J Ind Microbiol Biotechnol (2016) 43:517–523
DOI 10.1007/s10295-015-1722-6

FERMENTATION, CELL CULTURE AND BIOENGINEERING

Influence of methanol/sorbitol co-feeding rate on pAOX1 induction in a *Pichia pastoris* Mut+ strain in bioreactor with limited oxygen transfer rate

F. Carly[1] · H. Niu[2] · F. Delvigne[3] · P. Fickers[1,3]

Received: 9 September 2015 / Accepted: 11 December 2015 / Published online: 20 January 2016

Abstract High *Pichia pastoris* biomass density could be obtained using high co-feeding rate of methanol and sorbitol in a fed-batch or continuous culture, while further higher feeding rate finally leads to oxygen limitation in bioreactor. In the literature, there is lack of report about AOX1 promoter regulation with regard to dissolved oxygen level (DO). Therefore, in this work, chemostat cultures were performed to investigate the cell growth, metabolism and regulation of the AOX1 promoter (pAOX1) regarding co-feeding rate of optimized methanol/sorbitol mixture (methanol fraction 0.60 C-mol/C-mol) using a *P. pastoris* Mut+/pAOX1-lacZ strain. The oxygen transfer rates (OTR) in bioreactor were kept in the range of typical values of large bioreactor, i.e., 4–8 g/(L h) if DO equals 30 % saturation or 5–10 g/(L h) if DO nears zero. For DO >0, an increase of the carbon fed led to an increase of pAOX1 induction. By contrast, when dissolved oxygen was completely depleted, methanol accumulated, causing a 30 % decrease of pAOX1 induction. However, this decrease is more likely to be lined to methanol accumulation than to low level of dissolved oxygen (<4 % DO). Methanol/sorbitol co-feeding allowed cells to adapt to oxygen transient limitations that often occur at industrial scale with reduced effect on pAOX1 induction. The optimal feeding rate tested here was 6.6 mmol C (DCW h)$^{-1}$ at an OTR of 8.28 g O_2(L h)$^{-1}$ with over fivefold pAOX1 induction (probably directly associated with target protein productivity) compared with previous work.

Keywords *Pichia pastoris* · pAOX1 · Methanol/sorbitol feeding · Chemostat · Process optimization

Introduction

The methylotrophic yeast *Pichia pastoris* has become over the years one of the most extensively used expression systems for heterologous proteins production [9]. It presents many advantages over other yeast systems, such as availability of engineered strains capable of human-like glycosylation [7, 11], ability to grow in defined media at high biomass densities, and existence of strong promoters, e.g. pAOX1, tightly regulated by methanol. There are three types of *P. pastoris* host strains available that differ from their ability to metabolize methanol and thus from their oxygen consumption rate. These are the Mut+ phenotype (wild-type strain, high oxygen consumption), Mut^S (methanol utilization slow, resulting from AOX1 deletion, intermediate oxygen consumption) and Mut- (methanol utilization minus, resulting from the double AOX1-AOX2 deletion, low oxygen consumption) [9]. The Mut+ phenotype is mainly used for recombinant protein productions, although the Mut^S phenotype has been used in some other cases. However, the use of methanol as sole carbon source during the induction phase for protein production poses one major drawback for Mut+ strains (and Mut^S in a lower extent):

F. Carly and H. Niu contributed equally to the work.

✉ P. Fickers
pfickers@ulg.ac.be

[1] Unité de Biotechnologies et Bioprocédés, Université libre de Bruxelles, Av Roosevelt 52, CP152/51, 1050 Brussels, Belgium

[2] Department of Chemical Engineering, Centre for Process Systems Engineering, Imperial College London, South Kensington Campus, London SW7 2AZ, England, UK

