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Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process

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Abstract

Two strategies to increase the productivity of secreted Thai Rosewood β-glucosidase in *Pichia pastoris* processes were evaluated. Both methods were based on increasing the oxygen transfer rate (OTR) in the process by simple means. Increasing the driving force for the diffusion from the air bubbles to the medium by elevating the air pressure, from 1.2 to 1.9 bar increased the oxygen uptake rate (OUR) by 59% while increasing the driving force by accepting oxygen limitation increased the OUR by 35%. The OTR increased less than in proportion to the increased solubility in the high-pressure process, which indicates that air bubble compression reduces the volumetric oxygen transfer coefficient (K_La). Even though the methanol consumption increased almost in proportion to the OTR in both processes the biomass production did not increase as much. This is explained as a higher maintenance demand for methanol in the oxygen limited (0.027 g g^{-1} g^{-1}) and high-pressure processes (0.035 g g^{-1} g^{-1}), compared to 0.022 g g^{-1} g^{-1} in the methanol limited reference process. However, in spite of the low effect of increasing OTR on the biomass production the total β-glucosidase yield increased almost in proportion to the increased methanol consumption and reached highest value in the high-pressure process, while the β-glucosidase purity was highest in the oxygen-limited process due to release of less contaminating proteins.

Keywords: Aeration; Oxygen transfer; Dissolved oxygen tension; Enzyme production; β-Glucosidase; Oxygen-limited fed-batch

1. Introduction

The methylotrophic yeast *Pichia pastoris* is a common host for production of recombinant proteins. It is mostly used with the strong alcohol oxidase 1 (*aox1*) promoter to control the production of the recombinant protein, but this promoter also controls synthesis of the alcohol oxidase (AOX) enzyme that is used for oxidation of the carbon- and energy-source methanol [1,2]. The initial oxidation of methanol by the AOX enzyme is dependent on molecular oxygen as an electron acceptor resulting in formaldehyde and hydrogen peroxide (H_2O_2) [3]. *Pichia* processes are typically associated with relatively low specific product formation rate but this is compensated for by the low maintenance demand [4] that permits cultivations at very high-cell-density, typically in the range of 130–150 g L^{-1} cell dry weight [4,5]. However, the high-cell-density and the use of the reduced energy source methanol emphasize the demand for high oxygen transfer rate (OTR).

A common model showing parameters that influence the OTR is

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

To increase the OTR in *P. pastoris* processes beyond what is achieved by just increasing the aeration rate and the stirrer speed, which increases the volumetric oxygen transfer coefficient (K_La), several strategies have been used for increasing the driving force ($C^* - C$).

The driving force for oxygen transfer is increased if the process is permitted to run under oxygen limitation ($C \approx 0$). This may have serious drawbacks in processes with facultatively anaerobic organisms like *Escherichia coli* and *Saccharomyces cerevisiae*, which switch to fermentative metabolism under oxygen limiting conditions. Therefore, such organisms are mostly

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Nomenclature

aox1	alcohol oxidase 1 gene
AOX	enzyme alcohol oxidase
C	oxygen concentration in bulk liquid outside the film (mol L^{-1})
C^*	oxygen concentration in gas-liquid interface (mol L^{-1})
C^*_{ref}	C^* under 100% DOT electrode calibration (mol L^{-1})
CPR	carbon dioxide production rate (mol h^{-1})
DOT	dissolved oxygen tension (%)
K_La	volumetric oxygen transfer coefficient (mol L^{-1} h^{-1})
K_La_{ref}	K_La under 100% DOT electrode calibration condition (mol L^{-1} h^{-1})
MLFB	methanol-limited fed-batch
OLFB	oxygen-limited fed-batch
OTR	oxygen transfer rate (mol h^{-1})
OUR	oxygen uptake rate (mol h^{-1})
P	air pressure inside the bioreactor (bar)
PI	propidium iodide
P_{ref}	P under 100% DOT electrode calibration (bar)
q_m	maintenance coefficient (g g^{-1} h^{-1})
ROS	reactive oxygen species
Y_{em}	biomass yield on methanol, exclusive the maintenance (g g^{-1})
$Y_{x/s}$	biomass yield on methanol (g g^{-1})

cultivated at 20–30% air saturation. *P. pastoris* is however an obligately aerobic organism when grown on methanol. It cannot employ alternative metabolic reactions for methanol under oxygen limitation (DOT ≈ 0). Therefore, oxygen-limited fed-batch processes can be utilized [5–7]. About 33% OTR increase was obtained when oxygen-limited *P. pastoris* processes were compared with methanol-limited processes run at 25% DOT. Both growth and recombinant protein production was increased in the oxygen limited process [5].

