Combined Strategies for Improving the Production of Recombinant *Rhizopus oryzae* Lipase in *Pichia pastoris*

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We have developed a yeast *Pichia pastoris* system for the high-level expression of recombinant *Rhizopus oryzae* lipase (ROL), which is a potentially effective catalyst in the solvent-free production of biodiesel fuel. In the glycerol fed-batch phase, the combination of the dissolved-oxygen-stat and gradient-control glycerol feeding strategies resulted in a higher cell biomass in the *P. pastoris* culture, with shorter feed times. In the methanol fed-batch phase, a constant methanol concentration of 0.3-0.5% (v/v) was found to be optimal for high ROL activity, maximum protein concentration, and a maximum biomass. Cells were grown at 30 °C and induced by methanol at 22 °C and a pH of 6.0 with the addition of 0.5% (w/v) casein, which reduced the proteolytic degradation of ROL. Using a combined glycerol step-increasing, on-line methanol feeding strategy and these reducing proteolytic degradation methods, a maximum biomass of 96 g DCW/l, a maximum concentration of 2.0 g/L, and an activity of 1302.2 U/mL ROL were attained. The simple culture process is the highest level of ROL expression reported in *P. pastoris*. Our results strongly demonstrate that the efficiency of the recombinant ROL expression is directly dependent on feed strategies, lower induction temperature, maintaining the proper pH, and the addition of casein.

Keywords: Pichia pastoris; Rhizopus oryzae lipase; Feed strategy; Proteolytic degradation; Biodiesel

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INTRODUCTION

Biodiesel production is a very modern and technological area for researchers on account of the ever-increasing petroleum prices and the environmental advantages it entails (Marchetti *et al*. 2007). Conventional biodiesel technology is usually catalyzed by an inorganic base and acid, which makes the separation of the catalysts from the products difficult and consequently increases the cost of production. Recently, lipase-catalyzed methanolysis methods have become more attractive than the traditional chemical means in biodiesel production, since the recovery of glycerol is easy and transesterification of oil with high fatty acid content is available (Maceiras *et al*. 2009). The extracellular lipase from *Rhizopus oryzae* can induce methanolysis even in the presence of 4 to 30% water in oil, whereas other lipases are nearly inactive in the absence of water (Li *et al*. 2012). With these characteristics, *R. oryzae* lipase (ROL) is considered to be a potentially effective enzyme for a solvent-free system. However, the high cost of producing the enzyme is often the biggest obstacle to its widespread use in the industry.

The methylotrophic yeast *Pichia pastoris* is a promising candidate for the production of heterologous proteins. Lipases are widely produced by recombinant *P. pastoris* harboring ROL genes (*rol*). Complicated recovery and purification processes are

not required for recombinant ROL because few other proteins except recombinant ROL can be secreted into the broth (Li *et al*. 2011). The characteristics of high yield and high activity mean that the lipase product has greater market competitiveness (Shu *et al*. 2010). Optimization of high-level production of ROL in *P. pastoris* is considered to be a key factor to ensure successful lipase commercial production. *P. pastoris* is usually grown in fed-batch cultures that can reach very high cell density. The fed-batch culture process involves three stages: stage one (P1: glycerol batch phase), batch growth on glycerol to rapidly produce a significant amount of biomass, followed by stage two (P2: glycerol fed-batch phase), glycerol-limited fed-batch growth, and stage three (P3: methanol fed-batch phase), fed-batch growth on methanol to induce protein expression (Schenk *et al*. 2007).

It has been reported that the glycerol concentration in the second stage must be carefully controlled within a narrow range to avoid depressing *AOX1* (the alcohol oxidase gene one), and also to decrease the time duration of this stage (Arnau *et al*. 2011). Also, methanol accumulation in the fermenter is toxic to recombinant *P. pastoris* and can have a negative influence on protein induction (Jungo *et al*. 2007a). Therefore, the combined glycerol and methanol feed strategy is one of the most important factors for maximizing heterologous protein production (Cos *et al*. 2006). In addition to the glycerol and methanol feeding strategy, avoiding proteolytic degradation may also be another important factor that affects heterologous protein production. Proteolysis may lead to a decrease in lipase activity and reduced functional recombinant protein yields. Furthermore, protein degradation can also lead to added downstream purification costs. Optimizing the temperature, culture pH, and medium composition has also been proven to be effective in limiting proteolytic degradation of recombinant proteins (Wang *et al*. 2009; Korayashi *et al*. 2000; Ohya *et al*. 2002).

