Appl Biochem Biotechnol (2011) 165:559–571
DOI 10.1007/s12010-011-9275-2

Improvement of Porcine Interferon-α Production by Recombinant *Pichia pastoris* via Induction at Low Methanol Concentration and Low Temperature

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Received: 18 July 2010 / Accepted: 2 May 2011 /
Published online: 13 May 2011

Abstract Improved porcine interferon-α (pIFN-α) production by recombinant *Pichia pastoris* was achieved by culture conditions optimization in a 5-l bioreactor. The results indicated that the pIFN-α concentration, specific methanol consumption rate, specific activities of alcohol oxidase, formaldehyde dehydrogenase, and formate dehydrogenase could be significantly enhanced by decreasing induction temperature. The highest pIFN-α concentration (1.35 g l^{-1}) was obtained by simultaneously controlling methanol concentration at 5 g l^{-1} and induction temperature at 20 °C, which was about 1.6-fold higher than the maximum obtained with previous optimal methanol concentration level (about 10 g l^{-1}) when inducing at 30 °C. The potential mechanisms behind low temperature and low methanol concentration effect on pIFN-α production may be ascribed to higher cell metabolic activity, more carbon flux towards pIFN-α production, and less intracellular/extracellular protease release.

Keywords *Pichia pastoris* · Fed-batch cultivation · Induction · Metabolic flux · Porcine interferon-α

Introduction

The methylotrophic *Pichia pastoris* has become a popular yeast host for industrial production of milligram-to-gram quantities of heterologous proteins due to the availability of strong, tightly regulated methanol-inducible alcohol oxidase (AOX) promoter system and efficient high-cell-density fermentation protocols [1, 2]. As an example, the commercially important porcine interferon-α (pIFN-α), a vaccine adjuvant capable of

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attenuating occurrence of porcine foot-and-mouth disease as well as reducing the porcine reproductive and respiratory syndrome, could be effectively produced with the recombinant *P. pastoris* expression system [3, 4].

A typical three-phase operation has been commonly used for production of foreign proteins with *P. pastoris*: glycerol batch phase for initial cell accumulation, glycerol fed-batch phase to further increase biomass and to obtain a high pre-induction cell density (usually above 120 g dry cell weight (DCW) l^{-1}) by a limited glycerol/glycerol–methanol feeding strategy, and the induction phase by feeding methanol to induce the foreign protein expression [5]. Among the three phases, the optimization at the induction phase is actually most important; and at present, many studies have been carried out to achieve the maximum yield or activity of foreign proteins by optimizing process parameters including induction temperature, pH, and methanol concentration [1]. Recently, some authors reported that decreasing induction temperature to 20 °C or even lower levels could effectively improve the protein production [6–8]. Besides the positive effect of low induction temperature on reduction of cell lysis and proteolytic activity [6, 8], a much higher AOX transcriptional level and specific AOX activity were obtained by lowering induction temperature [7]. Likewise, methanol concentration has been reported to be another equally important factor for heterologous protein expression. Stratton et al. [9] suggested that maintaining methanol concentration at a constant level of 0.4–3% would be more desirable for the expression of foreign protein. Hong et al. [6] obtained a 5-fold higher volumetric laccase activity when the methanol concentration was kept at 0.5% instead of 1.0%. Whereas, Khatri et al. [10] reported that under oxygen-limited cultures, high methanol concentrations were required to compensate the lack of oxygen and to fully induce recombinant protein production, and single-chain antibody fragment concentrations of 60, 150, and 350 mg l^{-1} were obtained with methanol concentrations of 0.3%, 1%, and 3% (*v*/*v*), respectively.

The *P. pastoris* methanol metabolism process involves a series of key enzymes. Methanol is first oxidized to formaldehyde by AOX, and then a portion of formaldehyde enters into assimilatory pathway to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-monophosphate by dihydroxyacetone synthase (DHAS), and another portion of formaldehyde is further oxidized to formate and finally to carbon dioxide (dissimilatory pathway) by formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH), respectively. On one hand, it is necessary for part of formaldehyde to enter into dissimilatory pathway, as this pathway plays an important role in both energy production and detoxification of formaldehyde and formate [11–13]. On the other hand, if a large portion of formaldehyde enters into this pathway, the carbon flux towards assimilatory pathway (cell constitutes) will decrease correspondingly because of the loss of carbon source for release of CO_2 in dissimilatory pathway, which may ultimately lower the expression of foreign protein. Jahic et al. [7] reported that lowering induction temperature could increase AOX activity, but the enhanced AOX activity may result in the intracellular formaldehyde accumulation if the activities of detoxification pathway enzymes such as FLD and FDH could not be increased accordingly. However, except for few reports regarding the effect of temperature on AOX activity, until recently there is little information available in literature about the effect of temperature on activities of FLD and FDA.

From a view of methanol carbon metabolism point, an effective foreign protein production at least depends on the following two factors, namely, a fast methanol consumption rate and an enhanced assimilatory metabolic flow towards the targeted

protein synthesis. However, direct analysis of the assimilatory metabolic distribution is rather difficult because of the complexity of *P. pastoris* foreign protein synthesis pathway. On the contrary, the carbon flux towards dissimilatory pathway can be easily reflected and calculated by simultaneously monitoring the methanol consumption rate and carbon dioxide evolution rate (CER). In addition to the positive effects of induction temperature and methanol concentration on specific methanol consumption rate [14], carbon flux distribution may also be influenced by the induction temperature and/or methanol concentration, whereas no valuable information concerning this potential effect is available at present.

