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Regulation of alcohol oxidase 1 (*AOX1*) promoter and peroxisome biogenesis in different fermentation processes in *Pichia pastoris*

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ABSTRACT

Production of recombinant proteins is affected by process conditions, where transcriptional regulation of *Pichia pastoris* alcohol oxidase 1 (*PpAOX1*) promoter has been a key factor to influence expression levels of proteins of interest. Here, we demonstrate that the *AOX1* promoter and peroxisome biogenesis are regulated based on different process conditions. Two types of GFP-fusion proteins, Ub-R-GFP (short-lived GFP in the cytosol) and GFP-SKL (peroxisomal targeting GFP), were successfully used to characterize the time-course of the *AOX1* promoter and peroxisome biogenesis, respectively. The activity of the *AOX1* promoter and peroxisome biogenesis was highly subjected to different fermentation process conditions – methanol-limited condition at normoxy (ML), switched feeding of carbon sources (e.g., glucose and methanol) under carbon-limited condition at normoxy (SML), and oxygen-limited (OL) condition. The *AOX1* promoter was most active under the ML, but less active under the OL. Peroxisome biogenesis showed a high dependency on methanol consumption. In addition, the proliferation of peroxisomes was inhibited in a medium containing glucose and stimulated in the methanol phase under a carbon-limited fed-batch culture condition. The specific productivity of a monoclonal antibody (q_p) under the *AOX1* promoter was higher at 86 h of induction in the ML than in the OL (0.026 vs 0.020 mg g^{-1} h^{-1}). However, the oxygen-limited condition was a robust process suitable for longer induction (180 h) due to high cell fitness. Our study suggests that the maximal production of a recombinant protein is highly dependent on methanol consumption rate that is affected by the availability of methanol and oxygen molecules.

1. Introduction

Glyco-engineered *Pichia pastoris* is capable of producing recombinant proteins with highly uniform human *N*-linked glycans, and it has been reported that many therapeutic proteins including monoclonal antibodies were produced under the control of the *AOX1* promoter in glyco-engineered *P. pastoris* strains (Choi et al., 2003; Hamilton and Gerngross, 2007; Hamilton et al., 2006; Li et al., 2006; Potgieter et al., 2009). The specific productivity of a target protein is influenced by many factors including gene constructs, gene copy number, culture media, and fermentation methods (Akcapinar et al., 2011; Görgens et al., 2004; Hohenblum et al., 2004; Kim et al., 2007; Zhu et al., 2011). In order to maximize the volumetric productivity (titer) of recombinant proteins, *P. pastoris* needs to be cultivated to a high cell density using fed-batch methods. Typically the fed-batch culture consists of three growth phases – batch culture followed by a fed-batch using glycerol and lastly a second fed-batch using methanol for induction. Each growth phase shows different growth kinetics, which is influenced by media components and feeding protocols (i.e., methanol-limited or oxygen-limited methods). Methanol is utilized not only as an inducer for recombinant protein production, but also as a carbon source for cell growth. The addition of methanol and/or other carbon sources needs to be well controlled to minimize the formation of by-products (e.g., acetic acid), cell lysis, and inhibitory effects of known (e.g., glucose; glycerol) and unknown metabolites. In a given expression system, the titer is mostly dependent on cell growth and feeding methods of primary carbon sources including methanol as an inducer. The post-translational modifications (e.g., glycosylation) and the integrity of yeast cell structure are also affected by culture conditions.

The *AOX1* promoter is induced by methanol but repressed by other types of carbon sources such as glucose and ethanol

Abbreviations: ML, methanol-limited at normoxic condition; SML, switched feeding of carbon sources under carbon-limited at normoxy; OL, oxygen-limited condition; q_p, specific productivity of a monoclonal antibody (mg g^{-1} h^{-1}); q_m, specific consumption rate of methanol (mg g^{-1} h^{-1}).

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(Goodman et al., 1984; Zhang et al., 2010). As the first reaction step in methanol utilization metabolism in *P. pastoris*, the expression of the *AOX1* in the peroxisome depends on the fluxes of methanol and oxygen molecules in the presence of a cofactor, FAD, and generates formaldehyde and hydrogen peroxide as products. Formaldehyde is further metabolized for dissimilation and assimilation pathways in *P. pastoris*. As a reactive oxygen species (ROS), hydrogen peroxide induces oxidative stress, and is well known to be associated with cell fitness of *P. pastoris* during the induction phase (Hilt and Wolf, 1992; Xiao et al., 2006).

Peroxisomes are essential cellular organelles that play important metabolic functions in various organisms such as mammals, plants, and yeasts (Antonenkov et al., 2010). In *P. pastoris*, peroxisomes are central metabolism organelles for assimilation and dissimilation of methanol as a primary carbon source. A second typical function of peroxisomes is the β-oxidation of fatty acids (Antonenkov et al., 2010; Purdue and Lazarow, 2001).

Peroxisome biogenesis of a methylotropic yeast is known to be dynamic and is affected by changes in culture conditions such as carbon source (Sakai et al., 2006). Peroxisomes degenerate in the presence of glucose but proliferate within 15 h after transfer to methanol-containing medium in *Candida* yeast species (Goodman et al., 1984).

In this study, we investigated the transcription efficiency of the *AOX1* promoter using Ub-R-GFP (short-lived GFP) (Varshavsky, 1996; Dantuma et al., 2000) and peroxisome biogenesis using GFP-SKL (peroxisomal targeting GFP) (Monosov et al., 1996) in glyco-engineered *P. pastoris* under different fermentation processes – methanol-limited condition at normoxy (ML), switched feeding of carbon sources (e.g., glucose and methanol) under carbon-limited condition at normoxy (SML), and oxygen-limited (OL) condition.

