The effects of oxidative stress on bacterial behaviour, caused by oxygen, have been

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studied for mesophiles (Gregory and Fridovich 1973), as well as for thermophiles (MacMichael 1988). Knowledge about the role of the antioxidant enzymes [such as superoxide dismutase (SOD) and catalase, among others] in the defence of cells has significantly increased in recent years (Farr and Kogoma 1991; Hopkin et al. 1992; Moradas-Ferreira et al. 1996). Much of the work on pressure effects on microorganisms was done in static culture systems (solid medium) or with liquid cultures submitted to increased hydrostatic pressure (Nelson et al. 1992; Canganella et al. 1997). Therefore, there are still few studies of cell behaviour in aerated and pressurised bioreactors (Onken 1990; Yang and Wang 1992; Pinheiro et al. 1997; Belo and Mota 1998). Induction of antioxidant enzymes, like SOD, by raising oxygen partial pressure can also be used to obtain large quantities of these enzymes, which have potential interest for the pharmaceutical and food industries (Taniguchi et al. 1992).

The purpose of this research was to investigate whether total air pressure could be manipulated within a range of values easily applicable in an industrial environment, in order to improve cell cultivation and consequently intracellular enzyme productivities in thermophilic bacteria, using the portuguese isolated strain, *Thermus* sp. RQ-1 as an example. Batch and fed-batch operations were studied to simulate industrial processes. The ability of the cells to induce their defensive enzymes by exposure to higher oxygen partial pressure was investigated. Possible inhibitory factors that could limit the application of hyperbaric air were also explored, including the effect of increasing CO_2 partial pressure (Onken and Liefke 1990). Thus, experiments at increased total pressure and increased partial pressure of CO_2 were also performed.

Materials and methods

Bacterial strain and medium

Thermus sp. RQ-1 (DSM 9247) was used. This strain was isolated from shallow hot springs on S. Miguel, Azores, Portugal (Santos et al. 1989) and has already been characterised and compared with other thermophilic strains of the same genus (Manaia and Da Costa 1991; Manaia et al. 1994). This aerobic bacterium has an optimal temperature for growth at 70 °C. The strain was stored at −80 °C in *Thermus* medium with 20% (v/v) glycerol. From these stock cultures, agar plates [3% (w/v) agar] were inoculated. *Thermus* medium was composed of basal mineral medium 162 (Degryse et al. 1978) supplemented with 0.25% (w/v) tryptone, 0.25% yeast extract and 0.2% glucose. The pH was adjusted to 7.5 with NaOH 4 N and HCl 4 N, prior to autoclaving. For liquid cultivation phosphate buffer pH 7.5 was sterilised separately and added to the medium to a final concentration of 40 mM.

Batch cultivation

Thermus sp. RQ-1 cells were grown in a 2-l fermenter (Biolab from B. Braun) at atmospheric pressure, at 200 rpm and 400 rpm, as well as in a 500-ml stainless steel reactor (Parr 4563) at 200 rpm and increased pressure. Both reactors have turbine stirrers with two impellers of six blades each. The values of the ratio H/D for each reactor are 2.1 for the 2-l fermenter and 3.0 for the pressurised reactor. In the latter, the operating pressure was set by adjusting the compressed air pressure (inlet air) and the regulatory valve in the exit gas line. The reactor was equipped with a pressure transducer to monitor total internal pressure. The operating volume was 1.5 l in the fermenter and 300 ml in the pressurised reactor. Batch cultivations in each system were performed at 70 °C, 1 vvm of aeration rate (measured at standard conditions), without pH control but with buffered medium. For the inoculum, cells were used from a 16-h culture, grown in a flask in a shaker bath. Cells were harvested by centrifugation and resuspended in fresh medium before charging the reactors.

OTR and oxygen uptake rate of the cells in the fermenter were determined by the dynamic method using a polarographic probe (12/220 T-type, Mettler Toledo) and the respective meter (type 170) to measure the concentration of oxygen dissolved in the medium. In the pressurised reactor OTR was measured in blank assays using the sulphite method (Cooper et al. 1944).

