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Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes

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Abstract An oxygen-limited fed-batch technique (OLFB) was compared to traditional methanol-limited fed-batch technique (MLFB) for the production of recombinant Thai Rosewood β -glucosidase with *Pichia pastoris*. The degree of energy limitation, expressed as the relative rate of respiration ($q_O/q_{O,max}$), was kept similar in both the types of processes. Due to the higher driving force for oxygen transfer in the OLFB, the oxygen and methanol consumption rates were about 40% higher in the OLFB. The obligate aerobe *P. pastoris* responded to the severe oxygen limitation mainly by increased maintenance demand, measured as increased carbon dioxide production per methanol, but still somewhat higher cell density (5%) and higher product concentrations (16%) were obtained. The viability was similar, about 90–95%, in both process types, but the amount of total proteins released in the medium was much less in the OLFB processes resulting in substantially higher (64%) specific enzyme purity for input to the downstream processing.

Keywords Oxygen-limited fed batch (OLFB) · Methanol-limited fed batch (MLFB) · *Pichia pastoris* · β -glucosidase

List of symbols

AOX	Enzyme alcohol oxidase
<i>AOX1</i>	Alcohol oxidase gene 1
CPR	Carbon dioxide production rate (mol h ⁻¹)
DOT	Dissolved oxygen tension (%)

MLFB	Methanol limited fed-batch
OLFB	Oxygen limited fed-batch
OUR	Oxygen uptake rate (mol h ⁻¹)
PI	Propidium iodide
q_O	Specific oxygen uptake rate (mol g _{cell} ⁻¹ h ⁻¹)
$q_{O,max}$	Maximum specific oxygen uptake rate (mol g _{cell} ⁻¹ h ⁻¹)
q_P	Specific β -glucosidase productivity (U g _{cell} ⁻¹ h ⁻¹)
Q_i	Inlet air flow rate (L h ⁻¹)
Q_o	Outlet air flow rate (L h ⁻¹)
RRR	Relative rate of respiration
V	Medium volume (L)
V_m	Molar volume of gas (L mol ⁻¹)
X	Biomass concentration from dry weight (g L ⁻¹)
$Y^C_{CO_2/S}$	Carbon yield coefficient of carbon dioxide from methanol (mol mol ⁻¹)
$Y^C_{X/S}$	Carbon yield coefficient of biomass from methanol (mol mol ⁻¹)

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Introduction

Pichia pastoris is a methylotrophic yeast that is often genetically engineered to express proteins [1]. It is suited for foreign protein expression for three main reasons: it can be easily manipulated at the molecular genetic level; it can express and secrete proteins at high levels; and it can perform many of the 'higher eukaryotic' protein modifications such as glycosylation, disulfide-bond formation and proteolytic processing [2].

Pichia pastoris can be grown to very high cell densities (more than 130 g dry cell weight L⁻¹) [3, 4]. It also contains a tightly methanol-controlled alcohol oxidase (*AOX1*) promoter that is induced by methanol and can be used to drive expression of foreign genes [5]. The strong promoter, coupled with the high cell-density fermentation, has allowed production of recombinant product at very high levels. Product concentrations can

reach 22 g L^{-1} for intracellular production [6] and 14.8 g L^{-1} of clarified supernatant for secretion protein production [7].

In recombinant protein production by *P. pastoris*, a four-stage fermentation protocol has been suggested [4]. After an initial batch phase on glycerol to produce biomass, an exponential glycerol feed with glycerol-limiting concentration is applied for a short period to derepress the *AOX1* promoter [8]. Then a low but increasing methanol feed is applied matching the increasing AOX activity caused by the induction [9]. This results in increasing oxygen uptake rate, and when DOT reaches about 25% air sat., the methanol feed rate is kept constant during the main production phase to avoid oxygen limitation. Under these conditions the concentration of methanol is very low and growth-rate-limiting.