2. Materials and methods

2.1. Plasmid construction and strain generation

GFP-SKL was generated by placing the peroxisomal targeting signal 1, Ser-Lys-Leu (SKL), at the C-terminus of GFP. Ub-R-GFP was generated by fusing *P. pastoris* ubiquitin (Ub) (76 amino acids) to the N-terminus of GFP lacking the first amino acid (Met). Arginine (R) was placed between ubiquitin and GFP to make a short-lived GFP. The open reading frames (ORFs) encoding GFP-SKL (Monosov et al., 1996) or Ub-R-GFP (Varshavsky, 1996; Dantuma et al., 2000) were codon-optimized for *P. pastoris*, and cloned into both pGLY8369 and pGFI27t at *Eco*RI and *Fse*I sites, and pGLY6672 at *Not*I and *Pac*I sites, respectively. The resulting plasmids were designated pGLY11245 (*AOX1* Prom-GFP-SKL), pGLY13173 (*GAPDH* Prom-GFP-SKL) and pGLY10148 (*AOX1* Prom-Ub-R-GFP). The pGLY11245 and pGLY13173 are a roll-in (*URA6*)/expression plasmid, where the expression of GFP-SKL is under the control of the *P. pastoris AOX1* or *GAPDH* promoters. The pGLY10148 is a knock-in plasmid comprising two crossover regions to the *ADE4* to generate a single copy knock-in of Ub-R-GFP at the *ADE4* locus. The expression plasmids used in this study are shown in Fig. 1.

For the expression of GFP-SKL, pGLY11245 or pGLY13173 were transformed into yGLY14836 producing a monoclonal IgG1 antibody (YGLY27355 or YGLY31884). pGLY11245 and pGLY13173 were used for the study of the proxisomal localization (Fig. 2b) and biogenesis (Fig. 5), respectively. For the expression of Ub-R-GFP that is expressed in the cytosol, pGLY10148 was transformed into YGLY19309 producing a monoclonal IgG1 antibody (YGLY29325). A single copy of Ub-R-GFP was confirmed by colony PCR.

2.2. Transformation

Pichia transformation was performed as described previously (Choi et al., 2003). Briefly, 100 μL of overnight grown cells (OD600 nm, 0.2–6.0) were transformed with 10 μL of linearized DNA (5–10 μg) after 5 min incubation on ice. Electroporation was in a Bio-Rad GenePulser Xcell following the preset *P. pastoris* protocol (2 kV, 25 μF, 200 Ω), immediately followed by the addition of 1 mL YPDS recovery media (YPD media plus 1 M sorbitol). The transformed cells were allowed to recover for 4 h to overnight at room temperature (24 °C) before plating the cells on selective media.

2.3. Fed-batch culture conditions

Seed cultures were prepared by inoculation of a frozen stock vial (1 mL) into 200 mL of BMGY media. Methods and medium conditions were described previously (Potgieter et al., 2009).

Cultivations were performed in 3 L or 6 L bioreactor (Applikon, CA). Regulation of the *PpAOX1* promoter (YGLY29325) and peroxisome biogenesis (YGLY31884) were studied in three different fed-batch conditions – methanol limited condition (ML), methanol limited condition with switched feeding of glucose for 8 h and methanol for 20 h (SML), oxygen limited condition with bolus addition of 1% methanol at DO spikes indicating methanol depletion (OL).

Temperature was controlled at 24 °C and pH was controlled at 6.5 ± 0.1 with 14% ammonium hydroxide. Dissolved oxygen (DO) was maintained at 20% of air-saturation at atmospheric pressure and 24 °C by fixing the air flow rate at 0.7 vvm and cascading agitation and supply of pure oxygen. After batch period, exponential feeding of glycerol was initiated at 5.30 g L^{-1} h^{-1} and maintained at the specific growth rate of 0.08 h^{-1} for 8 h.

After the end of glycerol fed-batch period, three different feeding conditions were applied as follows: (1) For ML, methanol feeding was initiated at 2.6 g L^{-1} h^{-1} and exponentially increased at the specific growth rate of 0.0063 h^{-1}. DO level (%) was maintained at 20% by cascading agitation and supply of pure oxygen; (2) For SML, methanol was fed at the same condition as (1) for 20 h and feeding was switched from methanol to glucose (50%) at a constant rate of 15 g h^{-1} for 8 h per cycle. A total of three cycles of feeding were operated; (3) For OL, methanol was fed to achieve 1% in the reactor whenever DO rapidly increased, which indicates methanol depletion. DO cascade was turned-off and agitation speed was reduced down to achieve oxygen limitation.

2.4. Visualization and quantification of GFP expression in fixed cells

Fixation of *P. pastoris* cells was carried out according to the protocol (Swayne et al., 2009). Approximately 10^7 fixed cells were washed three times in 0.5 mL of PBS solution (pH 7.2 in g L^{-1}, 80 NaCl, 0.2 KCl, 1.44 Na_2HPO_4, 0.24 KH_2PO_4) and resuspended in 20 μL mounting solution (1 mg/mL of *p*-phenylenediamine, 90% [v/v] glycerol, PBS) containing the DNA-binding dye 4,6-diamidino-2-phenylindole, DAPI, (Invitrogen, Carlsbad, CA) at a final concentration of 0.5 μg/mL. 0.5–1 μL cell suspension was placed on a microscope slide and covered with coverslip. Images were acquired with an Axioscope 2 Plus microscope (Carl Zeiss, Oberkochen, Germany) by using a Plan-Apochromat 100× 1.4 numerical aperture objective lens, and a cooled charge-coupled device camera, Orca ER (Hamamatsu, Bridgewater, NJ). To detect the fluorescence of GFP, the excitation and emission wavelengths were 470 nm and 525 nm, respectively. Camera control and image collection were performed using OpenLab software (Improvision, Coventry, United Kingdom). The *z*-sections of cells were obtained at 0.4 μm intervals through the entire cell. The Measurement