Exposure to chemical oxidants

Cells of *Thermus* sp. RQ-1 from the same inoculum were cultivated simultaneously in the reactor at 0.5 MPa with continuous aeration and in a shake flask with micro-aeration provided by agitation. After 5 h growth, half the volume of each culture was collected and divided between two flasks: one with 50 mM H_2O_2 and the other with 1 mM paraquat (methyl viologen, a well known reductor of oxygen to superoxide anion). The cultures were incubated in a shaker bath for 2 h. The behaviour of the cells exposed to the oxidants was followed and compared with the behaviour of the cells remaining in the reactor and in the micro-aerated flask.

Fed-batch cultivation

Thermus sp. RQ-1 cells were grown in the pressurised reactor in batch mode as described above, with an initial volume of 100 ml. After 5 h growth, feeding was started (200 ml of five-times concentrated medium) through a high-pressure pump (Jasco 880-PU) at a constant flow of 5.4 ml/h or at a constant dilution rate of 0.05/h. Experiments at 0.1 MPa and 0.5 MPa were performed using air or a gas mixture composed of 21%(v/v) O_2, 4% CO_2 and 75% N_2. The composition of the exhaust gas was measured by mass spectrometry using a BIOQUAD spectrometer.

Analytical procedures

The biomass concentration of *Thermus* sp. RQ-1 was determined turbidimetrically at 600 nm and expressed as dry weight (DW). Glucose concentration was measured by the 3,5-dinitrosalycilic acid method (Miller 1959). ATP levels in the cells were analysed by a luciferase assay using the luminometer LUMAC 2500 with an appropriate ATP kit.

For the determination of the enzymatic activities of SOD and catalase, cells were harvested by centrifugation and resuspended in phosphate buffer 50 mM, pH 7.8, 0.1 mM EDTA. Disruption was achieved by vigorously shaking the cellular suspension mixed with 0.15-mm-diameter glass beads. Cellular extracts were obtained by centrifugation at 32 *g*, for 15 min at 4 °C and overnight dialysis (14,000 cut-off membrane) of the supernatant against buffer at 4 °C. Preliminary experiments showed there was no loss in the activity of SOD overnight at 4 °C. Total soluble protein was determined by Bradford's method (Bradford 1976), catalase activity was assayed according to Beers and Sizer (1952) and SOD activity was assayed by the pyrogallol method (Marklund and Marklund 1974). SOD was analysed in the presence or absence of 4 mM H_2O_2 (Fe-SOD inhibitor) to distinguish Mn-SOD from Fe-SOD.

Results

Batch culture

The strain *Thermus* sp. RQ-1 was isolated from hot shallow springs, an environment where the dissolved oxygen concentration is quite low. Nevertheless, batch cultivation of this bacterium at atmospheric pressure in a rich medium (*Thermus* medium) showed that both cell growth and substrate utilisation were stimulated by an increase in the oxygen supply rate (Fig. 1). This was due to the high value of specific oxygen uptake rate found for this strain under the experimental conditions used. During the exponential growth phase, a qO_2 value of 15.7 mmol $O_2/(g_{DW}$ h) was observed. The values of OTR and k_La measured at atmospheric pressure in the fermenter and in the pressurised reactor are presented in Table 1.

Figure 2 shows that *Thermus* sp. RQ-1 can withstand values of total air pressure up to 0.56 MPa and oxygen partial pressure of 0.12 MPa. In this range of pressure no inhibitory effects of pressure on cell growth were observed. On the contrary, cell productivity was improved by pressure (Table 1). As can be seen in Fig. 3, during the cultivation of *Thermus* sp. RQ-1 in the pressurised reactor, an induction of the antioxidant enzymes SOD and catalase was observed, mainly at the beginning of the exponential growth phase. This shows, as was expected, that the formation of reactive oxygen species, like O_2^- and H_2O_2, was stimulated by the rise in oxygen partial pressure. For thermophiles, the ability of cells to induce their antioxidant defences is very important, since at high temperatures greater production rates of oxygen radicals and consequent cell damage reactions are expected. From the results of Fig. 3, a pressure rise of 0.4 MPa led to a four-fold increase of SOD activity. An induction of this enzyme activity was also obtained for the late exponential phase with the highest pressure values.