[3] Microbial Processes and Interactions, Université de Liège – Gembloux AgroBio Tech, Passage des Déportés, 2, 5030 Gembloux, Belgium

as a high-degree reductant with high heat of combustion, methanol catabolism requires a high oxygen consumption in aerobic condition accordingly leading to a huge amount of heat production. To solve this problem, methanol co-feeding strategies have been developed to partially replace or at least complement methanol with other carbon sources such as glucose [22], glycerol [15, 28] or sorbitol [3, 26]. Among these, sorbitol was found the most promising co-substrate because it is not only a lower degree reductant than methanol but also a non-repressive carbon source for AOX1 promoter induction unlike glycerol or glucose [25]. The benefits of methanol/sorbitol co-feeding have been reported in regard to reducing oxygen consumption without loss of protein productivity [12]. Although some studies have been performed to quantitatively analyze cell metabolism, they only focused on the measurements of final products, i.e. secreted proteins, without any direct insights on quantifying the pAOX1 regulation [4, 16]. Recently, we have quantitatively characterized the pAOX1 induction of *P. pastoris* as well as the intracellular metabolic flux distributions during the methanol/sorbitol co-feeding process [20]. In detail, it was performed with chemostat cultures using a Mut+ strain in which the pAOX1-lacZ construct served as a reporter gene. Our results demonstrated that decreasing methanol fraction in the feeding media reduced the cell-specific oxygen consumption by 30 %. More interestingly, maximal β-galactosidase cell specific activities, and thus optimal pAOX1 induction, were achieved and maintained in the range of 0.45–0.75 C-mol/C-mol of methanol fraction.

As a follow-up, this work aimed to optimize the fermentation and protein production processes, i.e., mainly to achieve higher cell density, higher induction level and to characterize the effects of dissolved oxygen level in the co-feeding process [13, 18]. Anoxia management is one of the main challenges during the induction phase of pAOX1 due to limited oxygen transfer capacity of bioreactor. Moreover, inefficiency in mixing capacity could promote, especially at industrial scale, transient heterogeneities in the fermentation broth thus leading to transient anoxia as well as nutrient starvation. Accordingly, an improved understanding of the methanol/sorbitol co-feeding process, especially in respect of carbon feeding rate, oxygen limitation and pAOX1 induction, is of the utmost importance in the process optimization. Therefore, our strategy was to perform different chemostat cultures with increasing carbon concentrations in the methanol/sorbitol mixed feed while keeping dilution rate constant to exclude the variation of dilution effects amongst different experimental conditions. Traditional fermentation processes have OTR values ranging from about 100 mmol//(L h) (easy) through about 150–200 mmol//(L h) (average) to more than 300 g//(L h) (difficult) [2]. Correspondingly, the K_La values should be from 340 h^{-1} (easy) through 515–685 h^{-1} (average) to over 1030 h^{-1} (difficult) at 0.5 bar gauge pressure and DO = 30 %. In this work, we assume that the achievable values of K_La in large fermenters are in the range of 400–800 h^{-1} and accordingly typical values of oxygen supply are in the range of 4–8 g/(L h). The oxygen supply in bioreactor was kept in the range of typical values in large fermenters to provide experimental data for future industrial scale-up. Methanol and sorbitol uptake rates, cell growth and pAOX induction level were determined against carbon concentration (methanol and sorbitol) in the feed and oxygen level (oxygen limited or not).

Materials and methods

Strain and medium composition

A *P. pastoris* strain GS115 transformed with pSAOH5 vector bearing a pAOX1-lacZ construct was used [17]. The medium and feeding solutions were (per liter): YSG+ (6 g yeast extract, 5 g soy peptone, 20 g glycerol); BG (batch glycerol medium, 38 g glycerol, 25 mL H_3PO_4 85 %, 1.05 g $CaSO_4{\cdot}2H_2O$, 18.28 g K_2SO_4, 14.96 g $MgSO_4{\cdot}7H_2O$, 4.15 g KOH, and 12 mL PTM1 solution); FBG (glycerol feeding solution, 330 g glycerol and 12 mL PTM1 solution). The composition of the different methanol (FBM) and sorbitol (FBS) feeding solutions were (per liter) 12 mL PTM1 supplemented with different amounts of methanol (364, 459, 546 or 634 g) and sorbitol (152, 190, 230 and 266 g), respectively. PTM1 trace elements solution was from Invitrogen.

