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Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein

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Abstract A fusion protein composed of a cellulose binding domain from *Neocallimastix patriciarum* cellulase A and *Candida antarctica* lipase B (CBD-lipase) was produced by *Pichia pastoris* methanol utilization plus phenotype in high cell-density cultures. The genes expressing CBD-lipase were fused to the alpha-factor secretion signal sequence of *Saccharomyces cerevisiae* and placed under the control of the alcohol oxidase gene (*AOX1*) promoter. To control the repression and induction of *AOX1* and oxygen demand at high cell density, a four-stage process was used. Batch growth on glycerol was used in the first step to provide biomass (28 g L^{-1}) while product formation was prevented due to repression of the *AOX1*. The second stage was exponential fed-batch growth on glycerol, which caused a slight increase of the enzyme alcohol oxidase activity due to derepression of the *AOX1*. This procedure resulted in smooth transition to exponential fed-batch growth on methanol, the third stage, in which the *AOX1* was strongly induced. The fourth stage was constant fed-batch growth on methanol used to control the oxygen demand at the high cell density. A kinetic model was developed that could predict biomass growth and oxygen consumption in processes with and without oxygen-enriched air. With oxygen enrichment to 34% O_2 in the inlet air the methanol feed rate could be increased by 50% and this resulted in 14% higher final cell density (from 140 to 160 g L^{-1} cell dry weight). The increased methanol feed rate resulted in a proportionally increased specific rate of product secretion to the medium. After an initial decrease, the synthesis capacity of the cell was kept constant throughout the cultivation, which made the product concentration increase almost constantly during the process. The kinetic model also describes how the low maintenance demand of *P. pastoris* compared with *E. coli* enables this organism to grow to such high cell densities.

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Keywords Recombinant protein, *Pichia*, Modeling, Energy metabolism, Glycerol

List of symbols

AOX	enzyme alcohol oxidase
AOX1	alcohol oxidase gene one
AOX2	alcohol oxidase gene two
CAT	enzyme catalase
CAT	catalase gene
CBD	cellulose binding domain
C_S	carbon concentrations in the substrate (g g^{-1})
C_X	carbon concentrations in biomass (g g^{-1})
DOT	oxygen dissolved tension (%)
F	glycerol or methanol feed rate (L h^{-1})
K_s	saturation constant (g L^{-1})
Meth	methanol
NAD	nicotinamide adenine dinucleotide
OCR	total oxygen consumption rate (g h^{-1})
q_m	maintenance coefficient (g $g^{-1}h^{-1}$)
q_O	specific oxygen consumption rate (g $g^{-1}h^{-1}$)
q_S	specific rate of substrate consumption (g $g^{-1}h^{-1}$)
$q_{S,an}$	specific rate of substrate flux for anabolism (g g^{-1} h^{-1})
$q_{S,en}$	specific rate of substrate flux for energy metabolism (g $g^{-1}h^{-1}$)
$q_{S,max}$	specific maximum rate of substrate consumption (g $g^{-1}h^{-1}$)
S	limiting substrate concentration (g L^{-1})
S_i	inlet substrate concentration (g L^{-1})
t	time (h)
V	medium volume (L)
X	biomass concentration from dry weight (g L^{-1})
$Y_{O/S,an}$	coefficient for oxygen consumption per substrate used for anabolism (g g^{-1})
$Y_{O/S,en}$	coefficient for oxygen consumption per substrate used for energy metabolism (g g^{-1})
Y_{em}	biomass yield coefficient exclusive maintenance (g g^{-1})
$Y_{O/S}$	stoichiometric coefficient oxygen per substrate (g g^{-1})
$Y_{X/S}$	yield coefficient biomass per substrate (g g^{-1})
μ	specific growth rate (h^{-1})

Subscripts

an	anabolism
en	energy metabolism
gly	glycerol
i	inlet
max	maximum

meth	methanol
O	oxygen
S	substrate (glycerol or methanol)
X	biomass
0	initial value

1 Introduction

P. pastoris is frequently used for expression of heterologous genes [1, 2, 3, 4, 5, 6, 7, 8]. This organism can grow on methanol in fermentor culture at high cell densities (>130 g L^{-1} dry cell weight) [9]. The first step in the metabolism of methanol is the oxidation to formaldehyde, generating hydrogen peroxide, catalyzed by the enzyme alcohol oxidase using molecular oxygen [10, 11]. This step takes place within a specialized organelle, the peroxisome [12]. Alcohol oxidase has a poor affinity for oxygen, and *P. pastoris* compensates for this by generating large amounts of the enzyme. There are two genes in *P. pastoris* that code for alcohol oxidase: *AOX1* and *AOX2*. The *AOX1* gene is responsible for the majority of alcohol oxidase activity and *AOX1* and *AOX2* are regulated in a similar manner. The regulation involves both repression–derepression and induction mechanisms [13]. It is mainly the *AOX1* promoter that is used to control recombinant protein production in *P. pastoris*. The promoter is very strong but repressed by unlimited growth on glycerol [4, 14].

The productivity of *P. pastoris* in shake flasks is typically low and is improved greatly by fermentor culturing. The first reason is that only in the controlled environment of a fermentor is it possible to grow the organism to high cell densities. The second reason is that the level of transcription initiated from the *AOX1* promoter is greater in *P. pastoris* cells fed with methanol at growth-limiting rates in fermentor culture than in cells grown in excess of methanol [7]. Therefore improving the fermentation methodology is important for *P. pastoris* based processes. These improvements include substrate feeding strategies [15], oxygen supplementation to allow higher cell densities while avoiding oxygen limitation [16], and mixed-substrate feeding strategies [17, 18].