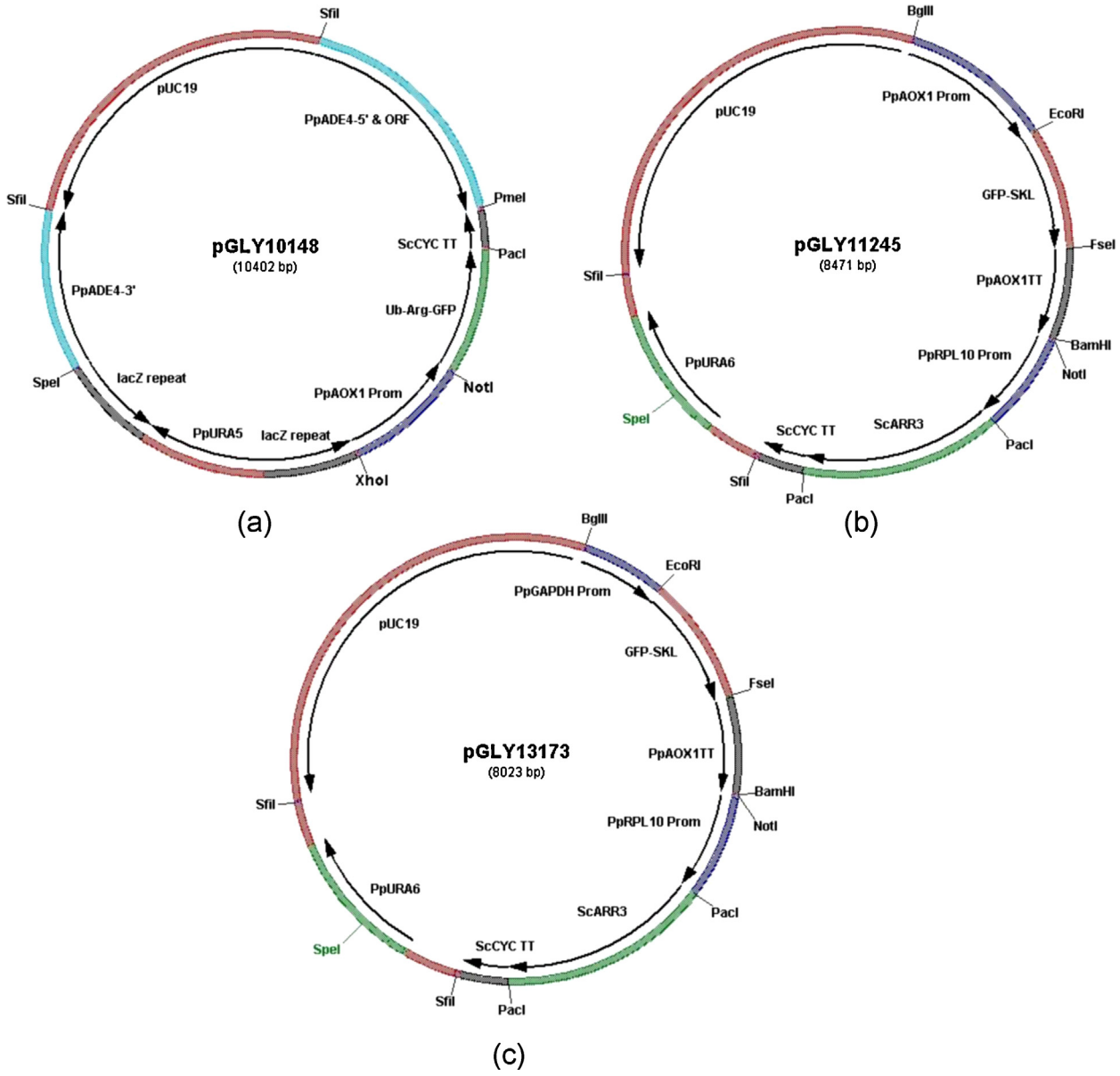

Fig. 1. Expression plasmids of (a) Ub-R-GFP (*AOX1* promoter-driven), (b) GFP-SKL (*AOX1* promoter-driven), and (c) GFP-SKL (*GAPDH* promoter-driven).

function of Volocity software (Improvision, Coventry, United Kingdom) was used to quantify the intensity of GFP in the cells. Individual cells were outlined for quantification using the transmitted light images. Afterwards, the mean pixel intensities of GFP signals in the outlined areas were measured, and corrected by subtracting the mean pixel intensities of the background. Statistical analyses were carried out using Microsoft's Excel software. Relative fluorescence was obtained by subtracting the pixel intensity of uninduced samples from that of induced samples, and it was reported as mean value from three measurements given in relative fluorescence units (RFU).

2.5. Determination of antibody titer

Antibody concentration in the culture supernatant was measured at 280 nm using a diode array detector and a protein A affinity column (Poros® A/20, Applied Biosystems, CA) and a HPLC system (Agilent Technologies, CA) at 25 °C. Buffer A (PBS, pH 7.4) was used as mobile phase at 1 mL min^{-1} of flow rate and Buffer B (PBS, pH 2.5) was used to elute the antibody.