Several papers describe the effect of methanol concentration on *P. pastoris*. At low concentration ($< 3\text{--}5 \text{ g L}^{-1}$) the specific growth rate exhibits typical Monod kinetics [10], but at higher concentrations substrate inhibition is observed [10–12]. However, in spite of the methanol-inhibiting effects on growth and substrate uptake observed by Katakura et al. [12], the specific rate of production of a human β_2 -glycoprotein I domain V fragment increased considerably. This might be due to a higher *AOX1* promoter activity at the higher methanol concentration but no AOX data were presented in this paper. However, high methanol concentration cannot be kept in high cell-density cultures without oxygen limitation or temperature limitation [9]. Facultative organisms like *Saccharomyces cerevisiae* and *E. coli* switch to anaerobic metabolism and accumulate toxic metabolites when exposed to oxygen limitation. Such responses have been suggested to play a major role in the scale-up responses of such organisms [13]. The information of *P. pastoris* response to oxygen limitation when growing on methanol is limited. Trentmann et al. [14] compared two *P. pastoris* cultivation techniques, methanol-limited and

methanol-saturated, with oxygen limitation cultures. The recombinant scFv protein quality and productivity were higher in the methanol-saturated processes. On the other hand, no significant difference was found when both techniques were applied for mouse endostatin production [15].

The methanol metabolism of *P. pastoris* has been reviewed by Lin Cereghino and Cregg [16] and the methanol and oxygen consumption has been modeled by Jahic et al. [4]. The pathways summarizing the metabolism of methanol in the methylotrophic yeast *P. pastoris* are shown in Fig. 1. Molecular oxygen is not only used for the respiration but also for the initial oxidation of methanol to formaldehyde. This reaction, catalyzed by the AOX enzyme, generates hydrogen peroxide that can also be used by AOX for methanol oxidation. Thus, two potentially toxic metabolites are generated in the cell during the initial methanol oxidation [5, 17, 18]. Of these components, formaldehyde could be expected to accumulate in the cells with detrimental effect when the cells are exposed to oxygen limitation.

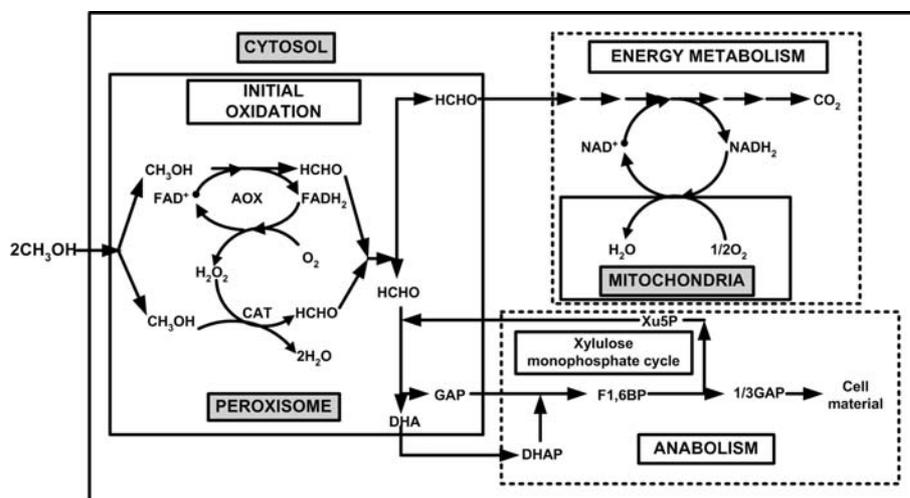
In this work we investigate the possibility to run fed-batch cultures with higher methanol concentration under oxygen limitation. Oxygen-limited fed-batch (OLFB) was compared with methanol-limited fed-batch (MLFB) for the production of the Thai Rosewood β -glucosidase.

Materials and methods

Strain and plasmid

The *P. pastoris* strain Y-11430 (wild-type strain) was a gift from J. Lin Cereghino [16]. β -glucosidase cDNA gene from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) [20] was cloned into the *pPICz α B* vector (Invitrogen). The *pPICz α B* with the β -glucosidase gene was then integrated in to *P. pastoris* Y-11430 at the *AOX1* promoter.