Protocols to study the recombinant ROL expression in *P. pastoris* are mostly focused on different strategies of glycerol and methanol substrate feeding (Surribas *et al*. 2007; Arnau *et al*. 2010). To the best of our knowledge, a systematic study of operational strategies to reduce preteolytic degradation has not been reported. The work presented here focuses on the application of alternative feeding strategies to improve the entire process of ROL production in the *P. pastoris* expression system. Another objective of this work is to investigate the effects of culture composition, temperature, and pH on ROL expression by a transformed methanol utilization slow (Mut^S) strain of *P. pastoris*; we also discuss the possible physiological mechanisms behind our findings.

EXPERIMENTAL

Materials

The Mut^S type *P. pastoris* KM71 (his4 aox1::ARG4) strain containing the vector pPICZαA-ROL was used for the heterologous expression of ROL under control of the *AOX1* gene promoter. *P. pastoris* strain KM71 and plasmid pPICZαA were obtained from Invitrogen (Carlsbad, CA, USA).

P. pastoris was grown on YPD (yeast extract peptone dextrose medium) plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, and 2% (w/v) agar and were stored at 4 °C. Long-term stocks were prepared as recommended by Invitrogen and stored frozen at -80 °C.

Shake Flask Culture

Inoculates for the batch cultures were grown on a buffered glycerol-complex medium (BMGY; 10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate, pH 6.0, 13.4 g/L YNB, 4×10^{-4} g/L biotin, 10 g/L glycerol). Cells were grown in 25 mL of BMGY in a 250 mL shake flask at 220 r/min and 28 °C until the OD_{600} was close to 2. Cells were then recovered by centrifugation and resuspended in 100 mL of buffered methanol-complex medium (BMMY; 10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate, pH 6.0, 13.4 g/L YNB, 4×10^{-4} g/L biotin, 5 g/L methanol) to an OD_{600} of 1.0, and shaken at 28 °C and 220 r/min in 1 L shake flasks for 120 h. Methanol (0.5%, 1%, 1.5%, 2%, 3%, v/v) was added to the shake flasks every 24 h in order to induce lipase production during the induction period. The cultures were then centrifuged at 8000 r/min for 10 min, and the supernatant was collected for SDS-PAGE, protein concentration, and lipase activity assays.

Glycerol Batch Phase Media and Operational Conditions

Inoculums for the batch culture were grown on buffered minimal glycerol medium (BMG; 13.4 g/L YNB, 100 ml/L of 100 mmol/L phosphate buffer pH 6.0, 400 mg/L biotin, and 10 g/L glycerol). Cells were grown in 100 mL of BMG in a 1 L shake flask at 200 r/min and 30 °C until the OD_{600} was close to 4; 400 mL of this culture was used to inoculate the bioreactor.

Cells were cultured in a 10 L bioreactor (GU/BJS-10AUTO, Zhenjiang Co., China) under the following conditions: initial volume: 4 L, stirring rate: 800 r/min, pH controlled at 5.5 by adding 28% (v/v) of NH_4OH during the batch transition and induction phases, and dissolved oxygen above 30% air saturation, with a constant air flow rate between 1 and 3 L/min. The temperature was set at 30 °C unless stated otherwise in some operational strategies. Foaming was controlled through the addition of antifoam AFE7510 [Dow Coring (China), Shanghai, P.R.C.]. For the batch cultures, the standard basal salt synthetic medium for *P. pastoris* contained per liter: 26.7 mL H_3PO_4 (85%), 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 40 g glycerol, and 4.35 mL of PTM1 solution. The PTM1 (*Pichia* trace metals) solution contained, per liter: 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, and 5 mL concentrated H_2SO_4. For the batch feeding, either glycerol or methanol was added by means of a peristaltic pump with regulated speed. The culture started with 40 g/L of glycerol for the batch phase.

Fed-batch Phase Alternative Operational Strategies

Glycerol fed-batch phase strategies

After the glycerol batch phase, a 50% w/v glycerol solution containing 12 mL/L PTM1 and 5 g/L of methanol was added to increase the cell biomass under three different feeding strategies in the fermenter as a glycerol fed-batch phase. In strategy A, glycerol was added at step-increasing feed rates from 12 to 15 mL/h/L broth. The feed rate increased 1 mL/h/L broth every 2 h until it reached 15 mL/h/L broth; then the feed rate was kept at 15 mL/h/L broth until OD_{600} reached 200. In strategy B, glycerol feeding rates were adjusted to control DO above 30%. When the DO level reached 30 to 35%, feeding of glycerol (50%, v/v) containing 1.2% PTM1 and 5 g/L of methanol was started. DO level fell to below 30 to 35%, at which point the feeding was stopped. In strategy C, glycerol feeding rates were adjusted by both controlling DO above 30% and at step-

increasing feed rates from 11 to 13 mL/h/L broth. The feed rate increased 1 mL/h/L broth every 2 h until it reached 13 mL/h/L broth; then the feed rate was kept at 13 mL/h/L broth until OD_{600} reached 200. The DO level stayed between 30 and 35% by controlling the aeration rate and agitation speed.