To our knowledge no model has been presented for simulation of growth and oxygen consumption in high cell-density fed-batch cultivations of *P. pastoris*. In this work a kinetic model for anabolism and energy metabolism was applied to *P. pastoris* grown sequentially on glycerol and methanol to simulate the growth and oxygen consumption in high cell-density fed-batch cultivations producing the fusion protein composed of a cellulose binding domain from *N. patriciarum* cellulase A and *C. antarctica* lipase B (*CBD*-lipase).

2 Materials and methods

2.1 Strain

P. pastoris SMD1168 is a protease-deficient strain (*his4 pep4*) available from Invitrogen, Carlsbad, Calif., USA. The design of the plasmid coding for the fusion protein composed of a cellulose-binding domain from *N. patriciarum* cellulase A and *C. antarctica* lipase B (CBD-lipase) is described elsewhere [19].

2.2 Medium

2.2.1 Shake flask medium

Inoculum was produced in a 1 L shake flask with 100 mL buffered minimal glycerol inoculum medium: yeast nitrogen base without amino acids, 134 g L^{-1}; phosphate buffer (132 mL of 1 M K_2HPO_4 and 868 mL 1 M KH_2PO_4), 100 mL L^{-1}; biotin, 400 μg L^{-1}; and glycerol, 10 g L^{-1}. The initial pH was 6. Cells were grown for 14 h at 30°C on a shaker with 260 rpm, which resulted in an OD_{600} between 2 and 5.

2.2.2 Fermentor medium

The shake flask culture was transferred to the fermentor with 3 L fermentor medium: H_3PO_4 85%, 26.7 mL L^{-1}; $CaSO_4{\cdot}2H_2O$, 0.93 g L^{-1}; K_2SO_4, 18.2 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 14.9 g L^{-1}; KOH, 4.13 g L^{-1}; glycerol, 40 g L^{-1}; and trace element solution, 4.3 mL L^{-1} of the fermentor medium. The trace element stock solution contained: $CuSO_4{\cdot}5\ H_2O$, 6 g L^{-1}; KI, 0.8 g L^{-1}; $MnSO_4{\cdot}H_2O$, 3 g L^{-1}; $Na_2MoO_4{\cdot}2H_2O$, 0.2 g L^{-1}; H_3BO_3, 0.2 g L^{-1}; $CaSO_4{\cdot}2H_2O$, 0.5 g L^{-1}; $ZnCl_2$, 20 g L^{-1}; $FeSO_4{\cdot}H_2O$, 65 g L^{-1}; biotin, 0.2 g L^{-1}; conc. H_2SO_4, 5 mL.

2.3 Cultivation conditions

When the glycerol was consumed after about 27 h, a feed containing 555 g L^{-1}glycerol and 12 mL L^{-1}trace element solution was started. The initial feed rate was 38.5 mL h^{-1} and was increased at a rate of 0.18 h^{-1} to force the cells to grow at a specific growth rate of about 0.18 h^{-1}. After 3–4 hours when cell concentration was approximately 42 g L^{-1} dry weight (about OD_{600}=120) the glycerol feed was replaced with a feed containing 780.6 g L^{-1} methanol and 12 mL L^{-1} trace element solution. The initial feed rate was 10.5 mL h^{-1} and was increased exponentially with 0.12 (0.07) h^{-1} up to 24 mL h^{-1} (36 mL h^{-1} in the oxygen enriched culture), which was then kept constant until the end of the process.

Fed-batch fermentation was carried out in a 10 L standard fermentor (Belach Biotek AB, Stockholm). The agitation, pH, temperature, dissolved oxygen tension (*DOT*), pressure, air-flow rate, oxygen flow rate, pump speed, and antifoam addition were automatically controlled. All these parameters and the signals for fermentor weight, feed reservoir weight, NH_4OH reservoir weight, accumulated antifoam pump run time, and outlet gas composition (CO_2 and O_2), were monitored and logged. The fermentation was carried out under the following conditions: temperature, 30°C; pH 5.0 controlled by the addition of 28% NH_4OH. *DOT* was controlled at the level of 30% air saturation by agitation up to 1,000 rpm with aeration rate 6 L min^{-1} and by increasing air flow rate or oxygen flow rate when rpm reached the maximum 1,000. Foaming was automatically controlled by means of a level

electrode and antifoam A (A5758, Sigma-Aldrich, Stockholm).