2.6. Cell lysis

Cell lysis was measured quantitatively in terms of the amount of extra-cellular DNA released from *P. pastoris* cells using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Grand Island, NY) according to the vendor's instructions.

3. Results

3.1. Subcellular localization of GFP-SKL and Ub-R-GFP

Green fluorescent protein (GFP) has widely been used for protein localization and quantification of gene expression (Albano et al., 1996; Gerdes and Kaether, 1996; Monosov et al., 1996; Hartner et al., 2008). Two different GFP fusion proteins, a short-lived Ub-R-GFP for cytosol expression and GFP-SKL for peroxisomal

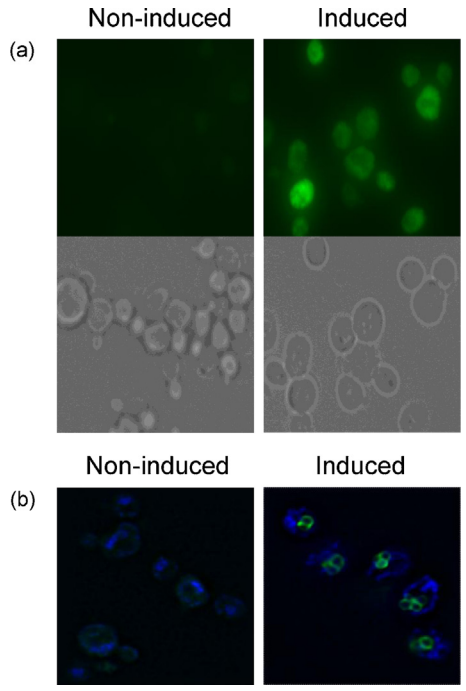

Fig. 2. (a) Ub-R-GFP (YGLY29325) and (b) GFP-SKL (YGLY27355) before and after methanol-induction. Ub-R-GFP is localized in the cytosol and GFP-SKL in peroxisome. Green – GFP fusion proteins; blue – DAPI-stained nuclei.

targeting, were generated to study the *AOX1* promoter and peroxisome biogenesis under three different process conditions, respectively. The expression of both Ub-R-GFP and GFP-SKL were under the control of the *AOX1* promoter. The short-lived Ub-R-GFP (2 min half-life) was used to investigate how the *AOX1* promoter is regulated in response to the availability of methanol and O_2 in the course of different methanol fed-batch conditions. In addition, peroxisome, a key organelle for methanol metabolism, was studied. Ub-R-GFP and GFP-SKL were correctly targeted to the cytosol and peroxisomes respectively, as expected. As shown in Fig. 2, the subcellular localization of Ub-R-GFP (a) and GFP-SKL (b) was confirmed by fluorescence microscopy.

3.2. Regulation of the AOX1 promoter under the ML, SML, and OL conditions

The *P. pastoris AOX1* promoter is a strong inducible promoter, thus it has successfully been used for recombinant protein production. Since the *AOX1* promoter is regulated by carbon sources (e.g., repression, de-repression, and induction), the availability of methanol in a bioreactor affects the *AOX1* promoter, resulting in the amount of recombinant protein production. Therefore, we investigated the regulation of the *AOX1* promoter in three different process conditions (see Materials and Methods for description). As shown in Fig. 3, during the induction phase, dissolved oxygen (DO) level was maintained at 20% of air saturation under the methanol-limited condition, normoxy (ML) (Fig. 3a), but was lower than 6% of air saturation under oxygen-limited condition, hypoxy (OL) (Fig. 3c). Methanol depletion in the OL process indicates a steep increase in DO, which triggered the next pulse of methanol (Fig. 3c) and led to a constant oxygen uptake rate (OUR) compared to a typical exponential OUR observed in the ML process (data not shown).

The fluorescence of Ub-R-GFP was detected after induction, showing changes of GFP intensity in different culture conditions (Fig. 4a). It has been demonstrated that the intensity of fluorescence of GFP is proportional to the expression level of GFP, which is directly correlated to the strength of the *AOX1* promoter (Hartner et al., 2008). The fluorescence intensity increased from a baseline of zero at the start of the fermentation to 630.7 ± 73 RFU at 36 h (T4) of induction but decreased down to 459.0 ± 45 RFU at 84 h (T6) of induction (Fig. 4b) in the ML. In contrast, the expression of Ub-R-GFP reached a plateau after 38 h of induction and maintained at around 160 ± 11 RFU in the OL, which was significantly lower level compared to that in the ML condition (Fig. 4b).