This work aims to investigate the effect of temperature on pIFN-α production by *P. pastoris* and some important enzymes involved in methanol metabolic pathway including AOX, FLD, and FDH, as well as the potential regulatory effect of temperature and/or methanol concentration on carbon metabolic flux towards assimilatory and dissimilatory distributions of *P. pastoris*. In addition, the on-line parameters monitoring analysis was conducted to provide new information for understanding the physiological characteristics of *P. pastoris*.

Materials and Methods

Strain and Fermentation Medium

P. pastoris KM71 (Muts his-, PAOXII, Invitrogen, Carlsbad, CA, USA) was used as host for pIFN-α expression. The expression plasmid pPICZ-αIFN was constructed by ligation of pIFN-α gene into pPICZα (Invitrogen, Carlsbad, CA, USA) at downstream of the promoter AOX1 and linearly integrated into the chromosome DNA of the host. The construction of the recombinant *P. pastoris* KM71H (IFNα-pPICZαA) was conducted at Animal Husbandry and Veterinary Research Institute, Shanghai Academy of Agricultural Science, China. Seed medium (in g l^{-1}, unless otherwise specified): glucose 20, peptone 20, and yeast extract 10. Initial batch medium: glycerol 20, $(NH_4)_2SO_4$ 5, H_3PO_4 2 (%, *v*/*v*), $MgSO_4$ 1, $CaSO_4$ 0.1, and K_2SO_4 1 and PTM_1 10 (ml l^{-1}), pH 6.0. Feeding medium for growth: glycerol 500, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.5, and $MgSO_4$ 0.03 and PTM_1 10 (ml l^{-1}), pH 6.0. Feeding medium for induction: methanol 500, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.5, and $MgSO_4$ 0.03 and PTM_1 10 (ml l^{-1}), pH 6.0. Mixed feeding medium for transition phase: growth medium/induction medium volumetric ratio of 25:1.

Analytical Methods

Measurements of Cell Density and Methanol Concentration

The cell concentration was determined by measuring the optical density at 600 nm (OD_{600}), and then DCW was calculated by a consistent calibration curve of DCW versus OD_{600}[$DWC(g\ l^{-1}) = 0.25 \times OD_{600}$]. Methanol and other by-metabolites such as ethanol were detected by a gas chromatography (GC112A, FID detector, Shanghai Precision & Scientific Instrument Co., China) with an Alpha-Col AC20 capillary column (SGE Int'l Pty. Ltd., Australia). A penetrative polymer-membrane type's on-line methanol electrode (FC-2002, Subo Co., China) was used for online methanol measurement. The on-line measured methanol concentration data were collected into a PC via a multi-channels A/D converter (PCL-812PG, Advantech Co., Taiwan).

Measurements of pIFN-α Concentration and Protease Activity

As for pIFN-α concentration measurement, an amount of 20 μl sample was placed in each cell of the electrophoretic plate. The SDS-page electrophoresis (15% resolving gel) was performed with the molecular weight standards until the bromophenol blue marker had reached the bottom of the gel. After SDS-page analysis, the pIFN-α concentration was quantified with a G:Box Bio Imaging System and GeneTools software (SynGene Co., Cambridge, UK); each band was scanned in triplicate and an average of three readings was obtained. The total protease activity (both extracellular and intracellular) was determined by the Pierce Colorimetric Quanticleave™ Protease Assay Kit [15].

AOX, FLD, and FDH Activity Assay

AOX activity was determined using the method described by Suye et al. [16]. Samples taken from the bioreactor with accurate volume were centrifuged at 10,000×*g* and 4 °C for 20 min. The cells were collected and washed twice with 50 mM phosphate buffer (pH 7.5), re-centrifuged, and then re-suspended in the same buffer with the volume ratio of 1:1 (buffer/sample). The suspension was sonicated by a sonifier (JY92-II, Scientz Biotechnology Co., China) at 0 °C for a total period of 10 min (pulse on, 5 s; pulse off, 10 s). The cell debris was removed by centrifugation at 8,000×*g* and 4 °C for 20 min, and the supernatant was used as the cell-free extract. The cell-free extract was then properly diluted by phosphate buffer (pH 7.0) to prepare the assay solution before activity measurement. AOX activity was assayed with a spectrophotometer (UV-2100, Unico, China) at 500 nm by measuring the optical increase within 10 min at 37 °C, with 3 ml colorized reaction mixture consisting of 100 μmol phosphate buffer (pH 7.0), 1 μmol 4-aminoantipyrine, 4.3 μmol phenol, 10 units of peroxidase, and 200 μmol methanol and the crude enzyme solution (diluted extract, supplemented until the mixture volume reached exactly 3 ml). One enzyme unit was defined as formation of 1 μmol H_2O_2 per 1 min under these conditions. The FLD and FDH activities were determined by measuring the rate of NADH formation at 340 nm at 30 °C for 10 min as described by Schütte et al. [17]. The assay mixture for FLD activity was composed of 100 μmol sodium phosphate buffer pH 8.0, 6 μmol GSH, 3 μmol NAD, 2.9 μmol formaldehyde, and properly diluted cell-free extract in a total volume of 3 ml. The FDH activity was measured in a mixture of 150 μmol potassium phosphate buffer pH 7.5, 5 μmol NAD, 500 μmol formate, and properly diluted cell-free extract in a total volume of 3 ml. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of NADH per min.