2.4 Calculation of feed profiles

Growth at high concentration of glycerol was assumed to repress the alcohol oxidase genes (*AOX1* and *AOX2)* and catalase gene (*CAT*) [12]. To permit derepression before the methanol feed was applied, an exponential but growth-rate limiting glycerol feed was applied for half a generation between the end of the glycerol batch phase and the start of the methanol feeding. The feed profile was calculated from a mass balance on glycerol,

$$\frac{dS}{dt} = \frac{F}{V}(S_i - S) - \frac{\mu}{Y_{X/S}}X \quad (1)$$

in which the limiting glycerol concentration was assumed to be insignificant compared to the inlet concentration, i.e., $S \approx 0$. Rearrangement of Eq. (1) for the condition $dS/dt=0$ gives the feed rate F_0 that corresponds to the substrate consumption rate for a certain amount of biomass (X_0V_0) at the time of the feed start.

$$F_0 = \frac{\mu}{Y_{X/S}S_i}X_0V_0 \quad (2)$$

The initial glycerol feed was calculated for $\mu=0.18\ h^{-1}$ and the biomass X_0V_0 at the end of the glycerol batch phase. To permit the biomass to increase with the exponent $0.18\ h^{-1}$, the feed profile was set to:

$$F(t) = F_0 e^{\mu t} \quad (3)$$

where t is time after the start of the exponential feed.

The *AOX1* and *AOX2* genes are not only repressed by glycerol but also induced by methanol. To avoid overfeeding in the beginning of the methanol feeding phase, a low initial methanol feed rate was used ($3.5\ mL\ L^{-1}\ h^{-1}$), corresponding to the consumption rate at $\mu=0.005\ h^{-1}$. This feed was also increased exponentially with exponent $0.12\ h^{-1}$ until the *DOT* approached 30% air saturation, after which the methanol feed was constant.

2.5 Analyses

2.5.1 Cell concentration

Cell concentration was monitored by measuring the optical density (OD_{600}) at 600 nm. Dry weight *(X)* of the cell suspensions was determined by centrifugation of 5 mL cell broth in a preweighed centrifuge tube, followed by drying to constant weight at 80°C in an oven. The correlation between X and OD_{600} was $X\ (g\ L^{-1}) = 10.76 + 0.258 \times OD_{600}$ over the whole cell concentration range.

2.5.2 Concentration of glycerol and protein in medium

Off-line analyses of glycerol were performed in quadruples by using an enzymatic kit (Boehringer Mannheim GMbH, Germany). The concentration of protein was analyzed according to Bradford [20].

2.5.3 Carbon concentration in the cells

The carbon concentration in the cells was analyzed with a carbon analyzer (TOC-5000, Shimadzu Corporation). In principle, total carbon was decomposed into CO_2 by oxidation catalyst and heating to 680°C, and then CO_2 was detected with a non-dispersive infrared gas analyzer.

2.5.4 Oxygen, carbon dioxide and air

The oxygen and carbon dioxide in the outlet gas was continuously analyzed with industrial emissions monitor Type 1311 (Innova, Denmark). The air-flow rate was measured with mass flowmeter CP111MF (Belach Biotek AB, Sweden). In experiments with oxygen-enriched air, the aeration rate was 5 L min^{-1} and oxygen (0–1 L min^{-1}) was mixed with the air before the inlet. The oxygen flow was measured with mass flowmeter CP111MF.

2.5.5 Methanol concentration in medium

The methanol concentration in the outlet gas was continuously analyzed with industrial emissions monitor Type 1311. Before the start of the fermentation, standard curves were performed by adding different amounts of methanol to the fermentor medium at the same airflow and same agitation as in real fermentation. From this data the concentration of methanol in liquid phase (fermentor broth) was recalculated.

2.5.6 Lipase hydrolytic activity

The specific lipase hydrolytic activity of the fusion enzyme towards tributyrin was measured at 25°C and pH 7.5 using pH-stat. The substrate solution (2% gum arabic, 0.2 M $CaCl_2$ and 0.2 M tributyrin) was emulsified by sonication for 2 min. To start the reaction, enzyme was added to 1.5 mL substrate solution under vigorous stirring and pH was titrated with 100 mM NaOH solution.

2.5.7 Alcohol oxidase activity

The specific alcohol oxidase activity towards oxygen was measured at 37°C and pH 7.5 using an oxygen electrode (Medelco AB, Sweden). The samples from the fermentor (5 mL) were centrifuged. The supernatant was removed and cells were washed with 3 mL 100 mM potassium phosphate buffer pH 7.5. After repeated centrifugation and removing of supernatant, 2 mL of 100 mM potassium phosphate buffer pH 7.5 were added to the cells. The cells were then disintegrated in a French press (SLM Aminco, USA) at 800 bar. The disintegrate was centrifuged and 10 μL were added to the sample reservoir with 3 mL of air saturated 100 mM potassium phosphate buffer pH 7.5. The reaction was started by adding 10 μL of 10 M methanol. The electrode was calibrated to 100% with air saturated 100 mM potassium phosphate buffer pH 7.5 and to 0% by adding sodium-dithionit.

3
Model of growth and oxygen consumption

The model is based on the metabolic flux of substrate carbon and the associated consumption of molecular oxygen for oxidation. While microorganisms growing on carbohydrates use molecular oxygen mainly for respiration, yeasts growing on methanol also require substantial amounts of oxygen for the initial oxidation of methanol to formaldehyde (Fig. 1) [12]. All methanol taken up by the cell is oxidized to formaldehyde in a coupled reaction involving enzyme alcohol oxidase (AOX) and catalase (CAT) in the peroxisomes. These reactions use molecular oxygen as the ultimate electron acceptor. The net stoichiometry of this reaction is

$$CH_3OH + 0.5O_2 \rightarrow HCHO + H_2O \quad (4)$$

which gives the stoichiometric conversion coefficient $Y_{O/S}$=0.5 g O_2 (g methanol)$^{-1}$ for the initial oxidation step.