Increased dissolved oxygen concentration in equilibrium with the gas phase (C^*) can be achieved either by using higher oxygen concentration in the inlet air or by increasing the total air pressure (P_{tot}) in the bioreactor. Mixing pure oxygen in the inlet air flow is effective on the small scale and it has been utilised to increase the methanol feed rate and the productivity [4,6] but on a large scale the supply and handling of pure oxygen can be unsafe. Alternatively, an oxygen enriching polysulphone membrane can be used for separation of regular air into nitrogen-enriched retentate and oxygen-enriched permeate [8]. An oxygen-enriched (35–38% oxygen) stream obtained with a polysulphone membrane has been used to increase the DOT, which enhanced production of elastase inhibiting peptide by recombinant *P. pastoris* [9]. Increase of the total air pressure, P_{tot}, has been applied in other yeast processes [10,11].

However, all these methods may have biological side-effects. The use of oxygen limitation in *Pichia* processes may theoretically result in elevated formaldehyde concentrations in the cell if it generates an imbalance between the initial oxidation of methanol, resulting in formaldehyde, and the formaldehyde consuming reactions in anabolism and catabolism. The high air pressure increases not only the partial pressure of oxygen but also that of carbon dioxide, which is a common antimicrobial agent [12]. Furthermore, increasing the oxygen equilibrium concentration C^* by high pressure may result in toxic side-effects of molecular oxygen. In the aerobic process, during the reduction of molecular oxygen to water through acceptance of four electrons, reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet -}$), H_2O_2 and hydroxyl radicals ($HO^{\bullet}$) are generated [13]. The initial oxidation of methanol by methylotrophic yeast is another reaction, which generates H_2O_2 [3]. These ROS attack almost all cell components, DNA, protein and lipid membrane [13,14].

In the present work, we investigate two simple methods for increasing the productivity of *Pichia* processes by increasing the OTR. Increased total air pressure is compared with the use of oxygen limitation to increase the driving force for oxygen transfer in a process for recombinant Thai Rosewood β-glucosidase production with *P. pastoris*. The growth, viability and product formation were compared with a traditional methanol-limited fed-batch process.

2. Materials and methods

2.1. Strain and plasmid

The β-glucosidase cDNA gene from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) [15] was cloned into the pPICzαB vector (Invitrogen) and then integrated into *P. pastoris* Y-11430 (wild-type strain) at the *aox1* promoter. A Mut^+, $zeocin^+$ transformant was selected and characterized for extracellular β-glucosidase production.

2.2. Inoculum preparation

The inoculum culture was prepared from one colony of *P. pastoris* on YPD agar (1 L contained 10 g yeast extract, 20 g peptone, 20 g dextrose, and 15 g agar) containing 100 μg zeocin mL^{-1} suspended in 100 mL BMGY medium (10 g yeast extract, 20 g peptone, and 10 g glycerol dissolved in 1 L 0.1 M potassium phosphate buffer with pH 6.0). The culture was incubated at 30 °C, with 200 rpm for 24 h.

2.3. Fed-batch fermentation

The fed-batch fermentations were carried out in a 10 L stirred tank bioreactor (Belach Bioteknik AB, Stockholm) with 3.0 L of glycerol basal salts (GBS) medium (containing per litre: H_3PO_4 85% 26.7 mL, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_4{\cdot}7H_2O$ 14.9 g, KOH 4.13 g, glycerol 40.0 g and PTM1 trace salts 4.35 mL). The PTM1 trace salt solution contains per litre: $CuSO_4{\cdot}5H_2O$ 6.0 g, KI 0.08 g, $MnSO_4{\cdot}H_2O$ 3.0 g, $Na_2MoO_4{\cdot}2H_2O$ 0.2 g, H_3BO_3 0.02 g, $ZnCl_2$ 20.0 g, $FeCl_3$ 13.7 g, $CoCl_2{\cdot}6H_2O$ 0.9 g, H_2SO_4 5.0 mL, and biotin 0.2 g. The

fermentations were controlled at the following conditions: temperature 30 °C, aeration 6 L min^{-1}, agitation 1000 rpm and pH 5.0. Ammonia solution 25% was used to control pH and addition of antifoam A (A-5758, Sigma) was controlled by a level electrode.