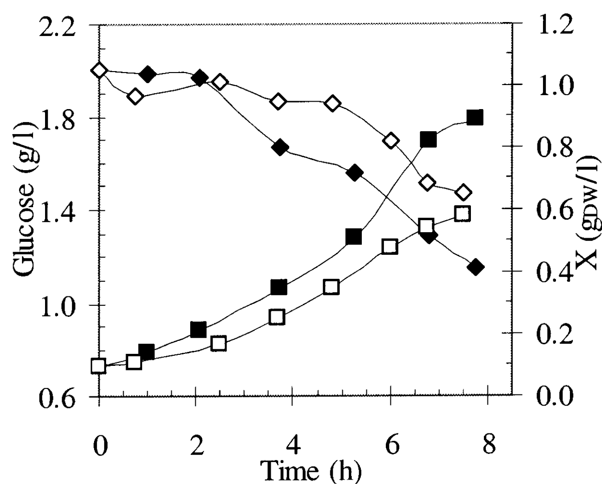

Fig. 1 Effect of stirring rate on cell growth (■, □) and substrate consumption (◆, ◇) in batch cultivation of *Thermus* sp. RQ-1 in a 2-l fermenter, at atmospheric pressure and different stirring rates: 200 rpm (□, ◇) and 400 rpm (■, ◆). *X* is the dry biomass per unit volume

Table 1 Effects of stirring rate in a fermenter at 0.1 MPa, and of pressure in a pressurised reactor at 200 rpm, on oxygen transfer rate (*OTR*), overall oxygen mass transfer coefficient (k_La), and cell productivity (*P*)

	Fermenter (rpm)		Pressurised reactor (MPa)			
	200	400	0.1	0.3	0.43	0.56
OTR [mmol/(l h)]	3	12	17	69	91	105
k_La (per h)	26	103	169	160	142	129
P [mg_{DW}/(l h)]	64	102	62	93	103	137

Exposure to chemical oxidants

In order to analyse the response of *Thermus* sp. RQ-1 (pre-cultivated under increased pressure) to an exposure to oxidant agents, H_2O_2 and methyl viologen were added to cultures obtained under different aeration conditions. The results are presented in Fig. 4 and Fig. 5.

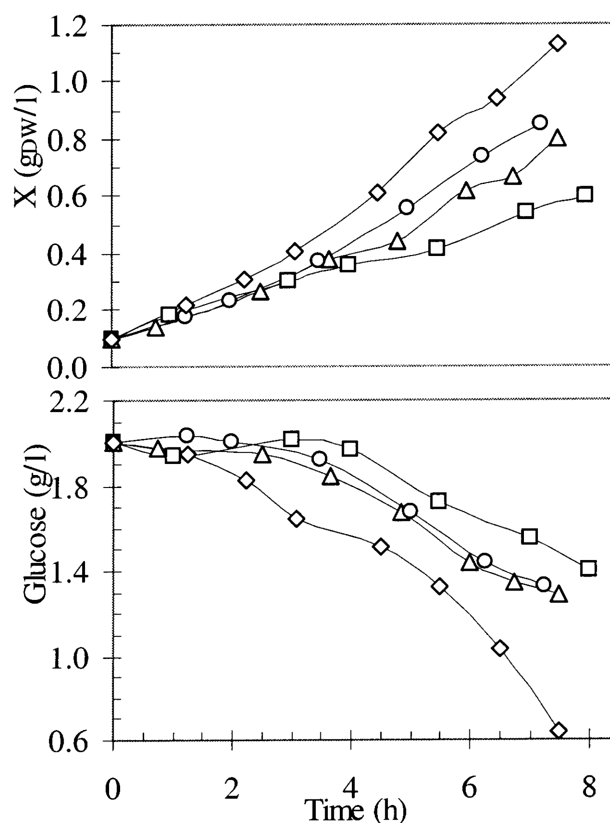

Fig. 2 Time course of cell and glucose concentration in batch cultivations of *Thermus* sp. RQ-1 in the pressurised reactor. The operating conditions used were a stirring rate of 200 rpm, an aeration rate of 0.3 l/min (at standard conditions) in 300 ml total volume and different air pressures: 0.10 MPa (□), 0.30 MPa (△), 0.43 MPa (○) and 0.56 MPa (◇)