The formaldehyde flux is then divided into a flux to anabolism and a flux to energy metabolism (Figs. 1 and 2). In the formaldehyde flux to anabolism the oxygen demand is considered insignificant. Thus, in the anabolism of methanol, oxygen is consumed only in the initial oxidation of methanol and the oxygen consumption per methanol in the anabolism is $Y_{O/S,an}$=0.5 g O_2 (g methanol)$^{-1}$.

The remaining part of the substrate carbon flux is used for energy metabolism of formaldehyde. In these reactions, formaldehyde is oxidized by NAD^+ to CO_2 and water with molecular oxygen as the ultimate electron acceptor (Fig. 1). The reactions for the methanol oxidation in the energy metabolism are

$$CH_3OH + 0.5O_2 \rightarrow HCHO + H_2O \quad (5)$$

in the initial oxidation of methanol, and

$$HCHO + O \rightarrow CO_2 + H_2O \quad (6)$$

in the energy metabolism of formaldehyde.

The net stoichiometry of these reactions is

$$CH_3OH + 1.5 \rightarrow O_2CO_2 + 2H_2O \quad (7)$$

which gives the stoichiometric conversion coefficient $Y_{O/S,en}$=1.5 g O_2 (g methanol)$^{-1}$ for the methanol flux to energy metabolism.

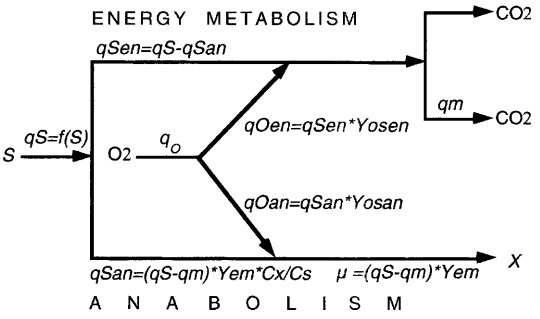

Fig. 2. Mathematical flux model for *P. pastoris* growing on methanol. A similar model was used for growth on glycerol with the modification that $q_{O,an}$ was set to zero. For abbreviations, see List of symbols. The equations are explained in Sect. 3 of the text

It is assumed that the specific rate of methanol consumption q_S, g methanol (g biomass)$^{-1}$ h^{-1}, in the methanol limitation regime follows the Monod model:

$$q_S = q_{S,\max}\frac{S}{S + K_S} \quad (8)$$

The assimilation rate of methanol, g methanol (g cells)$^{-1}$ h^{-1}, can be described with two carbon mass balances (Eqs. 9 and 10):

$$\text{Carbonfluxinanabolism} = q_{S,an}C_S(\text{gC(gcell)}^{-1}\text{h}^{-1}) \quad (9)$$

where C_S is the carbon concentration in the substrate (g carbon (g substrate)$^{-1}$) and $q_{S,an}$ is the specific rate of substrate flux for anabolism (g g^{-1}h^{-1}).

$$\text{Rate of carbon accumulation by growth} = (q_S - q_m)Y_{em}C_X(\text{gC(gcell)}^{-1}\text{h}^{-1}) \quad (10)$$

where C_X is carbon concentration in biomass (g carbon (g cells)$^{-1}$), q_m is maintenance coefficient (g g^{-1}h^{-1}). To take into account the gradually increasing relative use of substrate for maintenance in high cell-density fed-batch processes the concept of yield coefficient exclusive maintenance (Y_{em}) is used [21].

From Eqs. (9) and (10) the carbon flux to the anabolism is obtained as

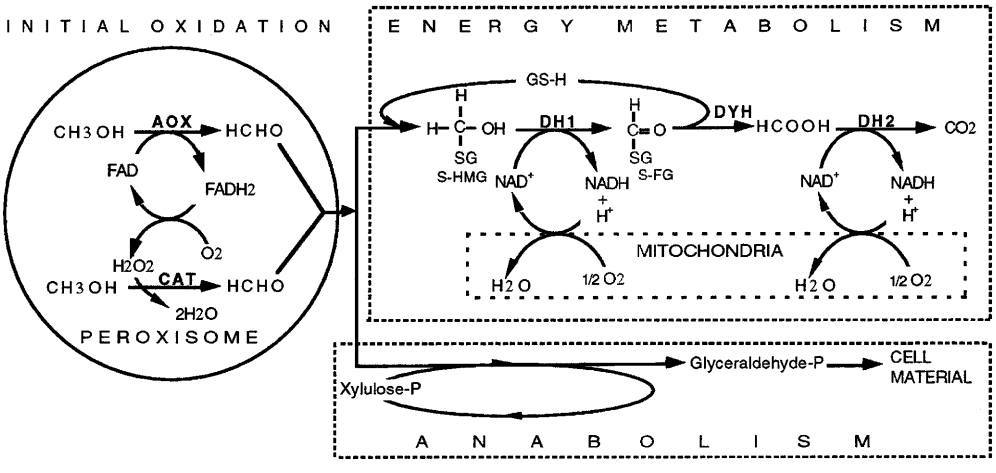

Fig. 1. Metabolism of methanol by *P. pastoris*. AOX: alcohol oxidase; DH2: formate dehydrogenase; CAT: catalase; S-HMG: S-hydroxymethylglutathione; DH1: formaldehyde dehydrogenase; S-FG: S-formylglutathione; DYH: S-formylglutathione-hydrolase; G-SH: reduced glutathione