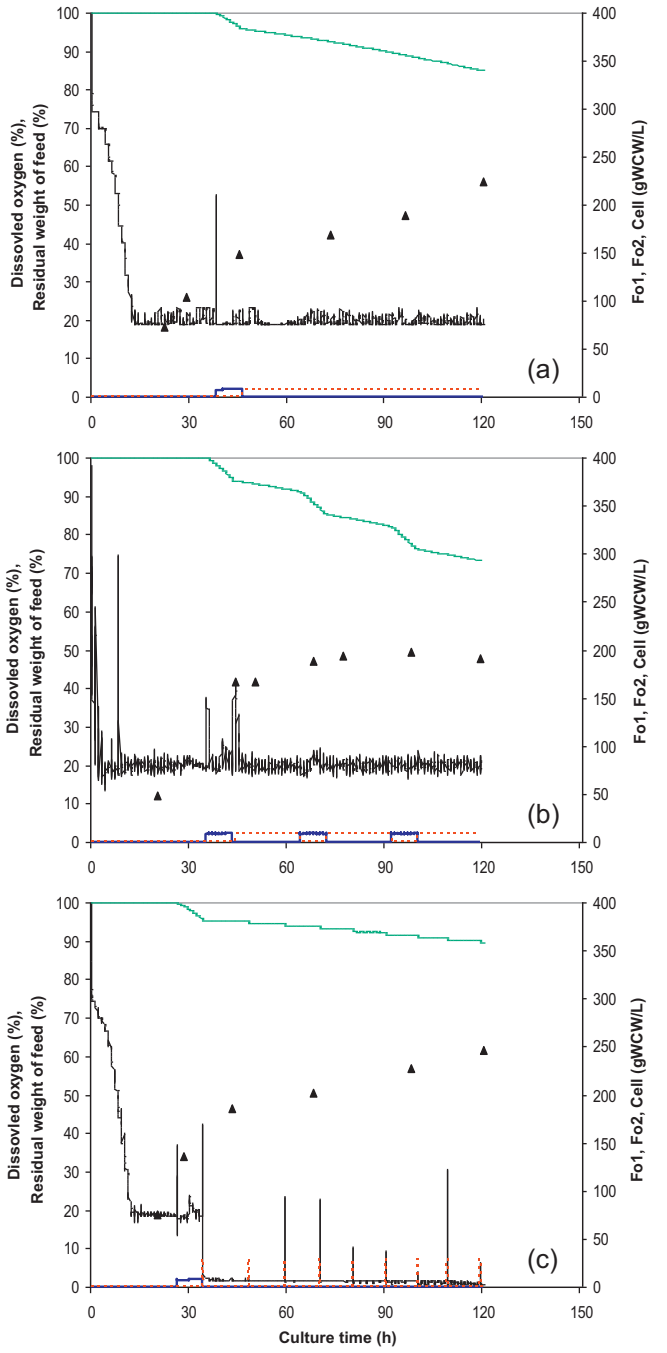

Fig. 3. Fermentation profiles of three different fed-batch conditions (YGLY29325): (a) methanol-limited (ML); (b) methanol-limited and switched with glucose (8 h) and methanol (20 h) (SML); (c) oxygen-limited with bolus additions of 1% methanol (OL) DO (%) in black solid line; glycerol or glucose feed (Fo1) in blue; methanol feed (Fo2) in red; residual weight of feed solution (%) in green; cell (wet cell weight, gWCW/L) in black solid triangle.

The *AOX1* promoter is known to be repressed in the presence of glucose (Goodman et al., 1984; Zhang et al., 2010). Switched feeding of glucose and methanol in the carbon-limited fed-batch