pIFN-α Expression by P. pastoris Fed-Batch Cultivation in 5 l Bioreactor

Bioreactor experiments were performed in a 5-l bench-scaled bioreactor (BIOTECH-5BG, Baoxing Co., China) with the initial working volume of 1.5 l. The entire operation details for growth, transition, and induction phases were exactly the same as those described in the previous report [18]. The previously proposed ANNPR-Ctrl approach [19, 20] was used for feeding glycerol and glycerol/methanol mixture during growth phase (26 h) and the short transition phase (3–4 h), allowing cells to reach high density (about 120–150 g DCW l^{-1}). The induction phase was started by feeding methanol-based medium at about 30 h after glycerol was completely consumed out. During induction phase, cell concentration

basically stayed constantly at a high density level (120–150 g DCW l^{-1}). Based on the measurement with the on-line methanol electrode, the PC drove a peristaltic pump (BT00-50M, Langer Co., China) via a D/A converter (PCL-812PG, Advantech Co., Taiwan) to maintain the methanol concentration at any required level with on–off control manner. An electronic balance (JA1102, Haikang Electronic Instrument Co., China) connected with the PC via the multi-channels A/D converter was used to on-line monitor and to calculate the methanol consumption rate by weighing the weight loss of methanol-feeding reservoir. The O_2 and CO_2 partial pressures in the exhaust gas, as well as the corresponding oxygen uptake rate (OUR) and CER, were on-line measured and calculated by a gas analyzer (LKM2000A, Lokas Co., Korea). These data were also collected into the PC via RS232 communication cable for storage in an interval of 1 min. If O_2 partial pressure exceeded the measurement range (0–30%) of the analyzer when sparging oxygen-enriched air, the exhaust gas measurements were stopped. Depending on requirements throughout the induction phase, temperature was controlled at 30 °C or 20 °C by using either tap water or water from a temperature-controllable circulating bath (MP-10, Shanghai Permanent Science and Technology, Co., China). Dissolved oxygen (DO) was maintained above 10% by adjusting agitation speed from 400 to 950 rpm. After shifting into induction phase, the aeration and agitation rates were fixed without further manual adjustment.

Results

Increased Cell Metabolic Activity and pIFN-α Concentration by Lowering Induction Temperature

Our previous study indicated that the optimal methanol concentration for pIFN-α production at 30 °C was about 10 g l^{-1} [18]. Therefore, the previous optimal condition (30 °C, 10 g l^{-1}) was adopted as control, and two comparable experiments were performed by controlling the same methanol concentration (10 g l^{-1}) and different induction temperatures (30 °C and 20 °C). The cells metabolic activity could be indirectly reflected by the changing patterns of apparent process parameters such as OUR and CER. As shown in Fig. 1, during the entire induction phase, OUR, CER, and cell concentration for both cases all exhibited a similar changing pattern. After shifting into methanol induction, OUR and CER began to decrease rapidly for the first few hours, then they rose gradually and finally reached a relatively stable level. However, when controlling temperature at 30 °C, the adaption period, indicated by a decrease in OUR, was no less than 6 h, but it was less than 1 h when inducing at 20 °C. In addition, the stable OUR at 20 °C was kept at a high level of about 300 mmol l^{-1} h^{-1}, which was 1.36-fold higher than the maximum obtained at 30 °C, reflecting a higher cell metabolic activity when induction was done at low temperature. Compared to induction at 30 °C, 20 °C induction yielded faster pIFN-α secretion level over the first 40 h after induction, and the highest pIFN-α concentration at 20 °C reached 1 g l^{-1}, which was 1.15-fold higher than the maximum obtained at 30 °C (Fig. 1c). Interestingly, nearly stable cell densities were observed for both cases throughout the entire induction time course (Fig. 1d).

Comparison of Specific Methanol Consumption Rate and Specific Activities of AOX, FLD, and FDH under Different Induction Temperatures

The *P. pastoris* methanol metabolic pathway involves a series of key enzymes such as AOX, FLD, and FDH. Among these enzymes, AOX is the first enzyme that catalyzes the