Culture conditions

Pre-cultures were performed at 30 °C for 24 h in a 1 L flask containing 150 mL YSG+ medium. Cultures in bioreactor were performed in a 2L Biostat Bplus (Sartorius AG, Germany). The temperature was maintained at 30 °C during growth on glycerol and shifted to 25 °C during methanol/sorbitol feeding phase according to previous work [21]. The pH was regulated at 5.8 by addition of 25 % ammonia solution. Dissolved oxygen (DO) was tried to maintain at 30 % of saturation using cascade control of agitation and airflow rate, while DO was not successfully maintained at the preset 30 % in the cultures with higher feeding rates. Culture and 100 % calibration of DO probe were performed at atmospheric pressure. Maximum stirring speed was set at 2000 rpm and maximum airflow was set at 1.5 L min^{-1}. The gauge pressure was set at 0.5 bar during fermentation. The MFCS/win 3.0 software (Sartorius AG) was used to record the culture history. Cultures started in batch mode in 750 mL of the BG medium at an initial OD600 of 0.3.

After depletion of the initial glycerol, indicated by a sudden increase in dissolved oxygen, the glycerol fed batch phase started at a feeding rate of 12 mL h^{-1} of the FBG solution. After 24 h of glycerol fed batch, feeding with the different FBM and FBS solutions started and lasted for 72 h at a volumetric flow rate of 12 and 18 mL h^{-1}, respectively, to obtain carbon feeding rates (CFRs) equal to 4.4, 5.5, 6.6 and 7.7 mmol C gDCW h^{-1} of a 0.6/0.4 C-mol/C-mol methanol/sorbitol mixture. For CFR calculation, the value of biomass obtained at the end of the glycerol-feeding phase. Culture volume was kept at 1.5 l by a pump controlled by a level probe.

Analytical methods

Cell growth was monitored by either optical density at 600 nm (OD_{600}) or dry cell weight (DCW) as previously described [20]. Glycerol, methanol and sorbitol were analyzed by isocratic RID-HPLC (Agilent 1100 series, Agilent Technologies) using an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm Bio-Rad, Hercules, USA) with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL min^{-1} at 30 °C. Ammonia assay was based on the phenol-hypochlorite method [24]. β-Galactosidase activities were determined using the Miller method [19]. In detail, the cell pellets were suspended in 500 µl of buffer Z (100 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$). The sample was then diluted 100–8000 times in buffer Z. Cells were then permeabilized by adding 30 µl of chloroform and 20 µl of 0.1 % SDS before being vortexed for 15 s. After 5–10 min, 200 µl of substrate solution was added (Z buffer + 4 mg/L ONPG), the samples were incubated at 37 °C, and the reaction was timed. When yellow color appeared, the reaction was terminated by adding 0.5 mL 1M Na_2CO_3 solution. Finally, the sample was centrifuged and the optical density at 420 nm (OD_{420}) of supernatant was measured. β-galactosidase specific activity (sGal) and β-galactosidase activity (XGal) were expressed in Miller unit (MU) and U mL^{-1}, respectively. One unit of β-galactosidase activity is defined as amount of enzyme releasing 1 µmol of nitrophenol per min in the assay conditions. All analyses were performed in triplicate.

Statistical tests

Statistical tests were performed using GraphPad Prism 6.01 software (GraphPad software, La Jolla, USA). Homoscedasticity of the biomass yield and β-galactosidase activities was tested using Bartlett's test [1] and their normal distribution was tested using Shapiro–Wilk's test [23]. β-galactosidase activities were compared using analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons [27], whereas biomass values were compared using Friedman's test (non-parametric equivalent of the repeated measures ANOVA) [10] followed by the Dunn multiple comparison test [8]. Statistical significance was accepted at $p < 0.05$.