$$q_{S,an} = (q_S - q_m) Y_{em} \frac{C_X}{C_S} \quad (11)$$

The carbon flux to the energy metabolism is described as

$$q_{S,en} = \left(q_S - q_{S,an}\right) \quad (12)$$

The specific oxygen consumption rate q_O, g O_2 (g biomass)$^{-1}$h^{-1}, can now be calculated from the two carbon fluxes (Eqs. 11 and 12) and corresponding stoichiometric conversion coefficients:

$$q_O = q_{S,an} Y_{O/S,an} + q_{S,en} Y_{O/S,en} \quad (13)$$

The specific growth rate is obtained from the yield coefficient exclusive maintenance and difference between specific rate of methanol consumption and maintenance:

$$\mu = (q_S - q_m) Y_{em} \quad (14)$$

The specific rate equations for μ, q_S and q_O (Eqs. 14, 8 and 13) were inserted in the corresponding mass balance equations (Eqs. 15, 16 and 17) to solve for the concentrations of biomass, substrate, and the oxygen consumption rate.

$$\frac{dX}{dt} = \left(-\frac{F}{V} + \mu\right) X \quad (15)$$

$$\frac{dS}{dt} = \frac{F}{V}(S_i - S) - q_S X \quad (16)$$

$$OCR = q_0 X V \quad (17)$$

In the first and second fermentation stages when the cells were grown on glycerol, the same model was used modified with the relevant stoichiometric conversion coefficients (Table 1). The volume change in a laboratory scale bioreactor has a considerable influence on the biomass concentration, which must be accounted for in the simulation. First, the volume is influenced by sampling. Second, the feed contains water, and part of the substrate is converted to water by the combustion in the energy metabolism. Finally, some water evaporates with the outlet air. The actual volume of medium was monitored by means of a balance under the vessel and used in the simulation.

4 Results and discussion

In the first process design, a shift from unlimited growth on glycerol (μ_{max} 0.26 h^{-1}) to methanol feed was performed at the end of the batch glycerol growth phase (cell density 52 g L^{-1}). The oxygen consumption rate then decreased below what was expected from the methanol feed rate, and the cell concentration declined by 3 g L^{-1} and did not increase for 12 h (data not shown). This was most likely due to lack of alcohol oxidase and catalase activity. Veenhuis et al. showed that both alcohol oxidase and catalase activities increased in other methanol utilizing yeasts (*Hansenula polymorpha* and *Kloeckera sp.*) when the dilution rate was below 0.2 in a glucose limited chemostat [12]. To permit derepression of the alcohol oxidase and catalase before the methanol feed was started, a short phase with exponential feed of glycerol was inserted when the initial glycerol had been consumed. The feed exponent used was 0.18 h^{-1}, permitting a growth rate of about 0.18 h^{-1} for about 0.5 generation. Figure 3 (expanded for the transition phase) shows that the exponential feed of glycerol resulted in a slight increase in alcohol oxidase activity. This is probably the reason why the transition to the methanol feeding was smooth compared to the case when no exponential glycerol feed was applied and a considerable cell death and 10-h long adaptation was needed (data not shown).

Figures 4 and 5 show the biomass concentration and the oxygen consumption rate in two cultures, with the highest possible methanol feed rate in the constant phase, to permit minimum 30% *DOT*. In Fig. 4 only air was used and the constant methanol feed rate was 24 mL h^{-1}, while in Fig. 5 the oxygen transfer rate was increased by supplying 1 L min^{-1} oxygen, which permitted a methanol feed rate of 36 mL h^{-1} with similar *DOT*. In both cases the biomass concentration profile exhibited the typical hyperbolic shape for a high cell-density fed-batch culture with a maximum cell density of 138 g L^{-1} and 158 g L^{-1} with the higher value for the oxygen enriched culture. In both cases, the total oxygen consumption rate (g h^{-1}) increased, during the constant methanol feed rate.

Table 1. Parameters used in simulation of *P. pastoris* growth and oxygen consumption

Symbol	Unit	Value		Origin and source	
		Glycerol	Methanol	Glycerol	Methanol
C_S	g g^{-1}	0.391	0.375	st. coeff.	st. coeff.[a]
C_X	g g^{-1}	0.396	0.96	expt	expt[b]
K_S	g L^{-1}	0.1	0.1	expt	expt
q_m	g g^{-1}h^{-1}	0[c]	0.013	–	expt
$q_{S,max}$	g g^{-1}h^{-1}	0.37	0.57	expt	expt
S_i	g L^{-1}	555	780	expt	expt
Y_{em}	g g^{-1}	0.7	0.36	expt	expt
$Y_{O/S,an}$	g g^{-1}	0	0.5	st. coeff.	st. coeff.
$Y_{O/S,en}$	g g^{-1}	1.217	1.5	st. coeff.	st. coeff.

[a]From stoichiometry.
[b]From experimental data.
[c]Not applied, no influence at the high growth rate.