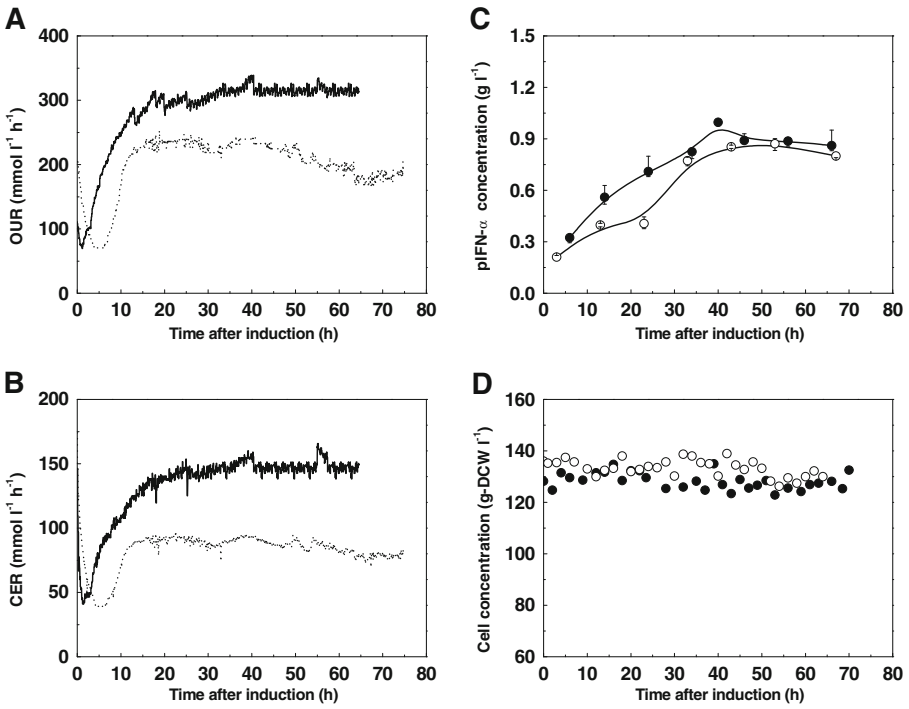

Fig. 1 Time courses of OUR, CER, cell density, and pIFN-α concentration during the entire induction phase at different induction temperatures but the same methanol concentration of 10 g l^{-1}. *Solid lines* and *filled circles*, 20 °C; *dot lines* and *open circles*, 30 °C

oxidization of methanol to formaldehyde, and its activity directly influence methanol consumption rate. Meanwhile, FLD and FDH, two enzymes in formaldehyde dissimilatory pathway, play important roles in both energy supply for cell growth on methanol and detoxification of formaldehyde and formate [11–13]. As shown in Fig. 2b, during the entire induction phase, the specific AOX activity at 20 °C was significantly higher compared to induction at 30 °C. Correspondingly, the specific methanol consumption rate in steady induction phase (20 h after induction) at 20 °C reached 0.15 h^{-1}, which was 2-fold higher than that observed at 30 °C. Besides the AOX activity, when implementing induction at 20 °C, both specific activities of FLD and FDH in dissimilatory pathway were also obviously enhanced, and the maximum activities of AOX, FLD, and FDH were about 5- to 6-fold higher compared to the maximum obtained at 30 °C (Fig. 2a–c). As formaldehyde that enters into dissimilatory pathway was finally oxidized to CO_2, the same methanol concentration (10 g l^{-1}) when inducing at 20 °C was also accompanied with more CO_2 release, and the stable CER at 20 °C was about 1.5-fold higher compared to that when inducing at 30 °C (Fig. 1b).

Further Enhancement of pIFN-α Concentration and Specific Methanol Consumption Rate by Optimization of Methanol Concentration at 20 °C

Methanol concentration, one of the most important parameters influencing fermentation performance, should be controlled at a constant and non-limiting level of 0.4–3% [9]. Our

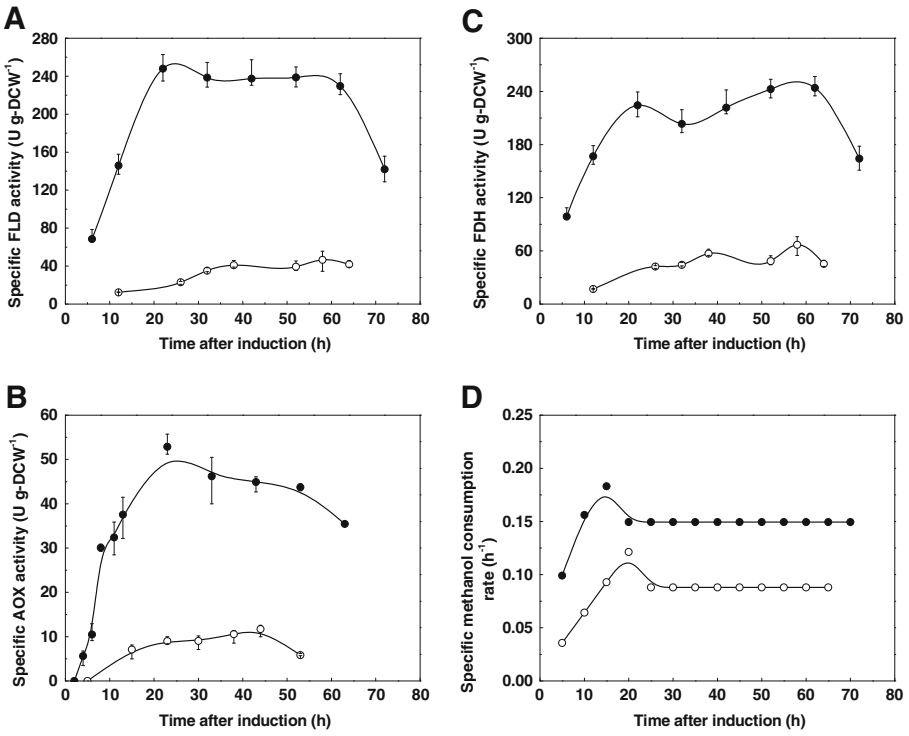

Fig. 2 Time courses of specific methanol consumption rate and specific activities of AOX, FLD, and FDH at different induction temperatures but the same methanol concentration of 10 g l^{-1}. *Filled circles*, 20 °C; *open circles*, 30 °C