Methanol induction fed-batch phase strategies

Upon completion of the 6-h glycerol fed-batch phase, when the OD_{600} reached a value of 200, the methanol fed-batch phase, in which methanol was fed as the sole carbon source in the culture, was initiated. Two methanol induction strategies were compared. In the DO-stat feeding strategy, methanol feeding rates were adjusted by controlling DO above 35%. The methanol feed rate was stepwise increased 10% per hour from 1 to 3 mL/h/L broth, then the feed rate was kept at 3 mL/h/L broth. In the methanol-limited feeding strategy, the methanol concentration was kept at 3 to 5 mL/L detected by an on-line methanol analyzer (FC2002, East China University of Science and Technology, China).

Strategies to Prevent Proteolytic Degradation

Effect of medium composition

0.5% (w/v) of corn steep powder or casein was added to prevent proteolytic degradation in the methanol induction phase. The glycerol feeding rates were adjusted by both controlling DO above 30% and at step-increasing feed rates from 11 to 13 mL/h/L broth. The methanol fed-batch induction was initiated at 30 h using an on-line methanol feeding strategy; the methanol concentration was maintained at ~3 to 5 mL/L. During the glycerol batch phase and the methanol induction stage, the culture was kept at 30 °C and at a pH of 5.0.

Effect of temperature on proteolytic activity

During the methanol induction stage, the temperature controller was set at 22 °C, 26 °C, or 30 °C. When the low temperature fed-batch was applied in the methanol induction phase, all conditions were as in (a) without the addition of 0.5% (w/v) corn steep powder or casein.

Effect of pH on proteolytic activity

During the methanol induction stage, the pH was kept at 5.0, 5.5, 6.0, or 6.5. When the different pH fed-batch conditions were applied in the induction phase, all conditions were kept as in (a) without the addition of 0.5% (w/v) of corn steep powder or casein.

Biomass Analysis

P. pastoris cell concentration was monitored by measuring the optical density at 600 nm (OD_{600}). For DCW (g/L) analysis, samples were collected every 12 h. A 5 mL sample of the culture was centrifuged in a pre-weighed centrifuge tube at 4,500×*g* (where *g* is gravitational acceleration) for 10 min. Supernatant was separated and the pellet was washed with distilled water once and dried to constant weight at 105 °C. Each sample was analyzed three times and the mean value was taken.

The experimental results showed that cell optical density (OD_{600}) of *P. pastoris* was linear with its dry cell weight and reached 100 OD_{600} = 394 g DCW/L.

Lipase Enzyme Activity Assay and Total Protein Concentration

Lipase activity determination was carried out using the olive oil emulsion method as described previously (Kohno *et al*. 1994). Supernatants were obtained by centrifuging culture samples in a microcentrifuge at 12,000 r/min. Aliquots of 10 to 50 μL of the supernatant were used in the assay. The fatty acids released were determined by titration with 50 mmol/L NaOH solution. Determinations were analyzed in triplicate and the mean value was taken. One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per minute at 35 °C at a pH of 7.5.

The total amount of protein in the culture broth was determined according to Bradford after removal of cells by centrifugation (Bradford 1976).

SDS-PAGE

SDS-PAGE analysis was carried out in a Mini-PROTEAN II (Bio-Rad) apparatus following the standard procedures recommended by the manufacturer and according to the protocol of Laemmli (1970). Low-range protein markers (BioRad) were used for molecular weight determination. Gels were stained using the Coomassie Brilliant Blue R-250 colloidal stain procedure. SDS-PAGE analysis using a 10% separation gel was performed using 1 μL samples of fermentation supernatants collected on days 1 to 5 after methanol induction.

RESULTS AND DISCUSSION

Optimization of Conditions in Shake Flasks

Lipase activity in *P. pastoris* KM71 carrying the ROL expression constructs reached a peak at 96 h after induction. SDS-PAGE analyses of supernatants from the shaking flasks demonstrated that recombinant protein was expressed and secreted by *P. pastoris*. Staining with Coomassie Brilliant Blue R-250 indicated that the molecular weight of the recombinant protein was approximately 32 kDa, which is the same as the molecular weight of mature ProROL (Li *et al*. 2011).