Fig. 1 Methanol metabolism in *P. pastoris*: AOX alcohol oxidase; CAT catalase; GAP glyceraldehyde-3-phosphate; DHA dihydroxyacetone; DHAP dihydroxyacetone phosphate; F1,6BP fructose-1,6-bisphosphate; Xu5P xylulose-5-phosphate. Adapted from Douma et al. [19]



Inoculum preparation

The first inoculum culture was prepared from one colony of *P. pastoris* on YPD agar (yeast extract 10 g, peptone 20 g and dextrose 20 g in 1 L of deionized water) containing 100 µg zeocin ml⁻¹ suspended in 20 ml YPD broth containing 100 µg zeocin ml⁻¹. The culture was incubated at 30° C, in a 100-ml baffled shake-flask on rotary shaker with 200 rpm for 24 h. A second inoculum culture was prepared by transferring the entire 20 ml of first inoculum into 1,000-ml baffled shake-flask that contained 80 ml BMGY medium (yeast extract 10 g, peptone 20 g and glycerol 10 g; dissolved in 1 L of 0.1 M potassium phosphate buffer pH 6.0). The culture was then incubated under the same condition as the first inoculum culture for 24 h.

Fed-batch fermentation

The fed-batch fermentation was carried out in a 10-L stirred tank bioreactor (Belach Bioteknik AB, Stockholm) which contained 3.0 L of glycerol basal salts (GBS) medium (containing H₃PO₄ 85% 26.7 ml; CaSO₄ 0.93 g; K₂SO₄ 18.2 g; MgSO₄·7H₂O 14.9 g; KOH 4.13 g; glycerol 40.0 g; PTM1 trace salts 4.35 ml in 1 L of deionized water). The PTM1 trace salts contained: CuSO₄·5H₂O 6.0 g; KI 0.08 g; MnSO₄·H₂O 3.0 g; Na₂MoO₄·2H₂O 0.2 g; H₃BO₃ 0.02 g; ZnCl₂ 20.0 g; FeCl₃ 13.7 g; CoCl₂·6H₂O 0.9 g; H₂SO₄ 5.0 ml; biotin 0.2 g in 1 L of deionized water. The fermentation was controlled under the following conditions: temperature 30° C, aeration 6 L min⁻¹, agitation 1,000 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 with a glycerol feed (GF) medium (glycerol 500 g L⁻¹ and PTM1 trace salts 12 ml L⁻¹) added 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 (OD₆₀₀ = 80), the process was switched to the third phase (methanol induction phase) by replacing the GF medium with the methanol feed (MF) medium (12 ml PTM1 trace salts per liter of methanol). The initial MF medium feed rate was constant at about 10 ml h⁻¹ for 2–3 h until the production phase. The MF medium was then fed into the bioreactor with different strategies (MLFB or OLFB).

Methanol feed control

In the MLFB, the DOT was kept at 25% air sat. by means of a feedback control of the methanol feed rate based on the DOT signal (Fig. 2a). In the OLFB, the

methanol concentration was kept at 350 mg L⁻¹ by means of feedback control of the methanol concentration from methanol analysis (Fig. 2b).

Analyses

Cell concentration and viability

Cell concentration was monitored by measuring the optical density at 600 nm (OD₆₀₀). Dry cell weight was determined by centrifugation of 5 ml of culture broth at 4,500 rpm for 10 min, and the supernatant was collected for the analysis of other compounds. The pellet was washed with distilled water once and dried at 105° C, till constant weight.

The viability was measured by staining with propidium iodide (PI; Sigma, P-4170). A Partec PAS flow cytometry (Partec GmbH, Münster, Germany) equipped with a 488-nm argon laser was used for this analysis. Samples taken from the fermentor were diluted with PBS (0.16 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄ and 0.001 M KH₂PO₄, pH 7.3). For staining, 25 µl of a stock solution containing 200 µg ml⁻¹ of PI dissolved in water was added to 975 µl of diluted sample at room temperature. Samples were then analyzed, at a data rate of about 1,500 counts s⁻¹. A count of 50,000 was collected in each measurement. The measurement was calibrated by using 3 µm diameter fluorescent beads (Standard 05-4008, Partec GmbH). PI-positive cells were considered as dead and the PI-negative cells were considered as viable.

Total protein concentration

The total protein concentration in the supernatant was analyzed according to Bradford [21]. Bovine serum albumin was used as standard protein.