Calculations at quasi steady states of cultures

The oxygen transfer rate (OTR) was calculated by the following equation:

$$\mathrm{OTR} = K_L a(C_O - C_L) \tag{1}$$

where C_O is the saturation concentration of dissolved oxygen and C_L is the dissolved oxygen concentration in fermentation broth. The $K_L a$ was estimated to be 712 h^{-1} from the original data from Niu et al. [21] by Eq. 1. At quasi steady states of the cultures, oxygen uptake rate (OUR) equals OTR. Accordingly, the specific oxygen consumption rate was determined by

$$q_{o_2} = \frac{\mathrm{OUR}}{X} = \frac{K_L a(C_O - C_L)}{X} \tag{2}$$

where X is the biomass (g DCW L^{-1})

The specific consumption rates of methanol and sorbitol were, respectively, determined by Eqs. 3 and 4,

$$q_{\mathrm{meth}} = \frac{F_{\mathrm{meth}} S_{\mathrm{meth\_in}} - (F_{\mathrm{sor}} + F_{\mathrm{meth}}) S_{\mathrm{meth}}}{XV} \tag{3}$$

$$q_{\mathrm{sorb}} = \frac{F_{\mathrm{sorb}} S_{\mathrm{sorb\_in}} - (F_{\mathrm{sorb}} + F_{\mathrm{meth}}) S_{\mathrm{sorb}}}{XV} \tag{4}$$

where F_{sorb} and F_{meth} are the feeding rates for sorbitol and methanol, respectively (L h^{-1}); $S_{\mathrm{sorb\_in}}$ and $S_{\mathrm{meth\_in}}$ are the sorbitol and methanol concentrations in the FBS and FBM feeding solutions, respectively (g L^{-1}); S_{sorb} and S_{meth} are the sorbitol and methanol concentrations in reactor, respectively (g L^{-1}).

Additionally, since the macroscopic balance of redox degree provided one-degree of redundancy in this study, data reconciliation and consistency tests were performed according to Niu et al. [21].

Results and discussion

Cell growth in regard to total carbon concentration in the mixed feed

In all experiments, the maximum biomass concentrations obtained at the end of the growth phase with glycerol feeding were similar and reached an average value of 50 gDCW L^{-1} (data not shown). To avoid any cell washout, methanol/sorbitol co-feeding was performed at a constant dilution rate of 0.03 h^{-1}, which is smaller than

Table 1 Mean values of biomass, β-galactosidase specific activity (sGal) and β-galactosidase activity (XGal calculated during the methanol/sorbitol feeding phase at different CRFs. Standard deviations were less than 10 % of the average values)

Feeding rate mmol C (DCW h)$^{-1}$	Biomass g DCW l^{-1}	sGal 10^4 MU	XGal 10^6 U ml^{-1}
4.4	49[a]	1.34[d]	2.25
5.5	56[b]	1.49[d]	3.32
6.6	59[b]	2.01[c]	4.75
7.7	63[b]	1.27[d]	3.23

[a] Significant difference in biomass between the different CRFs at $p < 0.05$

[b] No significant difference in biomass between the different CRFs

[c] Significant difference in sGal between the different CRFs at $p < 0.05$

[d] No significant difference in sGal between the different CRFs

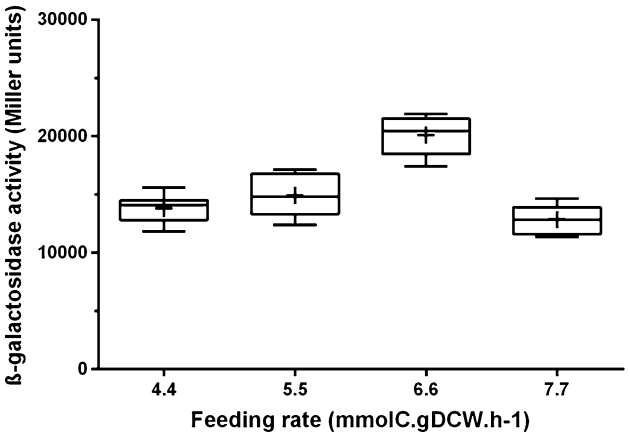

Fig. 1 β-Galactosidase specific activity (Miller unit) values between 45 and 72 h of methanol/sorbitol feeding. *Boxes* represent the 25th and 75th percentiles. *Whiskers* represent the 5th and 95th percentile. The "+" sign represents the mean value on eight different samples

the maximum specific growth rate observed either on methanol or on sorbitol (i.e., 0.10 and 0.04 h^{-1}, respectively) [21]. In our experimental set-up, CFRs increased stepwise in a narrow range of 25 %. In the range of 4.4–5.5 mmolC g DCW h^{-1}, there was a statistically significant increase of biomass (i.e. it increased from 49 to 56 gDCW L^{-1}, Table 1). However, in the range of 5.5–7.7 mmol C gDCW h^{-1}, no statistically significant differences in biomass were observed.