We found that methanol concentration was the most important factor for the expression of lipase in shake flasks. Experiments were performed at methanol concentrations ranging from 0.5 to 3% to induce lipase production in *P. pastoris* KM71. Results showed that the highest lipase activity was 256.2 U/mL (total protein concentration, 0.6 g/L) by 1% methanol induction at a pH of 6.0 and 30 °C. The lipase activity and protein concentration declined when methanol concentrations were higher than 1%. This result is consistent with the previous report (Jungo *et al.* 2007b). It may be attributed to the fact that transient methanol accumulation can lead to intoxication of cells and excessive methanol inhibits lipase production in the induction phase.

Effect of the Glycerol Feeding Strategies on Cell Growth

The glycerol batch phase and fed-batch phases are collectively referred to as the biomass (production) phase prior to methanol induction. Glycerol was used as the growth substrate at this stage because cells grown on glycerol have a higher specific growth rate than those grown on methanol. During the first phase, the *AOX1* gene promoter is completely inactive and no recombinant proteins are produced. The length of the glycerol fed-batch phase depends on the desired cell concentration prior to methanol induction (Potvin *et al*. 2012). In order to compare the oxygen uptake model and the glycerol

supplementation model, three glycerol feeding strategies (A, B, and C) were employed to control the glycerol supplementation rate. Figure 1 shows that Strategy A, the gradient-control glycerol feeding strategy can avoid carbon source repression of cell growth. However, the DO was extremely low and the OD_{600} (optical density at 600 nm) was 140 after 12 h of cultivation, which indicates that strategy A extends the glycerol fed-batch phase but also increases the cost. If the carbon source is supplied, the DO level falls because the metabolic pathways become active again. Using these phenomena, strategy B, which was a simplified indirect feedback protocol that couples substrate feeding with measurement of DO, can be designed. In this way, the DO level could be kept above 30% by regulating the glycerol feeding rate, and the culture required only 8 h until the OD_{600} reached 200. In contrast to strategy A, the cycle of glycerol fed-batch aeration using strategy B was dramatically shortened. However, the supply rate of glycerol in the DO-stat strategy was so unstable that the operation was complex during the glycerol fed-batch phase. In strategy C, which was the combination of the DO-stat and gradient-control glycerol feeding strategies, the DO level could be maintained at 30 to 35%, and the glycerol feed rate is lower than either strategy A or B. When the glycerol fed-batch phase was terminated after 6 h, a desired high cell density was reached (OD_{600} of 200). The glycerol supplies in strategy A, B, and C were 13:3:2 until the OD_{600} reached 200, indicating that strategy C has higher glycerol utilization efficiency and lower costs.

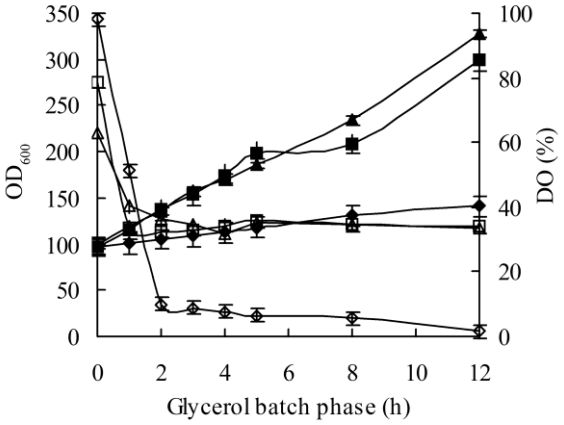

Fig. 1. Yeast cell growth and DO in glycerol fed-batch phase with *P. pastoris* (host KM71H, MutS, vector pPICZαA) using three glycerol feeding strategies. The DO (◇), the OD_{600} (◆) are indicated in strategy A; the DO (□), the OD_{600} (■) are indicated in strategy B; the DO (△), the OD_{600} (▲) are indicated in strategy C.

Effect of the Methanol Feeding Strategy on Lipase Production

During the methanol fed-batch (induction) phase, the feeding rate of methanol, which is both the carbon source and the pAOX1 inducer, directly impacts the residual methanol concentration, the specific growth rate of the culture, and heterologous protein expression levels. The most common methanol feeding strategies include constant DO feeding (DO-stat) and methanol limited fed-batch. Not limiting the oxygen is a vital factor for *P. pastoris* cells utilizing methanol through the oxidative pathway. In order to avoid methanol accumulation, proper DO control is an efficient methanol-induction strategy for optimum protein production in the *Pichia* expression system (Potvin *et al*. 2012). Furthermore, the on-line methanol electrode-based on-off control method effectively reduces the damage to cells or the ROL activity caused by the high concentration of methanol (Gurramkonda *et al*. 2009).