previous study [18] indicated that maintaining a moderate methanol concentration of 10 g l^{-1} was a prerequisite for effective pIFN-α production when inducing at 30 °C, and both low (0–5 g l^{-1}) and high (15–20 g l^{-1}) methanol concentration levels deteriorated cell metabolic activity and pIFN-α expression. Thus, a question whether the previous optimal methanol concentration was still the optimal one for induction at 20 °C was raised. To answer this question, three other fermentation runs were conducted at 20 °C by controlling methanol concentration at different constant levels of 5, 10, and 20 g l^{-1}. As shown in Fig. 3a, contrary to induction at 30 °C, the lowest methanol concentration seemed more beneficial for pIFN-α production when induction was done at 20 °C. Compared to induction at methanol concentration of 10 and 20 g l^{-1}, induction at 5 g l^{-1} not only advanced the beginning time of pIFN-α production, but also significantly improved the pIFN-α concentration. At 4 h after induction, the pIFN-α concentration reached 0.65 g l^{-1} at methanol concentration of 5 g l^{-1}, while it was only 0.37 g l^{-1} at 5 h and 0.32 g l^{-1} at 6 h when inducing at methanol concentration of 20 and 10 g l^{-1}, respectively. Moreover, at methanol concentration of 5 g l^{-1}, the observed maximum pIFN-α concentration was 1.35 g l^{-1}, which was 1.4-fold and 1.5-fold higher than the maximum obtained at methanol concentration of 10 and 20 g l^{-1}, respectively.

In addition, as shown in Fig. 4, the stable specific methanol consumption rate when inducing at methanol concentration of 5 g l^{-1} was obviously higher compared to induction at two other methanol concentrations (10 and 20 g l^{-1}). Being different from induction at

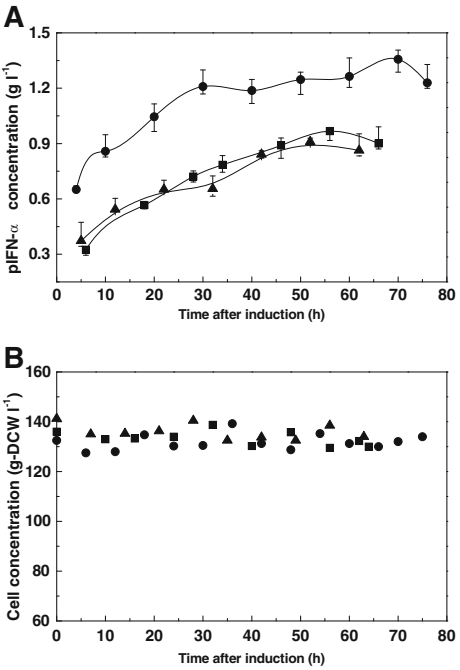

Fig. 3 Time courses of pIFN-α concentration and cell density under different methanol concentrations (MeOH) at 20 °C induction. *Circles*, MeOH 5 g l^{-1}; *squares*, MeOH 10 g l^{-1}; *triangles*, MeOH 20 g l^{-1}

30 °C, when inducing at 20 °C, a high methanol concentration of 20 g l^{-1} did not deteriorate cell metabolic activity and pIFN-α production, and the specific methanol consumption rate and pIFN-α concentration for both methanol concentration levels (10 and 20 g l^{-1}) during the entire induction phase were very close. When induction at methanol concentration of 5 g l^{-1}, specific activities of FLD and FDH increased continuously during the induction phase and the maximum activities of AOX, FLD, and FDH were the highest among the three cases. Moreover, despite of the continuous dilution effect by methanol feeding, cell concentrations for all cases basically stayed at a constant level of 130–140 g DCW l^{-1} without big variation throughout the whole induction phase.

Combined Effect of Temperature and Methanol Concentration on OUR, CER, and Carbon Ratio of CO_2 Evolution to Methanol Consumption Rate

As shown in Fig. 1, for both induction temperatures (30 °C and 20 °C), OUR and CER were basically stable after 15–20 h of induction, and the specific methanol consumption rate each also reach a stable value correspondingly (Fig. 2d). Figure 5 shows the changing patterns of OUR and CER under different experimental conditions during the first 24 h after induction. Regardless of the difference in methanol concentration at 20 °C, a higher OUR and shorter adaption phase (about 1 h) were observed compared to control (methanol concentration of 10 g l^{-1} at 30 °C). After adapting to methanol induction (at 1 h for 20 °C and 6 h for the control), OUR for all cases rose gradually and finally reached relatively stable levels at about 15–20 h after induction. The highest stable OUR level was observed with the low methanol concentration (5 g l^{-1}) at 20 °C; there was no obvious difference in OUR during 24 h when controlling methanol concentrations at 10 and 20 g l^{-1} (Fig. 5a). However, CER showed a completely different changing tendency from OUR; after adaption to methanol induction, the CER under high methanol concentration of 20 g l^{-1} was