Promoter induction in regard to carbon feeding rate

Induction levels of AOX1 promoter, quantitatively estimated either by means of β-galactosidase activity (XGal) and β-galactosidase specific activity (sGal), were measured at different methanol/sorbitol feeding rates. As shown in Fig. 1, increasing CFR from 4.4 to 6.6 mmol C g DCW h^{-1} resulted in a linear increase of induction levels. At a CFR of 4.4 mmol C g DCW h^{-1}, β-galactosidase specific activity and β-galactosidase activity (shown in bracket) were around 1.34×10^4 MU (2.25×10^6 U mL^{-1}) and increased to 1.49×10^4 MU (3.32×10^6 U mL^{-1}) and 2.01×10^4 MU (4.75×10^6 U ml^{-1}) for CFRs of 5.5 and 6.6 mmol C g DCW h^{-1}, respectively (Table 1). By contrast, at CFR of 7.7 mmol C g DCW h^{-1}, over 30 % decrease of β-galactosidase activity was observed, i.e., it reduced to 1.27×10^4 MU (3.23×10^6 U ml^{-1}) from the maximum of 2.01×10^4 MU (4.75×10^6 U ml^{-1}). To assess the significance of these results, statistical analysis was performed on the β-galactosidase activities measured between 42 and 72 h of feeding to make the assumption of the homoscedasticity and normality of the data. ANOVA and Tukey test analysis confirmed that CFR of 6.6 mmol C g DCW h^{-1} yielded to a significantly higher level of β-galactosidase activities ($p < 0.001$) and thus pAOX1 induction (Table 1). CFR of 6.6 mmol C g DCW h^{-1} led to a 35 % increased level of pAOX1 induction compared to the lower CFRs tested.

Cellular metabolism in regard to carbon feeding rate

In our experimental design, airflow, and thus oxygen transfer rate, was limited to be in the range of those applied at industrial scale, i.e., 5–10 g O_2/(L h). In those conditions, increasing the CFR may lead to oxygen starvation and accumulation of carbon sources in the culture medium such as the toxic methanol. For CFRs ranging from 4.4 to 6.6 mmol C gDCW h^{-1}, methanol was never detected in the culture medium, indicating that cells consumed all the methanol fed (Fig. 2). By contrast, at a CFR of 7.7 mmol C g DCW h^{-1}, methanol was only consumed partially and stabilized around 10 g L^{-1} in the bioreactor. This higher methanol supply than necessary has been linked to the oxygen starvation as indicated by the low DO value (Fig. 2). For all the CFRs tested, sorbitol was consumed to completion during the first 6 h of feeding. During this period, cells adapted their metabolism to methanol, and sorbitol was the main source of energy. After this period, sorbitol was only partially consumed. At a CFR of 4.4 mmol C g DCW h^{-1}, residual sorbitol concentrations were very low and in the range of those