A four-stage fermentation protocol was used in this study. The first stage was a glycerol batch phase. About 24 h after inoculation when the glycerol was completely consumed, as indicated by the DOT signal, the process was switched to glycerol fed-batch by feeding 500 g L^{-1} glycerol plus 12 mL L^{-1} PTM1 trace salts to the bioreactor at an exponentially increasing rate of 0.18 h^{-1} starting with 35.5 mL h^{-1}. After 3–3.5 h when the cell density reached 40 g L^{-1} ($OD_{600} \approx 80$), the process was switched to the methanol induction phase by replacing the glycerol feed with a feed of methanol plus 12 mL L^{-1} PTM1 trace salts. The initial methanol feed rate was constant at about 10 mL h^{-1} for 2–3 h. In methanol limited processes (designated ML below) the methanol was fed into the bioreactor by means of feedback control to keep DOT at 25% air saturation. The operating pressure was controlled with a regulatory valve in the exit gas line. The values of total air pressure studied were 1.2 bar (reference process ML1.2) and 1.9 bar (high-pressure process ML1.9). In the oxygen-limited fed-batch processes (OL1.2) the stirrer speed and aeration rate were the same as in the processes described above, but the methanol concentration was kept at 350 mg L^{-1} by feed rate control from the methanol analyser. The data in Table 1 are based on two processes of each kind.

2.4. Analyses

Analysis of cell dry weight, β-glucosidase activity in the medium and SDS-PAGE of the medium were performed with conventional methods as described previously [5]. Cell viability was analysed by flow cytometry counting of the frequency of propidium iodide (PI) stained (= dead) cells, as described earlier [5,6]. Total protein concentration in the medium was analysed according to Bradford [16] with BSA as reference.

2.4.1. Methanol feed rate

The methanol feed rate was calculated from the signal of a balance on which the feed solution was placed. The integrated value of this feed rate was used as the total methanol consumption, since the accumulation in the medium and the evaporation of methanol were insignificant in comparison to the feed.

2.4.2. Gas analyses

The concentrations of oxygen, carbon dioxide and methanol in the outlet air were continuously analyzed using Industrial Emissions Monitor Type 1311 (Brüel & Kjær, Innova, Denmark). The methanol signal was calibrated by addition of aliquots of methanol to the bioreactor in a 1 g L^{-1} NaCl solution before the fermentation. The inlet air flow rate was measured with a mass flow meter, and the outlet air flow rate was calculated from the inlet flow and the outlet oxygen and carbon dioxide concentrations, based on the mass balance of the inert nitrogen in the gas flow.

2.4.3. DOT electrode calibration

The calibration of the DOT electrode was performed under the same conditions for all experiments. The 100% air saturation point was calibrated in 3 L of glycerol basal salts medium at pH 5.0 and 30 °C, with aeration at 6 L min^{-1}, agitation at 1000 rpm, and a total air pressure of 1.2 bar. For the zero calibration, nitrogen gas was flown through the bioreactor instead of air.

3. Results and discussion

Three modes of operation with different maximum oxygen transfer rates were investigated: the reference methanol-limited process at 1.2 bar air pressure and DOT = 25% air saturation (ML1.2), the oxygen-limited process at 1.2 bar air pressure (OL1.2) and the methanol-limited process at 1.9 bar pressure and DOT = 25% air saturation (ML1.9). Due to the mode of calibration, the DOT at 1.9 bar was 164% air saturation before the inoculation. According to Eq. (1), the relative oxygen transfer capacities compared to the reference process (ML1.2) are 133%

Table 1
The relative OTR-values calculated from Eqs. (1) and (2) and experimental data expressed as absolute values and values relative to the control culture for the three process control strategies