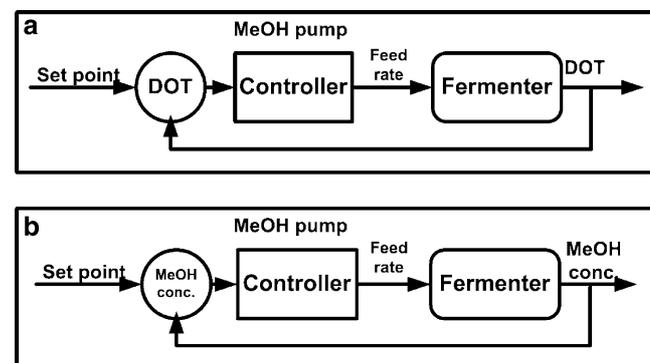


Fig. 2 Block diagram of the methanol feed control: **a** DOT regulation for a methanol-limited fed-batch (MLFB) process and **b** methanol concentration regulation for an oxygen-limited fed-batch (OLFB) process

β-glucosidase activity

β-glucosidase activity was assayed by Evans method [22]. This method used spectrophotometric assay to measure the release of *p*-nitrophenol (pNP) from *p*-nitrophenol-β-D glucopyranoside (pNP-Glu) by β-glucosidase reaction. The 3.3 mM pNP-Glu in 0.1 M sodium acetate buffer pH 5.0 was used as a substrate. One unit of enzyme was defined as the amount of enzyme releasing 1 μmol pNP per minute at 30° C, pH 5.0.

Methanol feed

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 when compared to the feed.

Outlet gas analysis

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 (at the stirrer speed, 1,000 rpm; aeration rate, 6 L min⁻¹; and temperature 30° C in liquid phase, applied in the process) by addition of aliquots of methanol to the fermentor in a 1 g L⁻¹ NaCl solution before fermentation, as described recently [9].

Calculation of relative respiration rate and carbon mass balances

The oxygen uptake rate (OUR) (mol h⁻¹) and carbon dioxide production rate (CPR) (mol h⁻¹) were calculated from:

$$\text{CPR} = \frac{Q_o \text{CO}_{2,o} - Q_i \text{CO}_{2,i}}{100 V_m} \quad (1)$$

$$\text{OUR} = \frac{Q_i \text{O}_{2,i} - Q_o \text{O}_{2,o}}{100 V_m} \quad (2)$$

$$Q_o = \frac{Q_i (100 - \text{O}_{2,i} - \text{CO}_{2,i})}{(100 - \text{O}_{2,o} - \text{CO}_{2,o})}, \quad (3)$$

where Q is air flow rate (l h⁻¹), O_2 is oxygen concentration in air (% v/v), CO_2 is carbon dioxide concentration in air (%v/v), and V_m is molar volume of the gas at the analysis temperature (24.04 l mol⁻¹). Subscripts *i* and *o* refer to inlet and outlet gas, respectively.

Relative rate of respiration (RRR) is a ratio between the observed specific oxygen uptake rate (q_o ; g g⁻¹ h⁻¹) and the maximum specific oxygen uptake rate ($q_{o,\max}$; g g⁻¹ h⁻¹).

$$\text{RRR} = \frac{q_o}{q_{o,\max}}, \quad (4)$$

where q_o was obtained from:

$$q_o = \frac{\text{OUR}}{XV}, \quad (5)$$

in which X is cell concentration (g L⁻¹) and V is culture volume (l). $q_{o,\max}$ was obtained from the maximum q_o value before methanol or DOT became limiting.

For the carbon mass balance calculations, a previously analyzed carbon concentration in *P. pastoris* (0.396 g g⁻¹) was used [4].

SDS-PAGE analysis

The sample, containing 60 μl of supernatant, 25 μl of sample buffer (NuPAGE LDS 4x sample buffer, Invitrogen), 10 μl of 0.5 M dithiothreitol and 5 μl of 3.5% PMSF in ethanol, was incubated for 10 min at 95° C. SDS-PAGE was performed on NuPAGE Novex 4–12% Bis-Tris Gel (1.0 mm×10 well; Invitrogen) using MOPS-running buffer. Ten μl of prepared sample was loaded to each well and run at 200 V for 60 min. The gel was stained with Coomassie Blue R-250 for 30 min and destained (with destain solution; 100 ml L⁻¹ methanol and 100 ml L⁻¹ glacial acetic acid in distillate water) for 1–2 h.