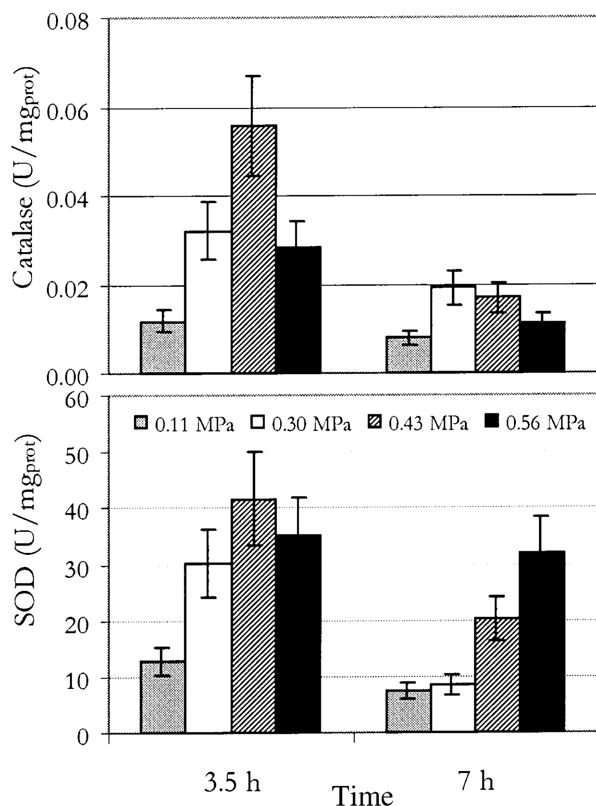

Fig. 3 Effect of hyperbaric air on the activities (expressed as units per unit mass of total protein) of the enzymes superoxide dismutase (SOD) and catalase, at the initial (3.5 h growth) and late exponential growth phase (7 h growth), for *Thermus* sp. RQ-1 cells. *Error bars* represent 95% confidence intervals

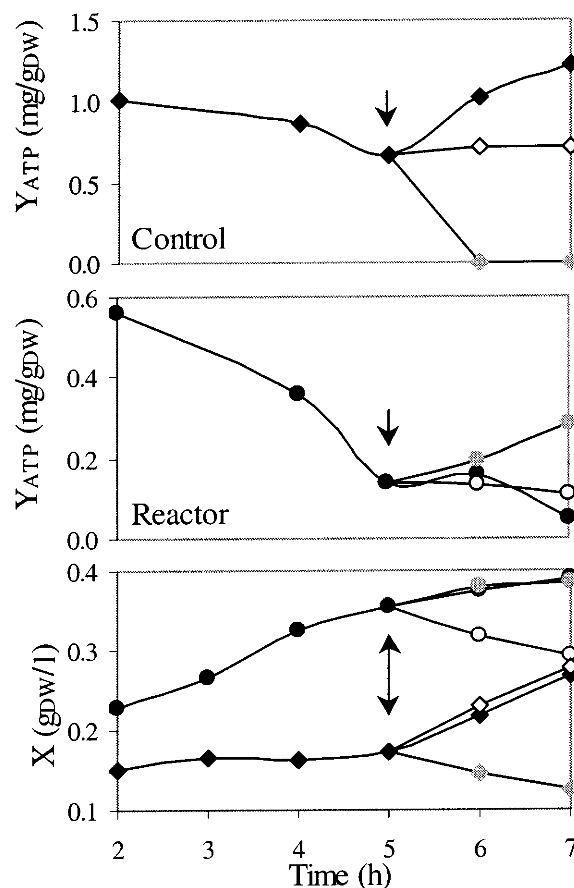

Fig. 4 Levels of ATP (Y_{ATP}) and cell concentration (X) of *Thermus* sp. RQ-1 cultivated in a pressurised reactor (●) and in a shake flask used as a control (◆). The *grey symbols* correspond to cells exposed to hydrogen peroxide and the white symbols corresponds to cells exposed to paraquat. *Arrows* indicate the time when part of each culture was exposed to oxidants

The cultures started with the same cell concentration but after 2 h it was clear that a greater cell density was achieved in the pressurised reactor than in the flask. H_2O_2 was lethal to the cells cultivated in the flask because an inhibition of growth and ATP synthesis was observed. Also, the cells were not able to increase their antioxidant activities and the levels of the enzymes were even lower after 2 h with H_2O_2 than under the initial conditions (5 h growth), and much lower than the levels of the cells not exposed, for the same time of incubation (7 h growth).