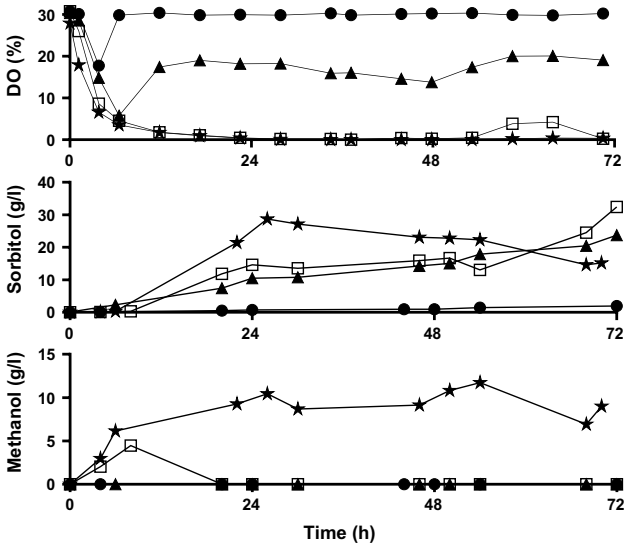

Fig. 2 Evolution of dissolved oxygen (DO), methanol and sorbitol concentrations in the culture medium during methanol/sorbitol feeding at different CFRs (*circles* 4.4 mmol C g DCW h^{-1}, *triangles* 5.5 mmol C g DCW h^{-1}, *squares* 6.6 mmol C gDCW h^{-1}, and *stars* 7.7 mmol C g DCW h^{-1})

observed previously with this strain [21]. For all the other CFRs tested, sorbitol concentration increased in the culture medium. However and in accordance with previous reports [3], sorbitol accumulation had no observably negative effect on pAOX1 induction. Indeed, Jungo et al. [16] reported the highest β-galactosidase activities at sorbitol concentration as high as 32 g L^{-1}. For CFRs of 5.5 and 6.6 mmol C g DCW h^{-1}, sorbitol concentration stabilized in the range of 13–16 g L^{-1} between 24 and 55 h of feeding (Fig. 2). It then increased at the end of the culture to 23 and 32 g L^{-1}, respectively. For CFR of 7.7 mmol C g DCW h^{-1}, sorbitol concentrations were generally in the range of 15–25 g L^{-1} in reactor for 24 h of feeding to the end of the culture (Fig. 2).

To assess the influence of oxygen starvation on sorbitol and methanol uptake capacity, q_{meth} and q_{sorb} were calculated for the different CFRs. As shown in Table 2, q_{meth} increased almost linearly in regard to CFRs from 0.06 to 0.077 g(g DCW h)$^{-1}$. The q_{meth} values in this work were in the range of those previously reported by Jordà http://www.ncbi.nlm.nih.gov/pubmed/?term=Jord%C3%A0%20J%5BAuthor%5D&cauthor=true&cauthor_uid=23845285 (see Table 4 in [14]), but a bit higher than the reported values in [6]. The q_{sorb} values obtained in this work are comparable to those in [3]. Despite the values of q_{meth} were nearly the same for CFRs of 6.6 and 7.7 mmol C g DCW h^{-1}, significantly different β-galactosidase activities and thus pAOX1 induction levels were obtained (Fig. 1; Table 1). Kim et al. [17] reported that oxygen-limited conditions led to a reduced induction level of pAOX1. However, at CFRs of 6.6 and 7.7 mmol C g DCW h^{-1}, q_{O2} values were higher than those when CFRs were 4.4 and 5.5 mmol C g DCW h^{-1}. This suggests that the lower value of β-galactosidase activities obtained at CFR of 7.7 mmol C gDCW h^{-1} was rather a consequence of methanol accumulation in the bioreactor than a result of an oxygen starvation. This is in accordance with Cregg [5] who reported that *P. pastoris* metabolism is negatively affected by the presence of methanol in the culture medium in this range of concentrations. However, in our experimental conditions methanol accumulation had no negative impact on cell growth, i.e., biomass slightly increased from 59 to 63 g DCW L^{-1} for CRFs of 6.6–7.7 mmol C (DCW h)$^{-1}$, respectively (Table 1). It was also observed that methanol accumulation did not affect oxygen consumption, i.e., q_{O2} did not change significantly (0.138 and 0.136 g DCW h^{-1} for CRFs of 6.6–7.7 mmol C DCW h^{-1}, respectively). Additionally, q_{O2} was found a 13 % increase from 0.122 to 0.138 g (DCW h)$^{-1}$ from oxygen-sufficient to oxygen-limited conditions. In this study, average OTR values found at industrial scale were used to determine the optimal carbon feeding rate (Table 2). As the OTRs felt into the value range of large reactors, the results provided a valuable basis for future industrial scale-up. The optimal feeding rate tested here was 6.6 mmol C (DCW h)$^{-1}$ at an oxygen transfer rate (OTR) of 8.28 g O_2(L h)$^{-1}$, which can be directly used to control co-feeding of the mixture during the induction phase after slight adjustment with optimized protein productivity using the dynamic model developed in our previous work [21].