Alcohol oxidase activity

Alcohol oxidase was assayed with the method described recently [9]. Sample from the fermentation (5 ml) were centrifuged at 4,200 rpm, 4° C for 10 min. The volume of supernatant was measured. The cell pellet was washed once with 5 ml of 0.1 M potassium-phosphate buffer pH 7.5 and finally suspended in the same buffer to the original supernatant volume. The cells were then disintegrated in a French press (SLM Aminco, USA) at 800 bars. One milliliter of 0.1 M potassium-phosphate buffer pH 7.5 with 2,000 units of catalase (from bovine liver, Sigma-Aldrich, Sweden) and 10 μl of cells homogenate were mixed. Catalase was added to assure that the hydrogen peroxide produced was converted to molecular oxygen, making the stoichiometry of the reaction 1 mol O₂ per 2 mol of CH₃OH. The reaction was started by adding 10 μl of 10 M methanol. Oxygen consumption was assayed polarographically with a Clark type oxygen electrode (Medelco AB, Sweden) at 37° C in air-saturated buffer. Alcohol oxidase units were expressed as a μmol of methanol oxidized per minute.

Results and discussion

Cell growth and product accumulation

The duplication processes of OLFB and MLFB processes showed the good reproducibility. The biomass

concentration profiles were quite similar for the two control strategies, but with a higher maximum value for the OLFB (129 g L^{-1}) than for the MLFB (122 g L^{-1} ; Fig. 3a). Also the β -glucosidase accumulation was higher in the OLFB with total $6,000 \text{ U L}^{-1}$ supernatant fluid, compared to $5,170 \text{ U L}^{-1}$ in the MLFB process. The specific β -glucosidase productivity (q_p) declined slowly during the process, but it was still about 52% of the initial value after 120 h of induction (Fig. 3b), indicating a capacity for extending the process even if the cell density does not increase. This is in agreement with earlier results on production of a fusion protein between a cellulose binding module and a lipase with *P. pastoris*, when the specific productivity was even better preserved during 160 h [4]. Thus, the oxygen limitation did not inhibit β -glucosidase accumulation which is similar to other protein productions [14, 23].

Effect of feed strategy on total methanol consumption and OUR

The methanol feed in the MLFB was regulated with the DOT electrode keeping the DOT constant at 25% air sat. (Fig. 4a). After a transient accumulation of methanol to 700 mg L^{-1} , the concentration decreased below the detection limit (about 25 mg L^{-1}) in 5 h (Fig. 4d). In the OLFB, the methanol concentration was automatically controlled at 350 mg L^{-1} (Fig. 4d), and it resulted in a rapid drop of DOT below the detection limit (Fig. 4a). In the OLFB, the OUR was about 40% higher than in the MLFB (Fig. 4b) and it was kept relatively constant in both processes.

Correspondingly, the total methanol consumption was about 40% higher in OLFB (Fig. 4d). Thus, the increased oxygen transfer rate obtained by the use of the

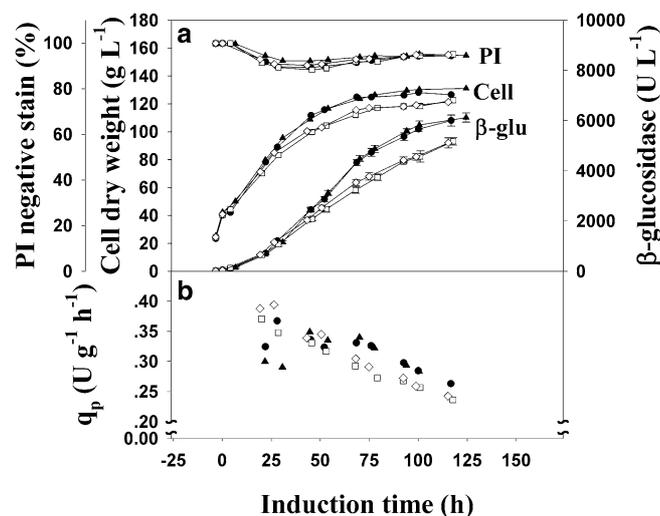


Fig. 3 a Fermentation profile of β -glucosidase production in MLFB (open symbols, thin lines) and OLFB (close symbols, thick lines); cell dry weight, β -glucosidase activity and PI-negative-stained cell b specific productivity (q_p)