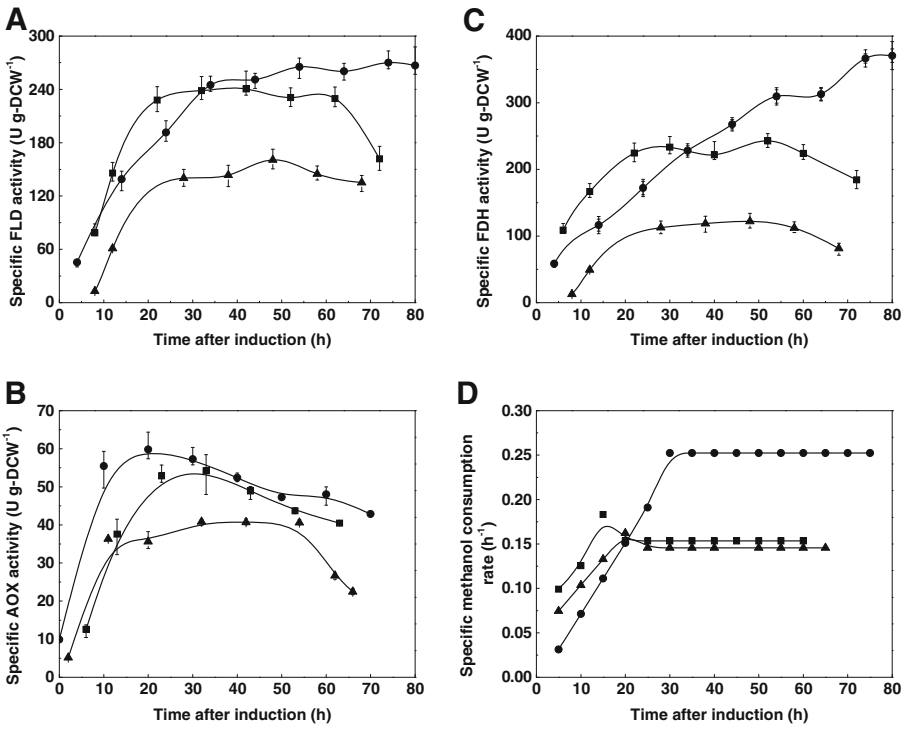

Fig. 4 The time courses of specific methanol consumption rate and specific activities of AOX, FLD, and FDH under different methanol concentrations (MeOH) at 20 °C induction. *Circles*, MeOH 5 g l^{-1}; *squares*, MeOH 10 g l^{-1}; *triangles*, MeOH 20 g l^{-1}

significantly higher compared to that observed at the other two methanol concentrations (5 and 10 g l^{-1}). More interestingly, CER at the lowest methanol concentration stayed at an almost invariable level of about 75 mmol l^{-1} h^{-1} after adaption to methanol induction (1 h after induction), which was even lower than that of the control (100 mmol l^{-1} h^{-1}).

As both CER and specific methanol consumption rate (q_{MeOH}) had already reached stable after 30 h of induction, the stable CER and q_{MeOH} could be used for evaluating carbon flux distribution. Figure 6 showed a comparison between the carbon mole ratio of CO_2 evolution to methanol consumption ($r_{CO_2/MeOH}$) and specific carbon consumption rate towards formaldehyde assimilatory pathway [$q_{MeOH-assim} = q_{MeOH} \times (1 - r_{CO_2/MeOH})$] during stable induction phase. The steady $r_{CO_2/MeOH}$ during induction phase increased with increase in methanol concentration when inducing at 20 °C (0.35, 0.24, and 0.08, respectively, at methanol concentration of 20, 10, and 5 g l^{-1}), indicating greater loss of carbon source caused by discard of CO_2 at higher methanol concentration. The value of $q_{MeOH\text{-}assim}$ could reflect the amount of carbon used for the synthesis of cell constituents and foreign protein. Compared to the control, although greater carbon losses (higher CER and $r_{CO_2/MeOH}$) were observed when inducing at 20 °C at both methanol concentrations (10 and 20 g l^{-1}; Figs. 5b and 6), the $q_{MeOH\text{-}assim}$ of the latter was still higher (Fig. 6) with a much higher q_{MeOH} (Figs. 2d and 4d), indicating that the carbon used for cell constituents and pIFN-α expression was more than that of the control. The highest $q_{MeOH\text{-}assim}$ was observed at methanol concentration of 5 g l^{-1} at 20 °C (0.23 h^{-1}), which was 2-fold and