For CFRs ranging from 5.5 to 7.7 mmol C g DCW h^{-1}, q_{sorb} and q_{meth} increased slightly (Table 2) from 0.036 to 0.044 g (g DCW h)$^{-1}$ and from 0.066 to

Table 2 Specific carbon uptake rates, oxygen transfer rate (OTR), and specific oxygen consumption rate at the different feeding rates

Carbon feeding rate mmol C (DCW h)$^{-1}$	q_{sorb} g (g DCW h)$^{-1}$	q_{meth} g (g DCW h)$^{-1}$	q_{sorb}/q_{meth}	OTR g_{O2} (L h)$^{-1}$	q_{O2} mg (g DCW h)$^{-1}$
4.4	0.037	0.060	0.61	5.99	122
5.5	0.036	0.066	0.55	6.83	122
6.6	0.041	0.074	0.55	8.28	138
7.7	0.044	0.077	0.57	8.54	136

For specific carbon uptake rates, standard deviations were less than 10 % of the average values

0.077 g (g DCW h)$^{-1}$, respectively. And from the ratio of q_{sorb} to q_{meth}, no significant difference was observed between the ratio of sorbitol and methanol consumed. For these CFRs, optimal methanol/sorbitol ratio is rather 0.64/0.36 C-mol/C-mol methanol/sorbitol than the 0.6/0.4 ratio observed at CFR of 4.4 mmol C g DCW h^{-1}.

For the different CFRs tested, nitrogen concentration was estimated after 4, 44 and 72 h of feeding. In all these conditions, nitrogen concentrations ranged between 225 and 425 mM for the different CFRs, indicating that the cultures were never nitrogen-limited in these conditions (data not shown).

Conclusions

In this work, it has been demonstrated that the feeding rate of total carbon concentration of an optimized methanol/sorbitol mixture influences directly the induction level of AOX1 promoter and consequently heterologous protein productivity. In aerobic or pseudo-aerobic condition (i.e., when DO > 0), an increase of the feeding rate of the methanol/sorbitol mixture yields to an increase of pAOX1 induction. By contrast, in anoxic or pseudo-anoxic condition (i.e., when DO is ≈0), pAOX1 induction level is decreased by more than 30 % from 6.6 to 7.7 mmolC g DCW h^{-1}. This decrease is more likely to be lined to methanol accumulation than to very low level of dissolved oxygen (DO < 4 %) by analyzing the results. However, there is no evidence of β-galactosidase-specific activity, and thus pAOX1 induction, being strictly lowered by extreme oxygen starvation. Nevertheless, this highlights that avoiding methanol high-level accumulation seems even more crucial than controlling dissolved oxygen at sufficient levels to an optimal process with higher protein productivity.