Parameters	ML1.2		OL1.2		ML1.9	
	Observed values	Relative values (%)	Observed values	Relative values (%)	Observed values	Relative values (%)
OTR (Eq. (1))	–	100	–	133	–	185
OTR (Eqs. (1) and (2))	–	100	–	133	–	161
OTR observed	608 ± 2 (mmol h^{-1})	100	821 ± 6 (mmol h^{-1})	135	966 ± 3 (mmol h^{-1})	159
Total methanol consumption	1414 ± 73 (g)	100	1887 ± 11 (g)	133	2224 ± 104 (g)	157
Cell DW	116 ± 1 (g L^{-1})	100	125 ± 1 (g L^{-1})	107	131 ± 3 (g L^{-1})	112
$Y_{x/s}$	0.37 ± 0.01 (g g^{-1})	100	0.33 ± 0.01 (g g^{-1})	89	0.29 ± 0.01 (g g^{-1})	78
β-Glucosidase	3761 ± 16 (U L^{-1})	100	4807 ± 72 (U L^{-1})	127	5204 ± 16 (U L^{-1})	138
Specific activity	6.04 ± 0.29 (U $mg^{-1}_{protein}$)	100	9.06 ± 0.53 (U $mg^{-1}_{protein}$)	150	7.28 ± 0.24 (U $mg^{-1}_{protein}$)	121
Total product yield	11404 ± 72 (U process^{-1})	100	16125 ± 195 (U process^{-1})	141	17082 ± 87 (U process^{-1})	150

Each experimental value is an average from two similar processes.

and 185% for the oxygen-limited (OL1.2) and high-pressure (ML1.9) processes, respectively (Table 1). The assumed value 185% at 1.9 bar air pressure and DOT = 25% is obtained if only the pressure effect on the partial pressure of oxygen is taken into account at the elevated pressure.

The resulting OUR in the three processes are shown in Fig. 1. Under the quasi-steady state condition during the methanol feed phase (50–100 h), the OUR also represents the OTR, which was 608 ± 2, 821 ± 6, and 966 ± 3 mmol h^{-1}, for ML1.2, OL1.2 and ML1.9, respectively. This gives the observed OTR 135% in the oxygen-limited process and 159% in the high-pressure process compared to the reference process (Table 1). Thus, while the OTR for the oxygen-limited process increased approximately according to the increased driving force (see Table 1), the corresponding values for the process with higher air pressure did not increase according to the increased driving force caused by higher oxygen solubility (Eq. (1) and Table 1).

An explanation to this may be the effect of air pressure on the parameter a (specific air bubble area m^{-1}) in $K_L a$, which may be reduced due to the gas compression. If the gas volume compression is taken into account $K_L a$ should be proportional to the cubic root of the square of the pressure ratio. Then the oxygen transfer rate becomes:

$$\mathrm{OTR} = K_L a_{\mathrm{ref}} \left(\frac{P_{\mathrm{ref}}}{P} \right)^{2/3} (PC^*_{\mathrm{ref}} - C) \qquad (2)$$

The effect of air pressure on the OTR was investigated by Yang and Wang [17] who showed that in the pressure range of 1.06–2.72 bar, the OTR both in sulfite solutions and in *E. coli* cultures was approximately directly proportional to the total pressure, indicating that $K_L a$ was not influenced by the air pressure. On the other hand, Pinheiro et al. [10] found that the sulfite oxidation rate increased only to 255% and 350% of the reference value at 1.2 bar when the pressure was increased by 333% and 500% (4 and 6 bar). This means that the OTR increased less than in proportion to the driving force increase caused by the increased oxygen solubility. This is in agreement with the measured OTR in ML1.9 in which the measured OTR (159%) agrees well with the value calculated by taking the air compression of the bubble interface into account (161%) (Table 1 and Eq. (2)). Obviously, different investigations have resulted in different conclusions regarding the effect of air pressure on $K_L a$. The reason may be that the parameter a depends also on the air dispersion and bubble coalescence, which are likely to depend on the medium properties and fluid dynamic situation.

The increased OTR was reflected by a correspondingly increased methanol consumption rate, which was approximately constant during the 80 h of methanol feeding (Fig. 2a). The relative values of total methanol consumption were almost proportional to the OTR, with 133% and 157% in the OL1.2 and ML1.9 processes, compared to the reference process (Table 1). However, the biomass production did not fully respond to the increased methanol utilization at higher OTR. Only 7% and 12% higher final cell mass was obtained in the OL1.2 and ML1.9 processes, respectively (Fig. 2b and Table 1). This indicates some sort of stress induced under the oxygen limiting and high air pressure conditions.