OLFB technique resulted in higher OUR and correspondingly higher methanol consumption rate. This was similar to a recent work on mouse endostatin production [15] and scFv production [14] when the methanol-saturation condition was applied for *P. pastoris* expression system.

The relative respiration rate (RRR), i.e., the current respiration rate divided by the maximum respiration rate is a parameter that can be used to describe the degree of oxygen limitation. It should also be a measure of the degree of methanol limitation, provided the organism is obligately aerobic and has no alternative pathways for the methanol metabolism. This analysis showed that both processes were quite similar with respect to degree of energy limitation (Fig. 4c). Initially, after the induction, both cultures were nonlimited with respect to oxygen and methanol and RRR was 1, but then this parameter dropped rapidly in both processes and approached about 0.20–0.25 with slightly a higher value (i.e., less degree of energy limitation) for the MLFB.

Maintenance demand and viability

Taking into account that considerably more methanol was consumed in the OLFB process but only slightly

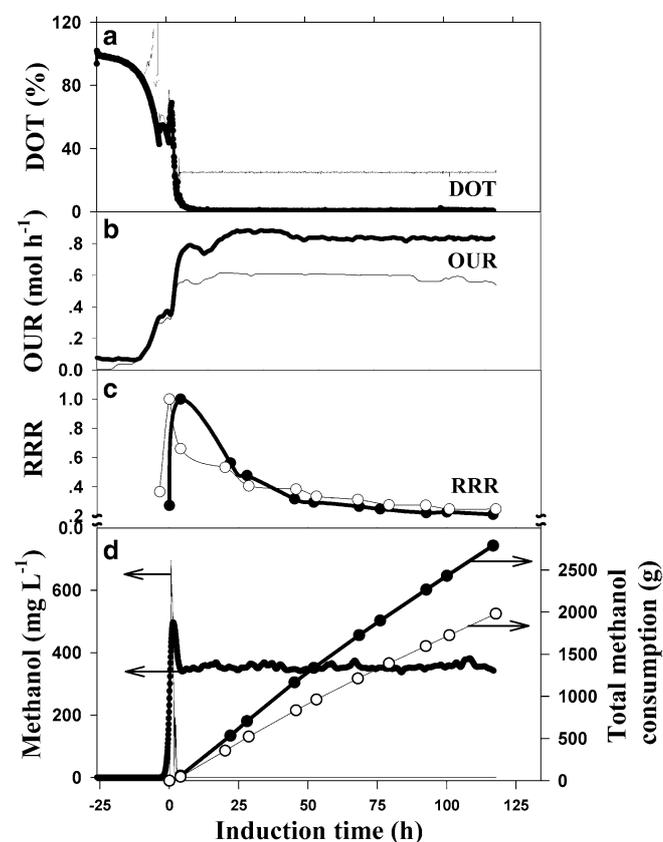


Fig. 4 Comparison of a DOT, b OUR, c RRR and d methanol concentration (lines) and total methanol uptake (symbols) in MLFB (open symbols, thin lines) and OLFB (close symbols, thick lines)

more cells were produced, the carbon yield coefficient of biomass from methanol ($Y_{X/S}^C$) must have been lower in the OLFB process. This was further investigated by carbon mass balances.