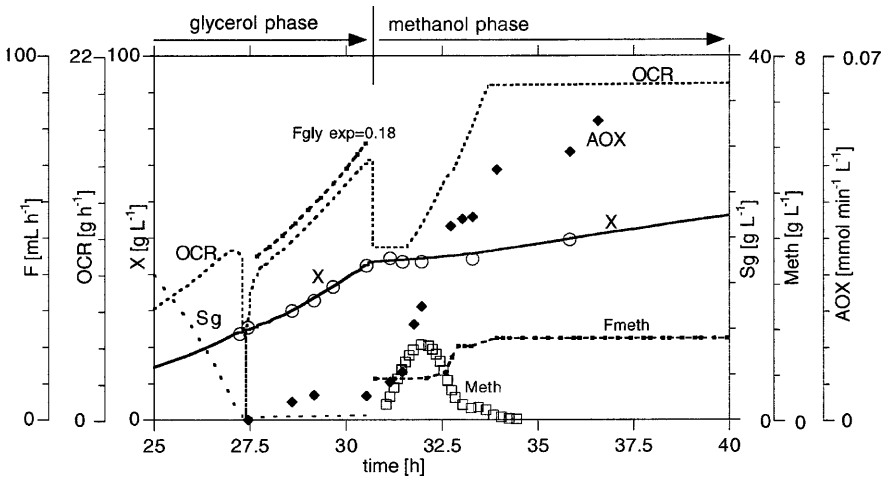

Fig. 3. Data from the transition from glycerol to methanol growth of a *P. pastoris* culture. *Lines* represent simulation and symbols represent measured data. Biomass concentration (*solid line*, *open circles*, X); glycerol concentration (*dotted line*, Sg); methanol concentration (*open squares*, Meth); alcohol oxidase activity (*filled diamonds*, AOX); oxygen consumption rate (*dotted line*, OCR); glycerol feed rate (*small filled squares*, Fgly); methanol feed rate (*small filled squares*, Fmeth)

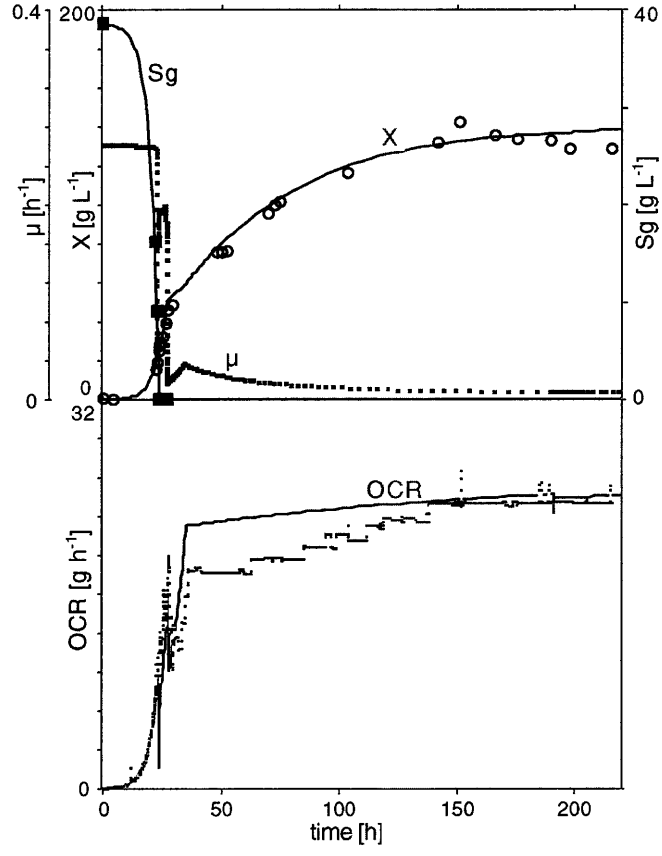

Fig. 4. Experimental data and simulation of *P. pastoris* control culture without oxygen enrichment. *Solid lines* represent simulation and *symbols* represent measured data. Biomass concentration (*open circles*, X); specific growth rate (*thick dotted line*, μ); glycerol concentration (*filled squares*, Sg); oxygen consumption rate (*thin dotted line*, OCR)

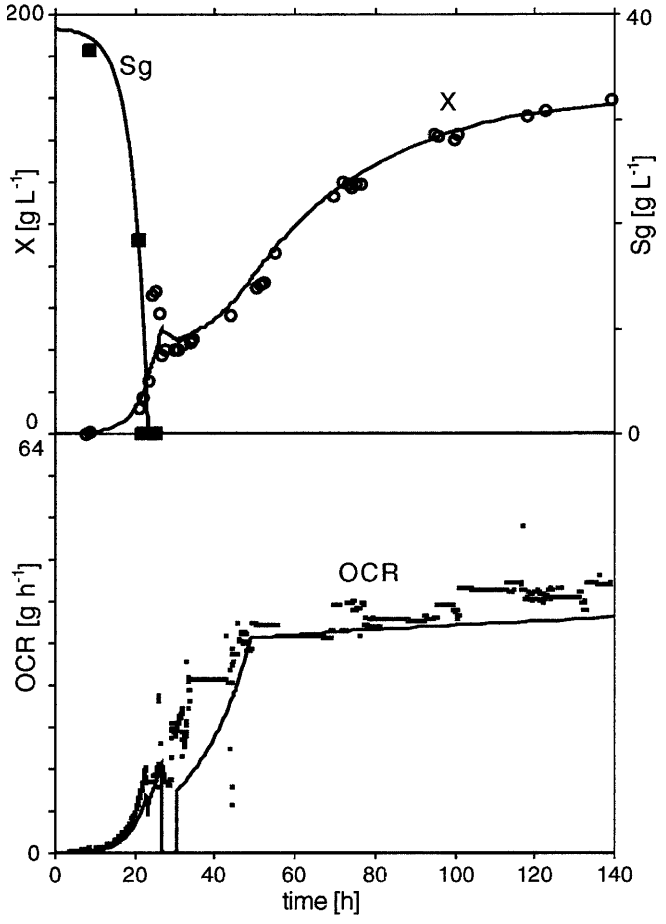

Fig. 5. Experimental data and simulation of oxygen-enriched *P. pastoris* culture. *Solid lines* represent simulation and *symbols* represent measured data. Biomass concentration (*open circles*, X); glycerol concentration (*filled squares*, Sg); oxygen consumption rate (*small filled squares*, OCR)