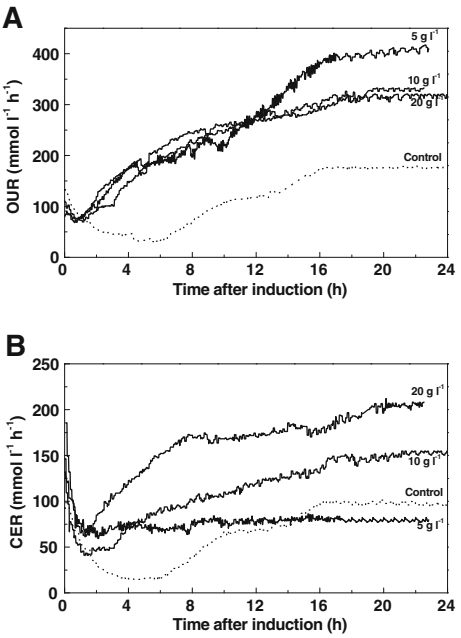

Fig. 5 Time courses of OUR and CER during the first 24 h after induction at different methanol concentrations (MeOH) and temperatures. *Control*, MeOH 10 g l^{-1}; *dot lines*, 30 °C; *solid lines*, 20 °C

2.5-fold higher compared to the other two methanol concentrations (10 and 20 g l^{-1}, respectively) at the same temperature (20 °C) and 3.4-fold higher than that of control.

Effect of Induction Temperatures and Methanol Concentrations on Protease Activity

Figure 7 shows the intra- and extracellular protease changing trends under different control strategies. At the beginning of induction, the intracellular protease activity was very close for all cases. But the intracellular protease activity of control increased significantly for the first 20 h (from 20 mg g DCW^{-1} at the beginning of induction to 55 mg g DCW^{-1} at 20 h after induction), and after entering into the steady stage (20 h after induction), the intracellular protease activity remained at a relatively stable level until the end of the fermentation. However, the intracellular protease activity at 20 °C for different methanol concentrations was obviously lower than that of the control, and it stayed at an almost invariable level during the entire induction phase when methanol concentration was

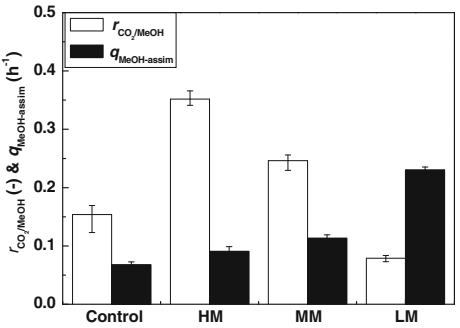

Fig. 6 Mole ratio of CO_2 evolution rate to methanol consumption rate ($r_{CO_2/MeOH}$) and specific methanol consumption rate towards assimilatory pathway ($q_{MeOH\text{-}assim}$) during the stable induction phase at different methanol concentrations (MeOH) and induction temperatures. Control, MeOH 10 g l^{-1} at 30 °C; HM, MeOH 20 g l^{-1} at 20 °C; MM, MeOH 10 g l^{-1} at 20 °C; LM, MeOH 5 g l^{-1} at 20 °C

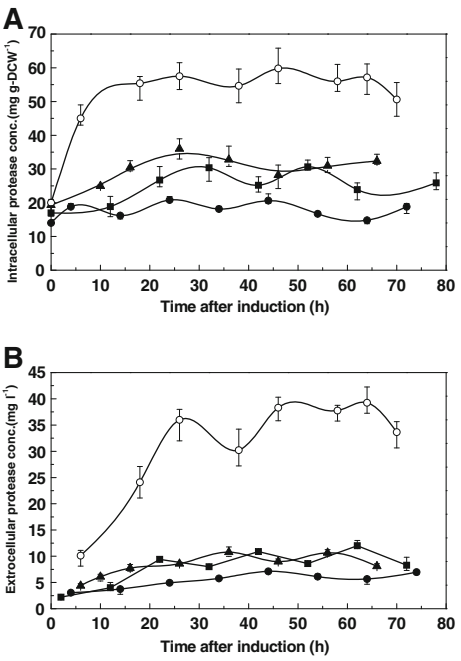

Fig. 7 Time courses of intra- and extracellular protease concentrations at different methanol concentrations (MeOH) and induction temperatures. *Filled circles*, MeOH 5 g l^{-1} at 20 °C; *filled squares*, MeOH 10 g l^{-1} at 20 °C; *filled triangles*, MeOH 20 g l^{-1} at 20 °C; *open circles*, control (MeOH 10 g l^{-1} at 30 °C)

maintained at 5 g l^{-1}. Meanwhile, the extracellular protease activity showed a similar changing tendency with intracellular protease activity. However, the extracellular protease activity was significantly lower at 20 °C compared to intracellular protease activity at the same time. No obvious difference in extracellular protease activity was observed at 20 °C for methanol concentrations of 20, 10, and 5 g l^{-1} during the entire induction phase.