The reduced biomass yield per methanol may reflect an altered metabolism, e.g., formation of by-products from the carbon source. This is often the case when cells are subject to oxygen limitation, but *P. pastoris* is not known to metabolise methanol in absence of oxygen. Furthermore, the biomass yield per methanol dropped even more in the high-pressure process (78%) than in the oxygen-limited process (89%). Alternatively, an increased maintenance demand, expressed as increased combustion of the energy source may reduce the obtained biomass. The maintenance coefficient has been suggested to be decisive for the level of cell density reached in fed-batch processes with constant feed [4]. Simulations of the biomass concentration profiles obtained in the present investigation were performed with

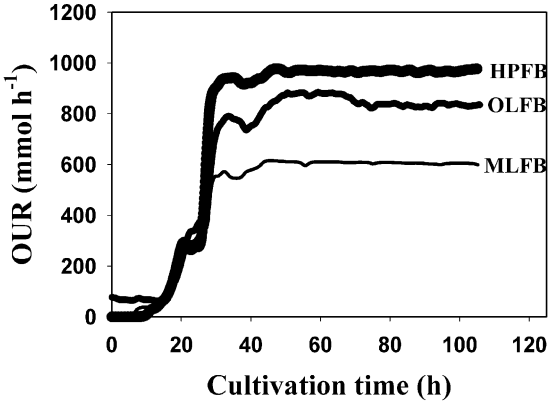

Fig. 1. Oxygen uptake rate (OUR) in the reference process (ML1.2), the oxygen-limited process (OL1.2), and the high-pressure process (ML1.9).

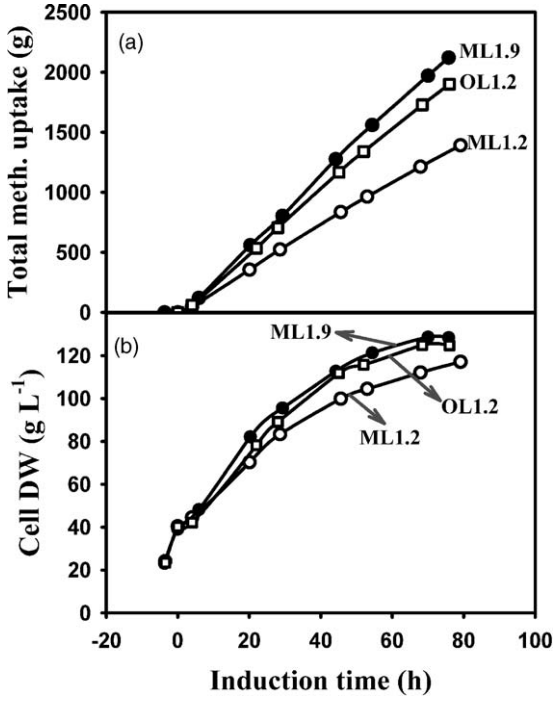

Fig. 2. Comparison of (a) accumulated methanol uptake and (b) cell growth in the reference process (ML1.2 (◯)), the oxygen limited process (OL1.2 (□)) and the high-pressure process (ML1.9 (●)).

the experimentally obtained methanol feed rates, using a model presented previously [4]. The simulation results show that the maintenance demand (q_m) increased from 0.022 g g^{-1} h^{-1} in the reference process (ML1.2) to 0.027 g g^{-1} h^{-1} in the high-pressure process (ML1.9) and to 0.035 g g^{-1} h^{-1} in the oxygen limited (OL1.2) process (Fig. 3). The alternative that the biomass composition or the yield exclusive maintenance (Y_{em}) changes does not give a fit to the experiments (thin line in Fig. 3 (middle panel) and (lower panel)).