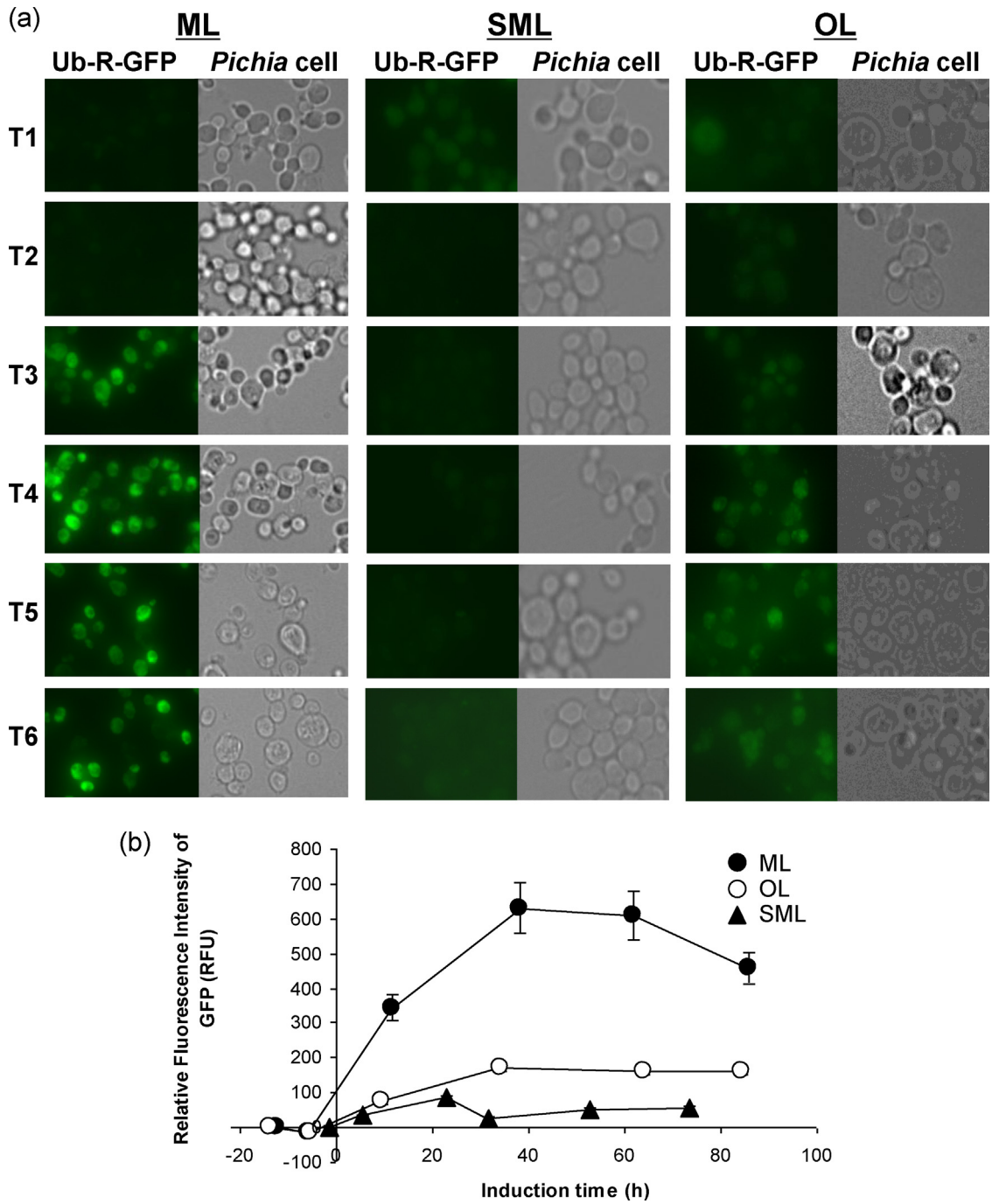

Fig. 4. Regulation of *P. pastoris AOX1* promoter (YGLY29325) under three different fed-batch conditions – methanol-limited (ML), switched with glucose and methanol (SML) and oxygen-limited (OL) fed-batch conditions. (a) Images of GFP expression; (b) relative intensity of fluorescence (RFU) with respect to induction time. For ML and OL, times indicate batch phase (T1), glycerol fed-batch phase before induction, (T2), and induction phase (T3, 10 ± 2 h; T4, 36 ± 2 h; T5, 62 ±2 h; T6, 84 ± 2 h). For SML, cell growth is in glucose phases at T2, T4, and T6, and in methanol phases at T3 and T5, respectively.

condition with 20%-air saturation (SML) was applied to see the degrees of repression and de-repression of the *AOX1* promoter (Fig. 3b). One cycle of switched feeding condition consists of glucose feeding for 8 h and methanol feeding for the following 20 h under carbon-limited feeding conditions. A total of three cycles of switched feeding was conducted in this study (Fig. 3b). The expression of Ub-R-GFP was significantly reduced under the SML condition, indicating that glucose repressed the transcription of the *AOX1* promoter tightly (Fig. 4) as expected. Interestingly, the *AOX1* promoter remained repressed during methanol phases even though residual glucose did not accumulate in the SML condition (Fig. 4b).

3.3. Peroxisome biogenesis

Alcohol oxidase (*AOX*), catalase (*CAT*), and dihydroacetone synthase (*DAS*) in peroxisomes play an essential role in methanol metabolism, where methanol is assimilated or dissimilated to produce cellular energy and biomass (Antonenkov et al., 2010; Purdue and Lazarow, 2001). Thus, peroxisomes allow the cells to grow

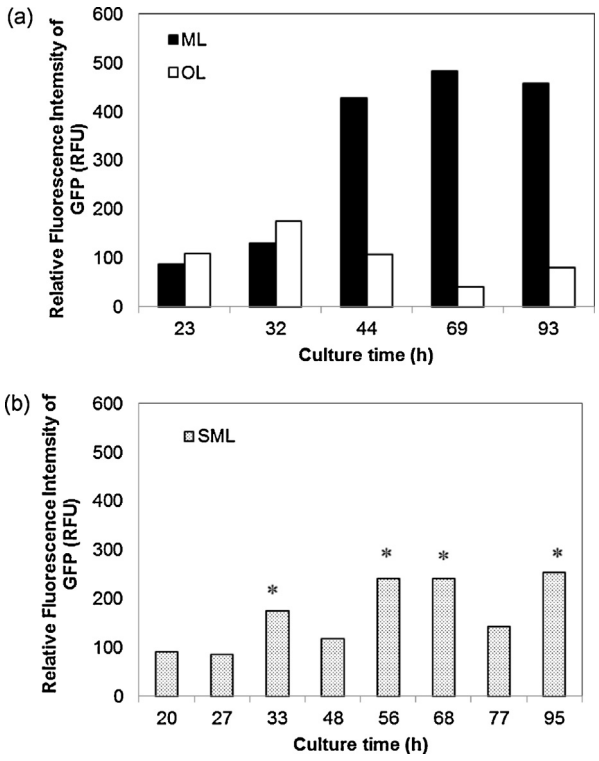

Fig. 5. Comparison of peroxisome biogenesis in three different culture conditions – (a) ML, OL, and (b) SML. SML: switched feeding of glucose and methanol. The peroxisomes were quantitatively measured using GFP-SKL (YGLY31884). Data points represent mean values in two independent runs. Asterisk (*) indicates methanol phase.

in the presence of methanol as the sole carbon source. To study peroxisome generation under different process conditions, peroxisomes were labeled with green fluorescent protein (GFP) that was fused to the peroxisomal targeting signal 1, Ser-Lys-Leu (SKL), where its expression was under the control of the *GAPDH* promoter. As shown in Fig. 5, the range of intensity of GFP fluorescence under the oxygen-limited condition was 41–107 RFU, whereas that under methanol-limited condition was 428–484 RFU. The relative intensity of fluorescence (RFU) of GFP was significantly higher in the ML than in the OL, indicating that peroxisome biogenesis was strongly dependent on methanol consumption that was controlled by DO level, where DO level was maintained at <6% in the OL, compared with 20% in the ML. Thus, peroxisome generation was influenced by specific methanol consumption rate (q_m). The higher methanol consumption rate (3.5 ± 0.2 mg g^{-1} h^{-1}) under the ML requires more peroxisomes than the lower one (2.5 ± 0.2 mg g^{-1} h^{-1}) under the OL. Under the methanol-limited condition, the peroxisomes were maximally generated at 34 h of induction but then declined afterwards. It is likely that the decrease in peroxisomes was caused by less methanol consumption due to limited availability of methanol relative to the cell biomass increase and cell lysis.