Discussion

An obviously positive effect of low induction temperature (20 °C) and low methanol concentration level (about 5 g l^{-1}) on pIFN-α production was observed by implementing a series of comparable experiments. Low induction temperature could significantly improve apparent process parameters such as OUR, specific methanol consumption rate, as well as the activities of some important enzymes for methanol metabolism including AOX, FLD, and FDH. As a result, compared to traditional 30 °C induction, cells under this low temperature induction environment (20 °C) exhibited stronger methanol endurance capability, and both specific methanol consumption rate and pIFN-α concentration observed at high methanol concentration of 20 g l^{-1} were very close to those of 10 g l^{-1}. However, compared to control, in spite of higher specific methanol consumption rate obtained at 20 °C, the same (10 g l^{-1}) or higher (20 g l^{-1}) methanol concentrations at 20 °C also accompanied with larger ratio of carbon loss (high CER) and resulted in the decrease of carbon flux towards assimilatory pathway (cell constitutes), which finally limited the further improvement of pIFN-α concentration. However, when inducing at 20 °C, the carbon loss could be decreased significantly by lowering methanol concentration (Fig. 5b). As a result, a low methanol concentration level (5 g l^{-1}) at 20 °C resulted in the highest specific methanol consumption rate and largest ratio of carbon flux towards cell constitutes

(Figs. 4d and 6), and ultimately achieved, the highest pIFN-α concentration (Fig. 3a). The enhanced protein production (low temperature and low methanol concentration) was similar to what was reported by Hong et al. [6].

The maximum AOX activity observed at 20 °C was 4- to 6-fold higher compared to that of the control (Fig. 2b), and this result was very similar to what was reported by Jahic et al. [7]. Moreover, when inducing at 20 °C, the activities of FLD and FDH in formaldehyde dissimilatory pathway were higher than the control (Fig. 2a, c), indicating that low temperature could simultaneously accelerate the transcription of AOX, FLD, and FDH. As FLD and FDH play an important role in detoxification of formaldehyde and formate [11–13], the accompanying high FLD and FDH activities were useful in eliminating possible accumulation of formaldehyde caused by high AOX activity at low temperature. Katakura et al. [14] reported that the specific methanol consumption rate decreased with the increase in methanol concentration. Likewise, when induction was done at 20 °C, the specific methanol consumption rate was significantly higher at methanol concentration of 5 g l^{-1} compared to 10 and 20 g l^{-1}, although the decreasing trend was not obvious when methanol concentration was above 10 g l^{-1}. The pIFN-α concentration level at methanol concentration of 10 g l^{-1} was slightly higher than that of 20 g l^{-1} during the entire induction phase, which may be ascribed to the very close specific methanol consumption rate but higher CER observed at high methanol concentration of 20 g l^{-1} (Figs. 3a, 4d and 5b).

An ideal heterologous protein production by methylotrophic *P. pastoris* depends on the following three factors: a fast methanol consumption rate, an increased carbon flux towards protein synthesis, and less protease release to reduce degradation of target protein. Compared to traditional 30 °C induction, low induction temperature of 20 °C could significantly enhance specific methanol consumption rate (Fig. 1c) and reduce both intra- and extracellular protease activities (Fig. 7). However, decreasing induction temperature alone could not significantly improve pIFN-α concentration, and there is still great space to increase pIFN-α concentration by further optimization of methanol concentration (Fig. 3a). Not only the specific methanol consumption rate, but also the carbon metabolism flux is influenced by methanol concentration.

Theoretically, higher FLD and FDH activities observed at 20 °C can increase the chance of formaldehyde towards dissimilatory pathway and finally cause more CO_2 release. Interestingly, when the methanol concentration was maintained at 5 g l^{-1}, although both the FLD and FDH activities were the highest, the CER was the lowest (Figs. 4a, c and 5b), indicating that much more methanol carbon entered into the assimilatory pathway at this condition and formaldehyde flux was not influenced by high activities of FLD and FDH at 20 °C. On the other hand, of the enzymes in assimilatory pathway, DHAS was the first key enzyme for formaldehyde metabolism, and its activity therefore may directly influence formaldehyde distribution towards this pathway. Sakai et al. [21] reported that the regulatory pattern of DHAS was very similar to that of alcohol oxidase, but distinct from that of two other enzymes in the formaldehyde dissimilation pathway enzymes, so it can be speculated that the above difference in carbon flux distribution under different methanol concentrations may come from the difference in DHAS activities. Since the specific AOX activity at 20 °C increased with the decrease in methanol concentration (Fig. 4b), so we could conclude that DHAS activities under low methanol concentration (5 g l^{-1}) was highest compared to the other two methanol concentrations (10 and 20 g l^{-1}), which resulted in the highest formaldehyde flux towards assimilatory pathway and highest pIFN-α concentration observed at 5 g l^{-1}; however, additional testing is also required to address whether DHAS or some other assimilatory enzymes were really stimulated by low

methanol concentration under low induction temperature in our following study. Our findings also confirmed the result reported by Sibirny et al. [22] that the main physiological role of FLD and FDH observed here was detoxification of formaldehyde and formate, and the high FLA and FDH activities at 20 °C indicated that *P. pastoris* possessed stronger detoxifying ability compared to 30 °C. Together with the special *P. pastoris* metabolic characteristics, the pIFN-α production performance could be evaluated by simultaneously monitoring specific methanol consumption rate and CO_2 evolution rate, which provided valuable information and may be applied to act as an important industrial production performance index for optimization of other foreign proteins.

Acknowledgments This study was supported by the key agricultural technology program of Shanghai Science & Technology Committee (no. 073919108) and Major State Basic Research Development Program (973) (no. 2007CB714303) of China. The authors would like to thank them for the kind financial support.

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