When the model was used to simulate the biomass concentration and the oxygen consumption rate, good agreement was obtained between the biomass data and the simulations (Figs. 4 and 5). Also, the oxygen consumption rate was reasonably well predicted with the model during the first and last part of the process in Fig. 4, while there was an overestimate during the first half of the constant feed phase. Such a deviation was observed in most oxygen consumption-rate simulations but without a systematic pattern (in Fig. 5 it is rather an underestimate). A possible explanation is an analytical error in the oxygen measurements, since the maximum deviation shown in Figs. 4 and 5 corresponds to a measurement error of about 0.7% oxygen.

The gradually increasing oxygen consumption rate in the constant methanol feed phase and the maximum cell concentration that was observed in all fermentations and simulations can be explained by the maintenance demand. Maintenance is here defined as energy substrate metabolism that does not result in biomass synthesis. This parameter is low for *P. pastoris* (0.013 g $g^{-1}h^{-1}$, in this study) compared to *E. coli*, with about 0.04 g $g^{-1}h^{-1}$ for growth on glucose [21, 22]. The low maintenance demand of *P. pastoris* is a requirement for the very high cell density that is achievable with this organism. The model can be used to illustrate this.

Under constant feed conditions, the maximum cell density is a function of the substrate feed rate and the maintenance coefficient. This is obtained from the mass balance on the substrate (Eq. 1) that can be written

$$\frac{dS}{dt} = \frac{F}{V}(S_i - S) - \frac{\mu}{Y_{em}}X - q_m X \qquad (18)$$

where Y_{em} (g g^{-1}) is the biomass yield coefficient exclusive maintenance and q_m (g $g^{-1}h^{-1}$) is the maintenance coefficient. Under high cell-density conditions in a constant-feed fed-batch culture, the substrate concentration becomes very low and the slope d*S*/d*t* becomes insignificant. Equation (18) can then be simplified to

$$FS_i = VX\left(\frac{\mu}{Y_{em}} + q_m\right) \qquad (19)$$

where FS_i is the constant feed of substrate (g h^{-1}). Since the biomass (XV) increases, the growth rate (μ) declines with time, and when the specific growth rate approaches the dilution rate F/V, the maximum cell concentration (X_{max}) is achieved. This is obtained from Eq. (19) by solving for X when $\mu = F/V$:

$$X_{max} = \frac{(F/V)S_i}{(F/V)(1/Y_{em}) + q_m} \qquad (20)$$

The drastic influence of the maintenance on the maximum cell density is illustrated in Fig. 6, where the process in Fig. 4 is simulated with three different maintenance coefficients 0, 0.013 (corresponding to *Pichia*) and 0.04 (corresponding to *E. coli*). Thus, the lower the maintenance, the higher is the maximum cell density achievable in a constant feed fed-batch process. The other parameter that has a large influence on the cell density is the feed rate (FS_i), but this parameter is in practice limited by the oxygen transfer capacity of the reactor.

The kinetic model and the hypothesis on the maximum cell density are further supported by the increasing total oxygen consumption rate (g h^{-1}) during the constant methanol feeding. According to the model, more oxygen is used per carbon unit in the energy pathway than for the anabolic flux, 1.5 and 0.5 mole O_2 (mole carbon)$^{-1}$, respectively. Since the growth rate gradually declines from about 0.18 to 0.005 h^{-1} and asymptotically approaches zero in the process (Fig. 4), more and more of the total carbon flux is directed to the energy flux, part of which is used for maintenance (see Fig. 2). In practice the maintenance coefficient is not a constant but depends on the process conditions [23]. At μ=0.033, about 33% of the carbon flux was used for anabolism, but at the end when μ was 0.005 only 20% was used for anabolism. This results in the increasing oxygen consumption rate observed in all cultures. The simulation in Fig. 6 shows that no such increase in oxygen consumption rate is expected if there is no maintenance at all (q_m=0). The very low value of the maintenance in *P. pastoris* is a prerequisite for its ability to grow to high cell densities in fed-batch cultures.