In the SML, the fluorescent intensity showed a decrease in glucose phase, and an increase in methanol phase, indicating that peroxisome biosynthesis was repressed in the presence of glucose and degraded via a selective peroxisome degradation pathway called pexophagy, but recovered in methanol phase (Fig. 5).

3.4. Antibody production

As a reporter protein in this study, a monoclonal IgG1 antibody was induced under the control of the *AOX1* promoter and secreted into the culture medium. The cell growth was higher in the ML than in the OL (Fig. 6a). That is, the cell specific consumption rate of methanol (q_m) was higher in the ML than in the OL (3.5 ± 0.2 vs 2.5 ± 0.2 mg g^{-1} h^{-1}). Cell lysis was quantified by the measurement of double stranded DNA released into the culture medium, and the cell lysis was higher in the ML than in other two conditions (Fig. 6b). It has been observed that the cell lysis negatively impacted the quality of recombinant proteins (i.e., proteolysis and miss-assembled antibodies). The product titer of antibody (mg/L) was 1.6-fold higher in the ML than in the OL for 87 ± 2 h of induction (Fig. 6c), however, the antibody yield was not significantly different between the ML and the OL in terms of methanol consumption ($Y_{p/m}$): 2.2 ± 0.3 vs 2.0 ± 0.1 mg g^{-1}).

Because of higher cell fitness in the OL than in the ML, it was possible to extend the induction time to 180 h in the OL condition as shown in Fig. 6d. The antibody titer increased continuously and reached 600 mg/L, which was 1.3-fold higher when compared to the final antibody titer obtained in the ML. The oxygen-limited process allowed for longer protein induction to achieve higher antibody production with additional benefits such as the better quality of protein (data not shown), compared to the methanol-limited process. This is in good agreement with the report by Potgieter et al. (2009) and Berdichevsky et al. (2011).

Overall antibody titer under the SML was much lower due to repression by glucose in switched mode than in other two conditions, and significant antibody increase was not observed during methanol phases of the SML condition (Fig. 6c).

4. Discussions and conclusions

Protein quality and yield are highly affected by process conditions, hence various process conditions have been developed in wild-type *P. pastoris* and glyco-engineered *P. pastoris* (Potgieter et al., 2009; Ye et al., 2011). In this study, we investigated how the *AOX1* promoter and peroxisomes are regulated in response to availability of the carbon source (e.g., methanol) and oxygen molecules during protein production phase to better understand process conditions commonly used in *P. pastoris* fermentation.

Two major process conditions (ML and OL) are largely differentiated by methanol consumption rate that is controlled by the availability of oxygen (DO: 20% vs <6%). The *AOX1* promoter should be fully activated during protein induction phase for the maximal protein production, but under the methanol-limited condition, we found that the activity of the *AOX1* promoter began to decline after 34 h. The decreasing activity of the *AOX1* promoter suggests that the available methanol as an inducer is much more limited as a result of increasing cell biomass and a feeding rate at 2.6 g L^{-1} h^{-1}. However, it seems that 20% of the dissolved oxygen (DO) is sufficient to get the maximal activity of the *AOX1* promoter at a given methanol feeding rate because >20% of the DO did not improve the *AOX1* promoter any further. In addition, we observed higher cell lysis with the ML over time in contrast to the OL, where the methanol supply was controlled by a feedback control. The lower cell lysis under the OL is likely due to the available methanol not being restricted by the lower oxygen level (<6% DO), which leads to the slower cell growth (Baumann et al., 2010). Therefore, cells do not undergo the carbon limitation under the OL as much as they do under the ML. The higher cell lysis observed is likely due to the higher carbon demand, which in turn, stresses the cells, but other factors cannot be excluded for this high cell lysis. Cell lysis is known to be correlated with the production of reactive oxygen species (ROS) such as hydrogen peroxide produced from methanol metabolism, which is toxic to the cell (van Zutphen et al., 2011) (Fig. 7). The accumulation of hydrogen peroxide adversely affects the negative energy balance by non-enzymatic oxidation of