Comparing the levels of the enzymes for both cultures before oxidant exposure (5 h) once again, there was a significant induction of SOD and catalase by an air pressure of 0.5 MPa (0.1 MPa of oxygen partial pressure). Furthermore, as was also reported for *Escherichia coli* (Fridovich and Gregory 1973; Schellhorn and Hassan 1988), a rise in oxygen tension particularly induced the manganese form of SOD.

H_2O_2 slightly induced SOD and catalase for the cells pre-cultivated at 0.5 MPa. For these cells, there was no effect on cell growth and ATP synthesis after exposure to this chemical oxidant.

The effect of methyl viologen on the *Thermus* sp. RQ-1 cells was opposite to the effect of H_2O_2. The addition of 1 mM paraquat had no effect on cell growth and ATP synthesis for cells grown in the shake flask. Cells pre-cultivated at 0.5 MPa showed a decrease in cell concentration after exposure to methyl viologen, but no effect on ATP levels was observed. Cells from both cultivation conditions were able to induce SOD (mainly Fe-SOD) and catalase.

Fed-batch cultivation

The effect of pressure on cell behaviour for each strain depends on several other factors. The mode of operation can influence the effect of hyperbaric air on final cell productivity. In this work, hyperbaric air at 0.5 MPa was applied to a fed-batch cultivation of *Thermus* sp. RQ-1. Cell behaviour was compared at atmospheric and increased pressure for two different feed strategies; constant dilution rate and constant feed flow rate. Also, a mixture of 21% (v/v) O_2, 4% CO_2 and N_2 was used to differentiate possible inhibitory effects of increased partial pressure of CO_2 from the effects of total air and oxygen partial pressures. From the results in Fig. 6, it

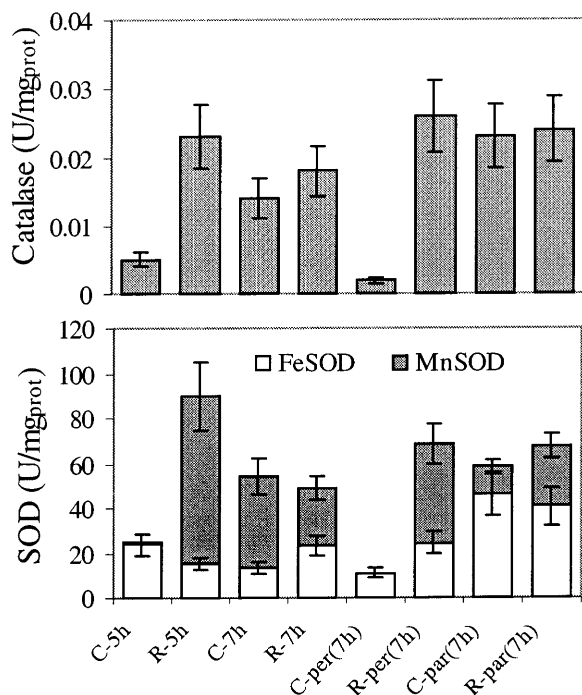

Fig. 5 Activity values of the antioxidant enzymes catalase and SOD (Mn-SOD and Fe-SOD) on *Thermus* sp. RQ-1 cells cultivated in a shake flask (*C*) and in a pressurised reactor (*R*) at 5 h and 7 h growth. The activities are also presented for the cultures after 2 h exposure to the chemical oxidants hydrogen peroxide (*R-per* and *C-per*) and paraquat (*R-par* and *C-par*). *Error bars* represent 95% confidence intervals