For a bioreactor with a K_La equal to 712 h^{-1}, which falls in the range of typical values for large industrial fermenters, the optimal carbon feeding rate was determined by the experimental results in this work to be 6.6 mmol C g DCW h^{-1}. However, higher K_La values would allow higher carbon feeding rates without or with low methanol accumulation, and thus to probably further increase pAOX1 induction. Accordingly, the characterization of the pAOX1 regulation in regard to K_La and the carbon feeding rate is a line of research that is worth studying in the future. However, compared to our previous study [20], the maximum biomass and β-galactosidase specific activity increased by 2.6 times (23–59 gDCW/L) and 5.2 times (0.92 × 10^6–4.75 × 10^6 U ml^{-1}) with the optimal CFR, respectively.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bartlett MS (1937) Properties of sufficiency and statistical tests. Proc R Stat Soc Ser A 160:268–282
2. Benz GT (2011) Bioreactor design for chemical engineers. Chem Eng Progr 107:21–26
3. Çelik E, Çalık P, Oliver SG (2009) Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. Yeast 26:473–484
4. Çelik E, Çalik P, Oliver SG (2010) Metabolic flux analysis for recombinant protein production by *Pichia pastoris* using dual carbon sources: effects of methanol feeding rate. Biotechnol Bioeng 105:317–329
5. Cregg JM (2010) Pichia protocols, 2nd edn. Humana Press, Totowa
6. Cunha AE et al (2004) Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnol Bioeng 86:458–467
7. De Schutter K et al (2009) Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat Biotechnol 27:561–566
8. Dunn OJ (1964) Multiple comparisons using rank sums. Technometrics 6:241–252
9. Fickers P (2014) *Pichia pastoris*: a workhorse for recombinant protein production. Curr Res Microbiol Biotechnol 2:354–363
10. Friedman M (1937) The use of ranks to avoid the assumption of normality implicit in the analysis of variance. J Am Stat Assoc 32:675–701
11. Hamilton SR et al (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313(5792):1441–1443
12. Inan M, Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. J Biosci Bioeng 92(6):585–589
13. Jin H, Liu G, Ye X, Shi Z (2010) Enhanced porcine interferon-α production by recombinant *Pichia pastoris* with a combinational control strategy of low induction temperature and high dissolved oxygen concentration. Biochem Eng J 52(1):91–98
14. Jordà J, de Jesus SS, Peltier S, Ferrer P (2014) Albiol J. Metabolic flux analysis of recombinant *Pichia pastoris* growing on different glycerol/methanol mixtures by iterative fitting of NMR-derived 13C-labelling data from proteinogenic amino acids. N Biotechnol 31(1):120–13215
15. Jungo C, Marison I, von Stockar U (2007) Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: a quantitative study based on concentration gradients in transient continuous cultures. J Biotechnol 128:824–837
16. Jungo C, Schenk J, Pasquier M, Marison IW, von Stockar U (2007) A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. J Biotechnol 131:57–66
17. Kim S, Warburton S, Boldogh I, Svensson C, Pon L, d' Anjou M, Stadheim TA, Choi BK (2013) Regulation of alcohol oxidase 1 (AOX1) promoter and peroxisome biogenesis in different fermentation processes in *Pichia pastoris*. J Biotechnol 166:174–181
18. Lorantfy B, Jazini M, Herwig C (2013) Investigation of the physiological response to oxygen limited process conditions of

Pichia pastoris Mut(+) strain using a two-compartment scale-down system. J Biosci Bioeng 116(3):371–379
19. Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, New-York
20. Niu H, Jost L, Pirlot N, Sassi H, Daukandt M, Rodriguez C, Fickers P (2013) A quantitative study of methanol/sorbitol co-feeding process of a *Pichia pastoris* Mut+/pAOX1-lacZ strain. Microb Cell Factories 12:33
21. Niu H, Daukandt M, Rodriguez C, Fickers P, Bogaerts P (2012) Dynamic modeling of methylotrophic *Pichia pastoris* culture with exhaust gas analysis: from cellular metabolism to process simulation. Chem Eng Sci 87:381–392
22. Paulová L, Hyka P, Branská B, Melzoch K, Kovar K (2012) Use of a mixture of glucose and methanol as substrates for the production of recombinant trypsinogen in continuous cultures with *Pichia pastoris* Mut+. J Biotechnol 157:180–188
23. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611
24. Solórzano L (1969) Determination of ammonia in natural waters by the phenol hypochlorite method. Limnol Oceanogr 14:799–801
25. Sreekrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsay JT, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene 190:55–62
26. Thorpe ED, d' Anjou MC, Daugulis AJ (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnol Lett 21:669–672
27. Tukey J (1969) Comparing Individual Means in the Analysis of variance. Biometrics 5:99–114
28. Zhang W, Hywood Potter KJ, Plantz BA, Schlegel VL, Smith LA, Meagher MM (2003) *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement. J Ind Microbiol Biotechnol 30:210–215