Cell death that is followed by cell lysis may be interpreted as low biomass yield and high maintenance demand. *Pichia* cultures are especially subject to cell death during the transition to the methanol phase [18,19]. Furthermore, lysis of dead cells can be responsible for substantial proteolytic degradation of products secreted to the medium [19,20]. In the present investigation, though, the percent of cell death was not influenced neither by oxygen limiting nor high-pressure conditions. From an initial value of 100% viable cells during the glycerol batch phase, the value declined to about 90% after transition to the methanol phase in all processes. During the methanol phase the viability remained constant and similar in all processes (Fig. 4a). However, this does not exclude that some of the dead cells lysed and escaped the flow cytometry count. Therefore also the total mass of dead cells was calculated from the flow cytometry and cell dry weight data. The total amount of dead cells was in all processes highest at about 45 h after induction and then stabilized or decreased (Fig. 4b). A decrease of total amount of dead

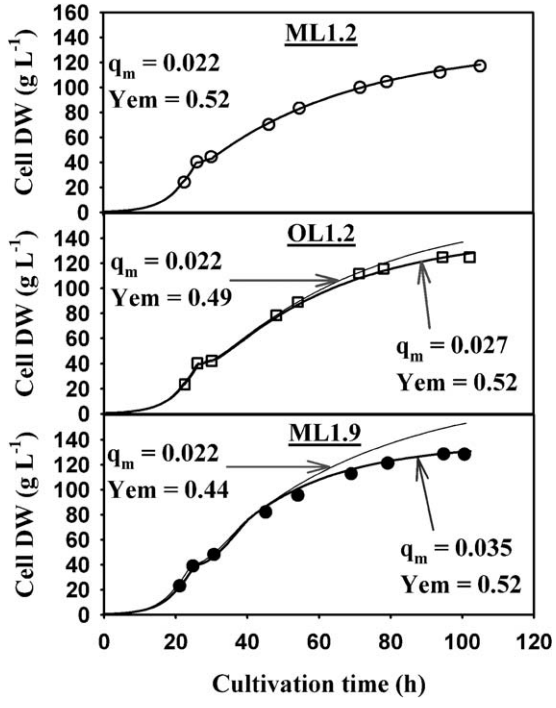

Fig. 3. Comparison of the experimental cell growth (symbols) and simulation (lines) in the reference process (ML1.2, upper panel), the oxygen limited process (OL1.2, middle panel) and the high-pressure process (ML1.9, lower panel). The thin continuous lines in the middle and lower panels represent simulations in which the maintenance coefficient (q_m) is kept unchanged and only the yield coefficient exclusive maintenance (Y_{em}) is reduced to fit the data.

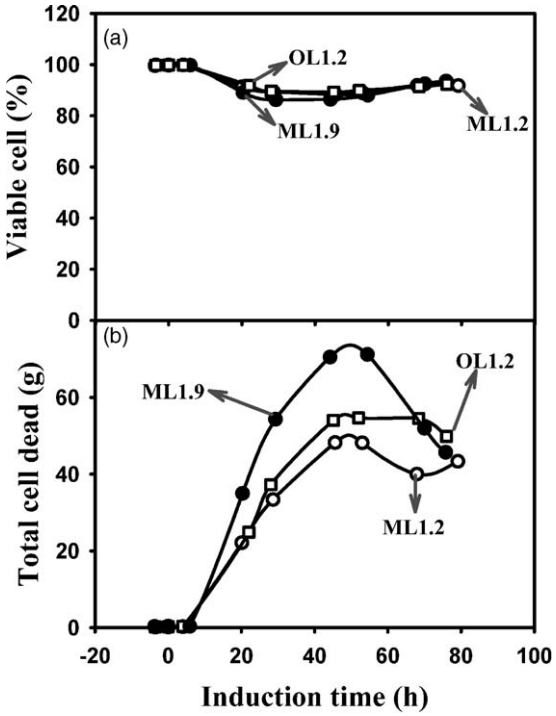

Fig. 4. (a) Viability and (b) total amount of dead cells during the methanol feed phase of the reference process (ML1.2 (◯)), the oxygen-limited process (OL1.2 (□)) and the high-pressure process (ML1.9 (●)).

cells, which was significant only in the high pressure process, means that cells must have lysed at least in this process, though it cannot be excluded that cell lysis also took place in the other processes.