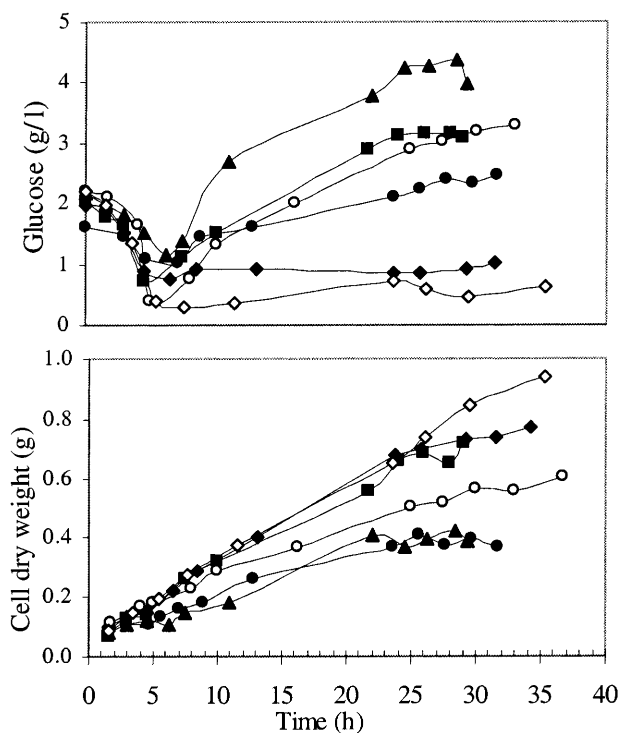

Fig. 6 Effect of a rise in pressure on glucose consumption and cell mass production for fed-batch cultivation of *Thermus* sp. RQ-1: Dilution rate (D) = 0.05/h at 0.1 MPa air (▲) and 0.5 MPa air (■); Feed flow rate (F) = 5.4 ml/h at 0.1 MPa air (●) and 0.5 MPa air (◆); F = 5.4 ml/h at 0.1 MPa (○) and 0.5 MPa (◇) of a gas mixture of 4% (V/V) CO_2, 21% O_2 and N_2

can be seen that for the same strategy of feeding, cell production was always better for the experiments at raised pressure, using either air or the mixture as sparging gas. This is a consequence not only of the enhancement of OTR by a rise in pressure, but also of the total elimination of liquid loss by evaporation which was around 40 ml at 0.1 MPa total air pressure. This liquid loss and the lower nutrient up-take at atmospheric pressure are the reasons for the observed glucose accumulation in the culture. Glucose concentration was always low during the fed-batch process at 0.5 MPa of air and 4% CO_2 mixture at constant feed flow rate. This feeding strategy was more suitable for this strain, since at raised pressure and constant dilution rate, an accumulation of glucose was also observed, although at a lower level than that observed at atmospheric pressure and the same dilution rate.

Also, a partial pressure of 20 kPa CO_2 had no inhibitory effect on cell growth. On the contrary, production of cell mass was slightly improved by the presence of CO_2. Also, CO_2 did not affect pH changes, which were due to the evaporation of liquid and consequently to the accumulation of acidic components in the medium. In fact, pH was close to 7.0 for all the experiments at 0.5 MPa and constant feed flow rate and decreased around 0.5 units for the experiments at 0.1 MPa. But there was no difference between the pH of the culture using air or 4% CO_2 gas mixture.

From the analysis of the exhaust gas composition, values of CO_2 partial pressure of 0.15 kPa and 1.15 kPa were attained for the experiments with air at 0.1 MPa and 0.5 MPa, respectively. The highest CO_2 production at raised pressure was followed by the highest oxygen consumption rate (measured by gas balance), which shows that cells at 0.5 MPa had a greater respiratory activity than those at 0.1 MPa. In fact, respiratory quotient (RQ) values close to 1.0 were found for the experiments at 0.5 MPa while higher values were obtained for the experiments at 0.1 MPa. However, the analysis of the RQ values obtained is very difficult due the increased error of this calculation for small reactors (Royce and Thornhill 1992), such as the 600-ml reactor used in this work. The highest respiratory activity at raised pressure is in agreement with the levels of ATP found in *Thermus* sp. RQ-1 cells cultivated with hyperbaric and atmospheric air (Fig. 7). In general, the amount of ATP per unit cell dry weight was higher for the fed-batch cultivation at increased pressure than at atmospheric pressure. For this latter case, a decrease in ATP yield was also observed at the end of the cultivation period.