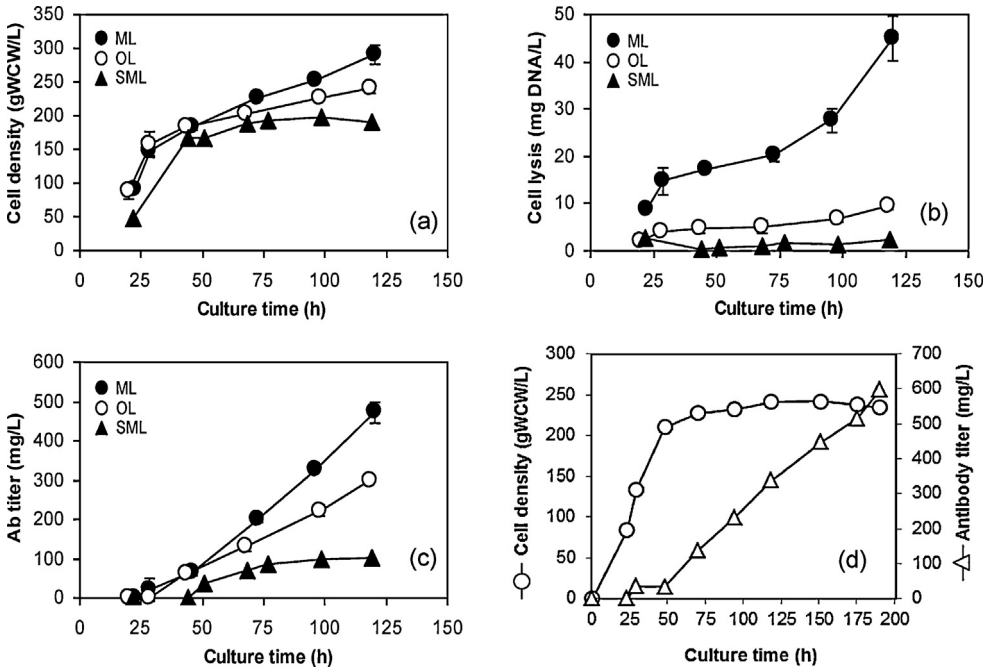

Fig. 6. Fermentation profiles cultivated in three different process conditions (YGLY29325) – ML, OL, and SML. (a) cell density profile (gWCW/L); (b) cell lysis index (mg DNA/L); (c) antibody titer (mg/L); (d) cell density and antibody titer for extended induction time under OL.

glutathione (GSH), which leads to a reduction of dissimilation due to reduction of GSH available to condense with formaldehyde to form formaldehyde dehydrogenase substrate and causes increasing level of free formaldehyde in the cytosol (Fujimura et al., 2007; Klei et al., 2006).

In this study, the methanol-limited condition demonstrated the most productive process for the production of the test monoclonal antibody within 87 h induction although the activity of the *AOX1* promoter is suboptimal after 25 h induction. This indicates that the cell biomass is a critical factor influencing the final protein yield when three different processes are compared as a function of the cell biomass, where the higher cell biomass was generated with the higher methanol uptake rate ($3.5\ mg\ g^{-1}\ h^{-1}$) under the ML than OL ($2.5\ mg\ g^{-1}\ h^{-1}$). These results demonstrated that the strength of the *AOX1* promoter was higher in the ML than in the OL due to stoichiometrically higher levels of methanol and oxygen molecules in the ML than in the OL, as schematically shown in Fig. 7. However, since the cell lysis continued to increase over time, the ML process was not adequate for the longer induction (180 h).

Peroxisomes are actively generated when grown in methanol or oleic acid as the sole carbon source (Sakai et al., 2006; Wriessnegger et al., 2007). We found that peroxisome biogenesis is highly affected by methanol consumption as demonstrated in the ML and the OL processes. Under the SML condition, peroxisome biogenesis was dynamic in the presence of glucose or methanol. They were degraded at glucose fed-batch phase and re-generated at methanol fed-batch phase (Fig. 5b). In addition, the high levels of peroxisomes during methanol induction are the result of higher methanol consumption because the ML feeding strategy was employed. However, in spite of the high amounts of peroxisomes, the antibody titer was the lowest among the three process conditions (Fig. 6c). One of the possible explanations would be the repression of the *AOX1* promoter by glucose as shown in the activity of the *AOX1* promoter under the SML (Fig. 4b), where the *AOX1* promoter was maintained at the weakest levels.

In conclusion, two types of methanol-inducible GFP-fusion proteins, Ub-R-GFP and GFP-SKL, were successfully used to characterize three bioprocess conditions in terms of the regulation of the

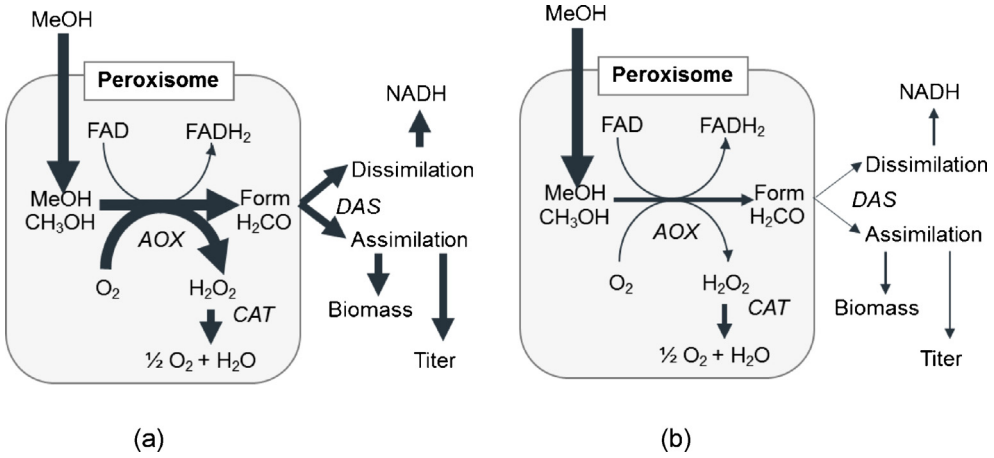

Fig. 7. Proposed metabolic fluxes in methanol phases of (a) methanol-limited (ML) and (b) oxygen-limited (OL) fed-batch cultures of *P. pastoris*.

AOX1 promoter and peroxisome biogenesis, respectively. Among three different fed-batch processes, it was demonstrated that the oxygen-limited process (OL) was a robust process with minimal cell lysis for antibody production, and one suitable for longer induction (180 h), whereas the methanol-limited process (ML) was more productive for shorter induction (<100 h). Additionally, methanol consumption rate was found to determine the expression level of a recombinant protein. A number of process conditions have been developed to optimize the production of recombinant proteins in *P. pastoris*, depending on proteins of interest or strains (wild type and glyco-engineered). In general, the process of optimization has been focused on process parameters rather than the understanding of regulation of the *AOX1* promoter during protein production. However, this GFP application provides a useful tool to identify inefficient processes, allowing us to fine-tune process conditions and maximize productivity of protein production.

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