An indication that lysis of dead cells in the initial methanol feed phase may be involved to different degree is obtained from the data on the specific enzyme activity. Fig. 5 shows the accumulation of β-glucosidase activity and total protein in the medium. While the β-glucosidase activity accumulated in a pattern resembling that of cell growth, the total protein accumulated much faster during the first hours after start of the methanol feed, when the cell viability dropped. Unfortunately the specific activity of pure β-glucosidase is not available and therefore it is not possible to translate the activity curve (Fig. 4a) into a mass curve. However, SDS-PAGE analyses show that the product seems to be the main part of the total protein in the medium (data not shown).

Even though the biomass production increased only slightly when the methanol consumption was increased, the enzyme productivity responded more favorably to the increased oxygen transfer. The final enzyme yield (U process^{-1}) increased with 41% and 50% in the OL1.2 and ML1.9 processes, respectively (Table 1).

The specific activity of the β-glucosidase in the broth was calculated from the β-glucosidase activity and total protein data (Fig. 6 and Table 1). It showed that the specific activity was highest in the oxygen-limited process even though the total activity was highest in the high-pressure process. One possible explanation is that the higher specific activity is caused by less cell lysis in the oxygen limited process but it cannot be ruled out that

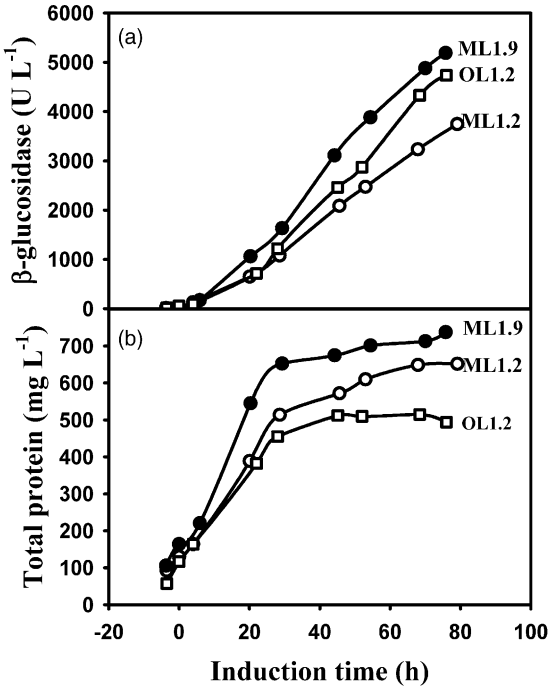

Fig. 5. Accumulation of total protein and β-glucosidase in the *P. pastoris* processes. (a) β-Glucosidase activity in the medium, (b) total protein in medium. Reference process (ML1.2 (◯)); oxygen-limited process (OL1.2 (□)); high-pressure process (ML1.9 (●)).

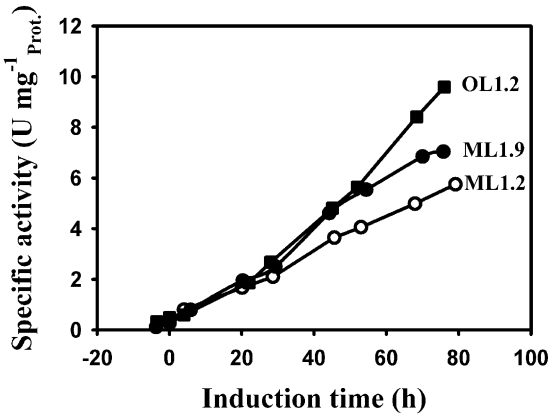

Fig. 6. Specific activity of the β-glucosidase ($U\,mg^{-1}_{protein}$) in the reference process (ML1.2 (◯)), oxygen-limited process (OL1.2 (□)) and the high-pressure process (ML1.9 (●)).

there is a difference in secretion of endogenous proteins from *P. pastoris* under the different process conditions.

4. Conclusion

This work shows that the cell productivity of *P. pastoris* processes can be improved by increasing the oxygen transfer rate by two simple and inexpensive means: application of elevated air pressure and/or accepting oxygen limitation. The increased oxygen transfer rate resulted in approximately correspondingly increased methanol consumption and total product yield, while the biomass yield did not increase in proportion to the increased methanol consumption rates. The latter is actually an advantage, since the high biomass concentration in *Pichia* processes complicates the downstream processing.

Acknowledgments

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