Discussion

This work demonstrated the usefulness of increased pressure for aerobic thermophile cultivation. Utilising

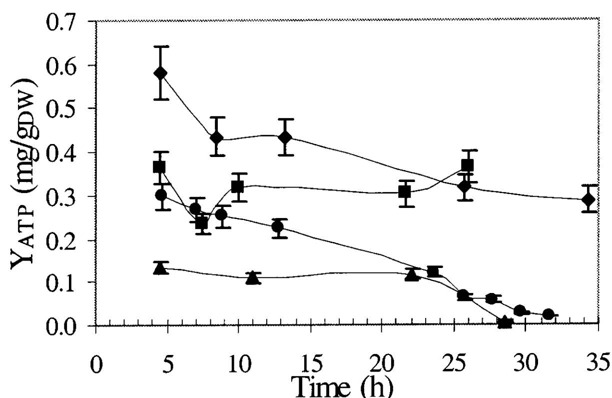

Fig. 7 ATP content in the cells (Y_{ATP}) of *Thermus* sp. RQ-1 cultivated in a pressurised reactor: D = 0.05/h at 0.1 MPa air (▲) and 0.5 MPa air (■), F = 5.4 ml/h at 0.1 MPa air (●) and 0.5 MPa air (◆). *Error bars* represent the 95% confidence intervals

pressure up to 0.3 MPa has been proposed in the literature mainly as a tool for preventing evaporation on cultures of thermophilic microorganisms (Edwards 1990). With the study presented here, it was concluded that it is possible to use higher values of air pressure (up to 0.56 MPa) in batch and fed-batch cell cultivation. Besides the advantage of reducing liquid loss, pressure was shown to be an important tool for the enhancement of oxygen supply to the culture with a concomitant cell growth improvement. This has also been proposed for other microorganisms, like for instance, the mesophile *E. coli* and a fragile alga (Yang and Wang 1992) for pressures up to 0.27 MPa; and it would probably be applicable for other aerobic microorganisms which have high oxygen consumption rates. The value of qO_2 found for the strain studied in this work was similar to the values reported for other thermophilic strains (Kuhn et al. 1980; Cometta et al. 1982). These values were found to be greater than those reported for mesophiles. Even for the low cell concentrations reached in batch cultivation at atmospheric pressure, a total depletion of oxygen from the culture was observed at the lowest value of stirring rate. A minimal value of k_La for preventing oxygen exhaustion from the culture was also reported by Pham et al. (1998) for batch cultivation of *Bacillus* sp. I-1018 at 50 °C. Raising oxygen solubility through the increase in total air pressure enhanced OTR in the pressurised reactor and decreased k_La. In fact, for the same inlet air flow (measured at standard conditions), the actual volumetric air flow inside the reactor decreases with pressure, thus decreasing superficial gas velocity and consequently k_La. The differences in the measured OTR (and k_La) values observed in each of the reactors should be carefully analysed because different methods were used for their determination. As a matter of fact, the OTR measurement in the fermenter by both methods (dynamic and sulphite) under the same operating conditions showed that the values obtained by the dynamic method are around 43% of the values obtained by the sulphite method. Thus, OTR measured by oxygen consumption in a sulphite solution is an over-estimation of the oxygen transfer capacity of the system using real medium. Also, the experimental errors for each method of OTR determination are different. The values of OTR in Table 1 obtained by the sulphite method are affected by a 20% standard error and the values determined by the dynamic method have a smaller standard error, about 10%. Nevertheless, the determination of OTR by the sulphite method in the hyperbaric reactor is very useful because it gives an idea about the effect of pressure on both k_La and OTR.

With reactor pressurisation, it is possible to supply oxygen at the required rates with low k_La values. This could represent important savings in power input costs due to reduced agitator consumption. Bioreactor pressurisation can also be useful to produce antioxidant enzymes, as was demonstrated with SOD in this work. The induction of SOD and catalase activities was firstly demonstrated by MacMichael (1988) for several thermophilic strains, by changing the conditions of growth from static to slightly aerated (orbital rotation). It was also reported that the level of induction depends on the strain.