Carbon mass balances based on methanol input and outputs of biomass, carbon dioxide and protein in the medium showed good agreement in both processes. The methanol loss in the outlet air is insignificant since the methanol analyzer signal from 500 mg L⁻¹ in the fermentor is kept stable for at least 2 days under calibration condition (data not shown). During the main part of the process time the carbon recovery was 98–99% (Fig. 5). The extracellular protein in the medium accounted for less than 0.4 % of the total carbon (data not shown). Thus, no major by-products from the methanol metabolism were produced. The $Y_{X/S}^C$ (molC molC⁻¹) gradually declined from 0.33 to 0.24 for the OLFB and from 0.38 to 0.27 for the MLFB (Table 1). Corresponding carbon yield coefficients of carbon dioxide from methanol ($Y_{CO_2/S}^C$) increased from 0.60 to 0.74 in the OLFB and from 0.59 to 0.72 in the MLFB. The decreasing biomass yield and the increasing carbon dioxide yield reflects the increasing total maintenance demand at declining specific growth rate [4] but it also shows that the maintenance demand is larger in the OLFB.

Methanol is known to be toxic for many species. However, *P. pastoris* is quite resistant to methanol and 10 g L⁻¹ methanol exhibited only a slight reduction of the specific growth and methanol uptake rates [12]. Not until the methanol concentration reached 30 g L⁻¹ was the growth almost completely inhibited. Therefore the higher methanol concentration in the OLFB process (350 mg L⁻¹) was not expected to be inhibitory. However, the low oxygen concentration in the OLFB might cause an accumulation of intracellular methanol if it limits the oxidation rate of methanol. This is especially intriguing since in vitro assays of the AOX kinetics showed a very high K_m (0.7 mM) for oxygen at 10 mM methanol [5].

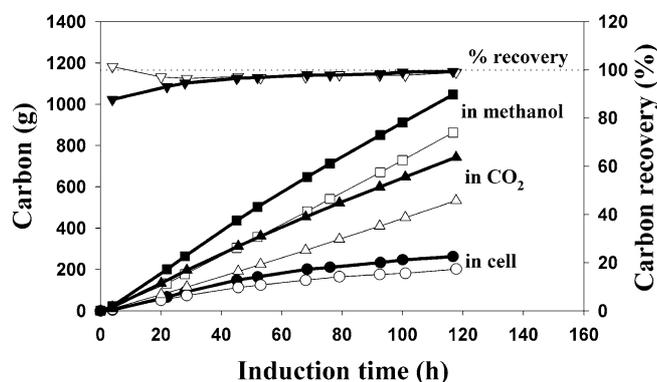


Fig. 5 Carbon mass balances of a MLFB (open symbols) and an OLFB (close symbols): carbon in cell (circle), carbon in CO₂ (triangle), carbon in methanol (square) and carbon recovery (inverted triangle)

The possible toxic effect of methanol in the OLFB process was investigated by comparing the viability analyzed as frequency of dead cells according to propidium iodide (PI) staining. This method has previously been used to reveal large differences in viability of *P. pastoris* cultures under different process conditions [9]. However, no difference in viability was observed between the OLFB and the MLFB processes (Fig. 3a). In both cases the frequency of viable cells dropped to about 90% during the first 22 h of induction but then it stayed within 90–95% throughout the processes.

The intracellular AOX activity

The β -glucosidase accumulation during the transition phase after the induction was similar to both cultivation techniques, but after 20 h higher level of β -glucosidase accumulation was observed in OLFB process (Fig. 3a). To investigate whether there were differences in the activity of the AOX promoter, the intracellular AOX activity was compared. Figure 6 shows that the AOX activity initially showed a similar rapid increase to a maximum of about 2,000–2,300 U g_{cell}⁻¹ after 20 h of induction in both techniques. Then during the main production phase a gradual decrease of the AOX activity was observed in both processes, but the decrease was less pronounced in the OLFB and at the end of the process, the OLFB exhibited 37% higher AOX activity. This might be due to the fact that the methanol concentration was higher and not growth-limiting in the OLFB. It is also plausible that the higher β -glucosidase production, at least partly, could be due to a higher activity of the *AOX1* promoter since the deviation between the q_p curve (Fig. 3b) and the AOX curve for the two processes follows a similar pattern after about 50 h (Fig. 6).

Product purity

One of the advantages of the *P. pastoris* system for the production of recombinant proteins is that it often permits secretion of the product to a defined mineral salt medium contaminated with only a few host proteins. At the end of the processes, at about 120–130 g L⁻¹ cell dry weight, the β -glucosidase activity was higher in an OLFB process (6,000 U L⁻¹) than in a MLFB process (5,200 U L⁻¹). On the contrary, the total protein in the medium (data not shown) was lower in the OLFB (final concentration about 470 mg L⁻¹) than MLFB (about 660 mg L⁻¹). The combined effect of these parameters was that the specific activity of the β -glucosidase became much higher in the OLFB processes (Fig. 7).