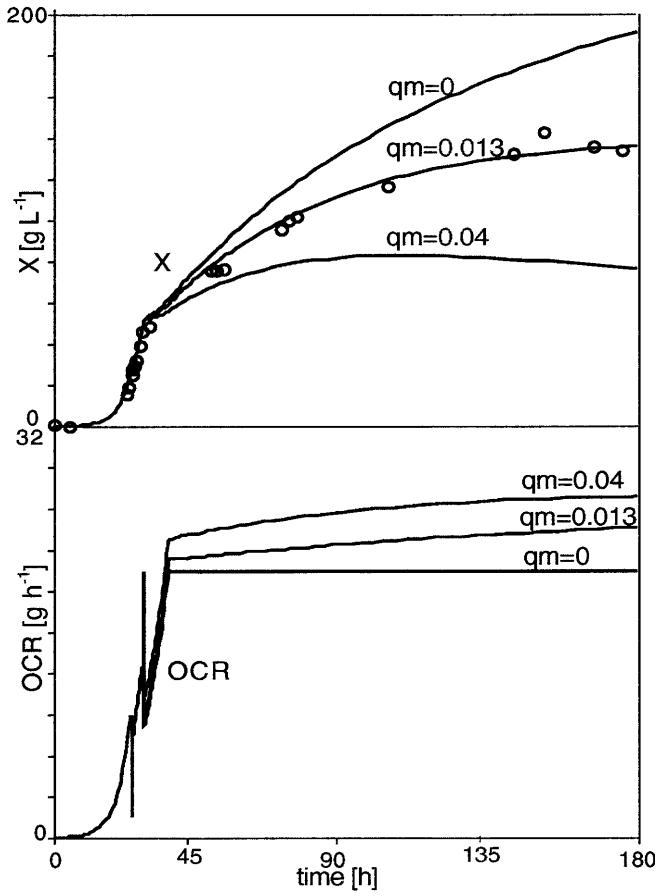

Fig. 6. Simulation of the process in Fig. 4 with three dierent maintenance coecients q_m=0, 0.013 (corresponding to *P. pastoris*), and 0.04 (corresponding to *E. coli*). *Open circles* represent measured data for biomass concentration. *Upper panel*: biomass concentration (X); *lower panel*: oxygen consumption rate in whole reactor (OCR)

The total protein concentration in the medium increased at a constant rate up to the 140th hour and then leveled off at about 1.5 g L^{-1} (data not shown). The *CBD*-lipase, measured as lipase activity, also increased at a constant rate and did so for a longer period, up to the 200th hour in the control culture (Fig. 7). The final product concentration was about 1.5 g L^{-1} estimated on the basis of the lipase activity, which means that most of the protein in the medium constituted product. When lipase activity was analyzed in a cell-free medium that was incubated for 48 h, no decrease in the activity was observed, indicating that the protein was not degraded by proteolysis to the extent that the enzyme activity was impaired.

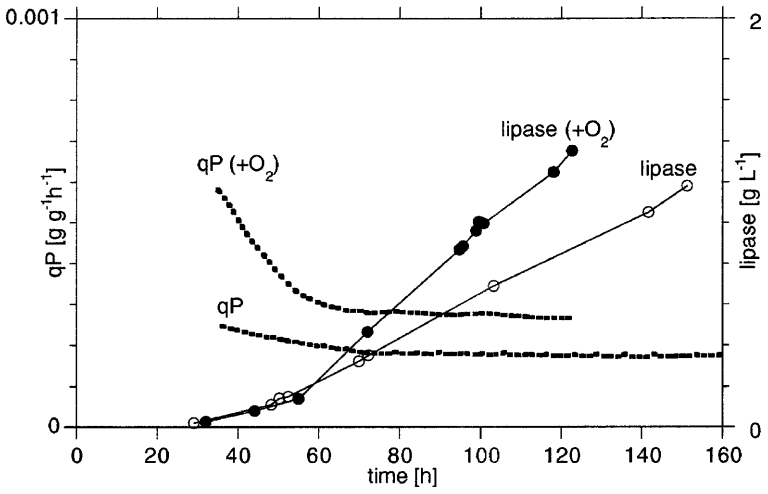

Fig. 7. Lipase concentration (●, ○) and the specific product accumulation rate (*thick dotted lines*) in control and oxygen-enriched ($+O_2$) culture of *P. pastoris*

In the oxygen-enriched culture the accumulation pattern was similar, but total protein and lipase accumulated at a rate that was about 35 and 52% higher, respectively, than in the control fermentation. This is due to the difference in methanol feed rate, which was 50% higher in the oxygen-enriched process. However, since the dilution rate was different due to the different methanol feed rates, these figures do not describe the performance of the cells correctly. Therefore the specific product accumulation rate was calculated. This rate was almost constant throughout the process, except for a higher value directly after induction (Fig. 7). This pattern differs greatly from *E. coli*, where the specific rate of product formation often drops quite quickly, although from a much higher value. Figure 7 also shows that the specific productivity was almost in proportion to the methanol feed and stabilized at a 48% higher value when the feed rate was increased by 50%. Further increased oxygen enrichment (2 L oxygen min^{-1}, 5 L air min^{-1}) permitted a methanol feed rate of 65 mL h^{-1} (170% higher then the control) but this resulted in only 21% increased biomass concentration and did not result in any further increase in specific product formation rate (data not shown).

5
Conclusion

This work shows that a kinetic model can be used to simulate growth and oxygen consumption and explain two limiting factors for maximum cell density in fed-batch cultures of *P. pastoris*: the maintenance demand and the feed rate, which is limited by the oxygen transfer capacity. Furthermore, oxygen enrichment in the inlet air to 34% could be utilized to increase the methanol feed rate by 50%, which resulted in a proportionally increased specific productivity, which remains constant at the level up to the maximum cell density of about 160 g L^{-1} cell dry weight.

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