From the results obtained for cells in the shake flask at 5 h and 7 h growth, it seems that the proportion of each SOD iso-enzyme could depend on the growth phase of the cells, since the Mn-SOD activity increased from 5 h to 7 h and during this period an acceleration of cell growth was observed. As the cells cultivated in the pressurised reactor had a shorter lag phase, the cells were already at the initial stationary phase during the same period of time (5 h–7 h), and therefore SOD and catalase activities decreased. Despite the observed induction of catalase by pressure, the activity values of this enzyme were very low for *Thermus* sp. RQ-1 cells. This probably indicates that there are other peroxidases more important to this strain for the elimination of H_2O_2. For example, in the case of the mesophile *E. coli*, it was reported that catalase is not very important in defending the cell against oxidative stress (Fridovich and Gregory 1973).

In the present study it was also shown that cell cultivation at raised pressure affects the cell's sensibility to chemical oxidants. The increased resistance to H_2O_2 associated with the low values of catalase observed may be additional evidence of the existence of peroxidases other than catalase. The greater sensibility to methyl viologen observed for cells in the pressurised reactor could be attributed to a probable increase in the production of the hydroxyl radical ($OH^\bullet$) which is a very reactive and harmful species. Since the cells in the reactor had more SOD, there could simultaneously be a high intracellular concentration of H_2O_2 and O_2^-, leading to hydroxyl radical formation by Fenton's reaction (Farr and Kogoma 1991). For *E. coli* it was reported that cells overproducing SOD were more sensitive to generators of superoxide anions (Schellhorn and Hassan 1988).

As already mentioned, thermophiles have potential applications in industry and the production of thermostable enzymes is one of the most important. Some

extracellular enzymes from thermophilic bacterial sources have been reported (Manelius et al. 1994) but many are intracellular (Hjörleifsdottir et al. 1997; Mizobata et al. 1998) and in these cases high-density cell cultivation systems will be required. Recently, new bioreactor systems have been proposed to grow thermophilic microorganisms to cell concentrations up to 130 g/l (Krahe et al. 1996). The fed-batch mode of operation in pressurised bioreactors with an optimised feed strategy could also be an interesting way for obtaining large amounts of cell mass from thermophiles without significant investment in new reactor designs.

In the present case of thermophilic microorganisms, the positive effect of pressure on cell growth in batch cultivation would also be expected for a fed-batch process, since it is possible to obtain higher cell densities and thus attain higher oxygen uptake rates. Oxygen is an important limiting factor in most fed-batch processes and it is common to use oxygen-enriched air to ensure the right OTR for the cultures (Pan et al. 1987).

In this work, hyperbaric air was successfully applied to fed-batch cultivation of *Thermus* sp. RQ-1. Moreover, metabolically produced CO_2 will not be a constraint for hyperbaric air utilisation in high cell density cultures of this thermophile, since the exit gas of an aerobic culture in an industrial bioreactor commonly does not have more than 3% (v/v) CO_2 (Onken and Liefke 1990) and there were no negative effects observed by the use of a sparging gas with 4% CO_2 at raised pressure (20 kPa CO_2 partial pressure). This is very important since at raised air pressure metabolically produced CO_2 will be kept in the culture at concentration values higher than atmospheric pressure. Also, the concentration of the anion HCO_3^- will be significantly increased, because at neutral pH its concentration is 2.5-fold higher than the aqueous carbon dioxide concentration (Jones and Greenfield 1982).

In conclusion, it is important to say that, from an economic point of view, raised pressure can offer new perspectives for industrial processes, since important savings could be obtained in power costs due to the decrease in the stirring rate of the cell cultivation systems. The increment in capital cost of the construction material of the equipment needed to cope with the range of pressures used in this work would be rapidly compensated by the reduced operating costs, as well as by the significant increase in productivity. Indeed, normally all the fermentors can withstand pressures up to 6 bar or more and so the operating air pressures proposed in this work are totally feasible.

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