Conclusions

The obligately aerobic *P. pastoris* did not respond to severe oxygen limitation with major negative responses.

Table 1 Carbon yield coefficient of biomass and carbon dioxide for MLFB and OLFB

MLFB			OLFB		
Induction time (h)	$Y_{X/S}^{C*}$ (molC molC ⁻¹)	$Y_{CO_2/S}^{C**}$ (molC molC ⁻¹)	Induction time (h)	$Y_{X/S}^{C*}$ (molC molC ⁻¹)	$Y_{CO_2/S}^{C**}$ (molC molC ⁻¹)
20.1	0.38	0.59	22.0	0.33	0.60
28.6	0.38	0.59	28.0	0.34	0.60
45.6	0.36	0.61	45.1	0.33	0.63
53.0	0.34	0.62	52.0	0.32	0.64
67.9	0.33	0.65	68.4	0.3	0.67
79.1	0.32	0.66	76.0	0.29	0.68
92.4	0.29	0.68	92.6	0.27	0.71
100.9	0.28	0.70	100.0	0.26	0.72
117.7	0.27	0.72	116.7	0.24	0.74

** $Y_{CO_2/S}^{C}$ Carbon yield coefficient of carbon dioxide from methanol

* $Y_{X/S}^{C}$ Carbon yield coefficient of biomass from methanol

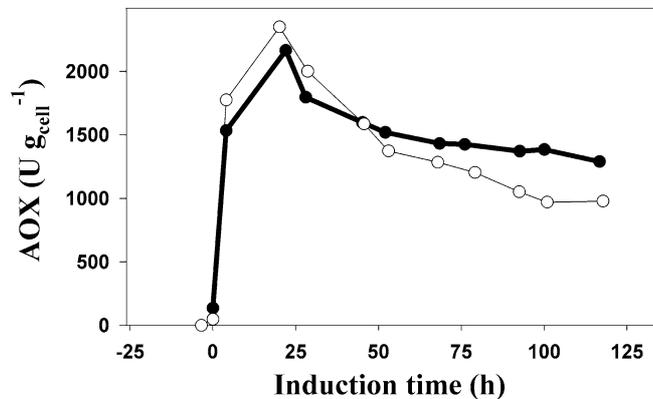


Fig. 6 Comparison of the AOX activity in MLFB grown cells (open symbols) and OLFB grown cells (close symbols)

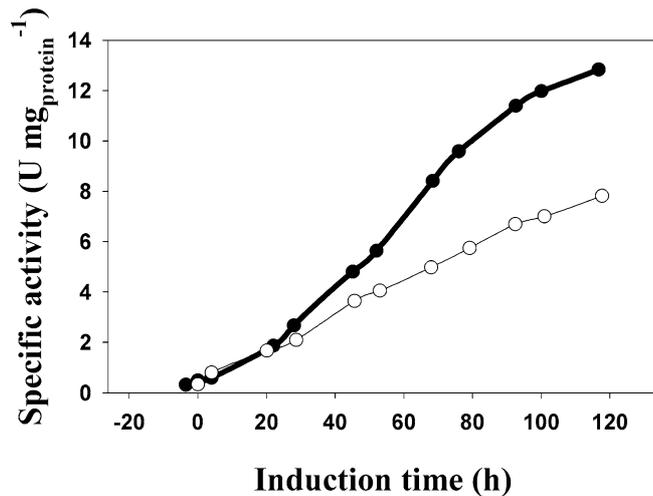


Fig. 7 Specific activity of β -glucosidase in the culture supernatant from one MLFB (open symbols) and OLFB (close symbols) process

Therefore, an oxygen-limited fed-batch technique could be used to improve the oxygen transfer rate and productivity. An additional advantage was that less total proteins were released to the medium making the specific

product concentration in the broth much higher. On the other hand, the oxygen limitation caused an increasing maintenance demand which resulted in a lower biomass yield per methanol.

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