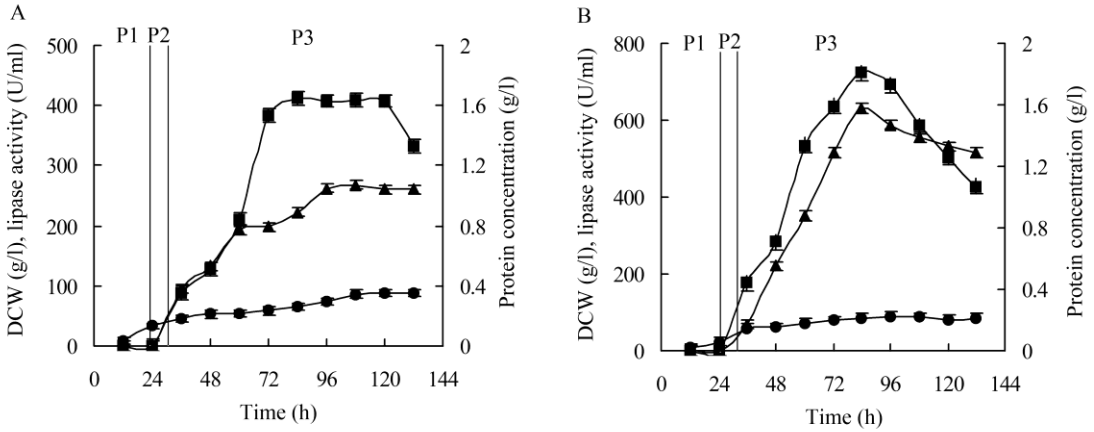

Fig. 2. Typical time-course profiles of dry cell weight (●), lipase activity (■), and protein concentration (▲) in different methanol fed-batch strategies. (A) DO-stat method; (B) on-line methanol control method. P1: Glycerol batch phase; P2: Glycerol fed-batch phase; P3: Methanol fed-batch phase. The glycerol fed-batch phase was initiated at 24 h. The glycerol feeding rates were adjusted by both controlling DO above 30% and with step-increasing feed rates from 11 to 13 mL/h/L broth. The feed rate increased 1 mL/h/L broth every 2 h until it reached 13 mL/h/L broth; then the feed rate was kept at 13 mL/h/L broth until OD_{600} reached 200 and the cultivation was kept at 30 °C. The methanol fed-batch induction was initiated at 30 h. During the methanol induction stage, the culture was kept at 30 °C and at pH 5.0 without medium additives.

The methanol fed-batch phase was initiated at 30 h for both the DO-stat method (Fig. 2A) and the on-line methanol control method (Fig. 2B) as the methanol feeding strategy. In the DO-stat method, cell biomass was approximately 66.3 g DCW/L at 84 h, and the highest lipase activity and protein concentration were 411.6 U/mL and 0.9 g/L at 84 h, respectively. There was practically no increase in lipase activity after 84 h. In the DO-stat strategy, the methanol concentration may become higher than is required due to the later DO concentration reaction, and the Mut^S recombinant strain is sensitive to high methanol concentrations. When the methanol concentration was kept at 0.3 to 0.5% (v/v), the cell biomass was 85.5 g DCW/L and the protein concentration was 1.6 g/L during the final induction phase in the on-line methanol electrode-based on-off case (Fig. 2B); the lipase activity was 1.8-fold (723.7 U/mL) higher than that achieved in the DO-stat case. Therefore, the on-line methanol electrode-based on-off control method is more effective than DO-stat for lipase production in the induction phase using a Mut^S strain. Although the maximum activity of ROL and protein concentration were increased using the on-line methanol control method, the activity of ROL decreased quickly after 84 h. This phenomenon may be attributed to the fact that cell autolysis can lead to the damage to cells and proteolytic degradation of ROL.

Strategies to Reduce Proteolytic Degradation

One major drawback of the *P. pastoris* expression system is the post-secretory proteolytic degradation of recombinant products (Idiris *et al*. 2010). In our study, the phenomenon of proteolytic degradation was observed for ROL in the bioreactor, but not in the shake flask (data not shown). In Figure 3, panels B1 and B2 show that the recombinant ROL was degraded increasingly after 24 h of induction in the bioreactor, and that the rate of degradation increased significantly later in the fermentation.

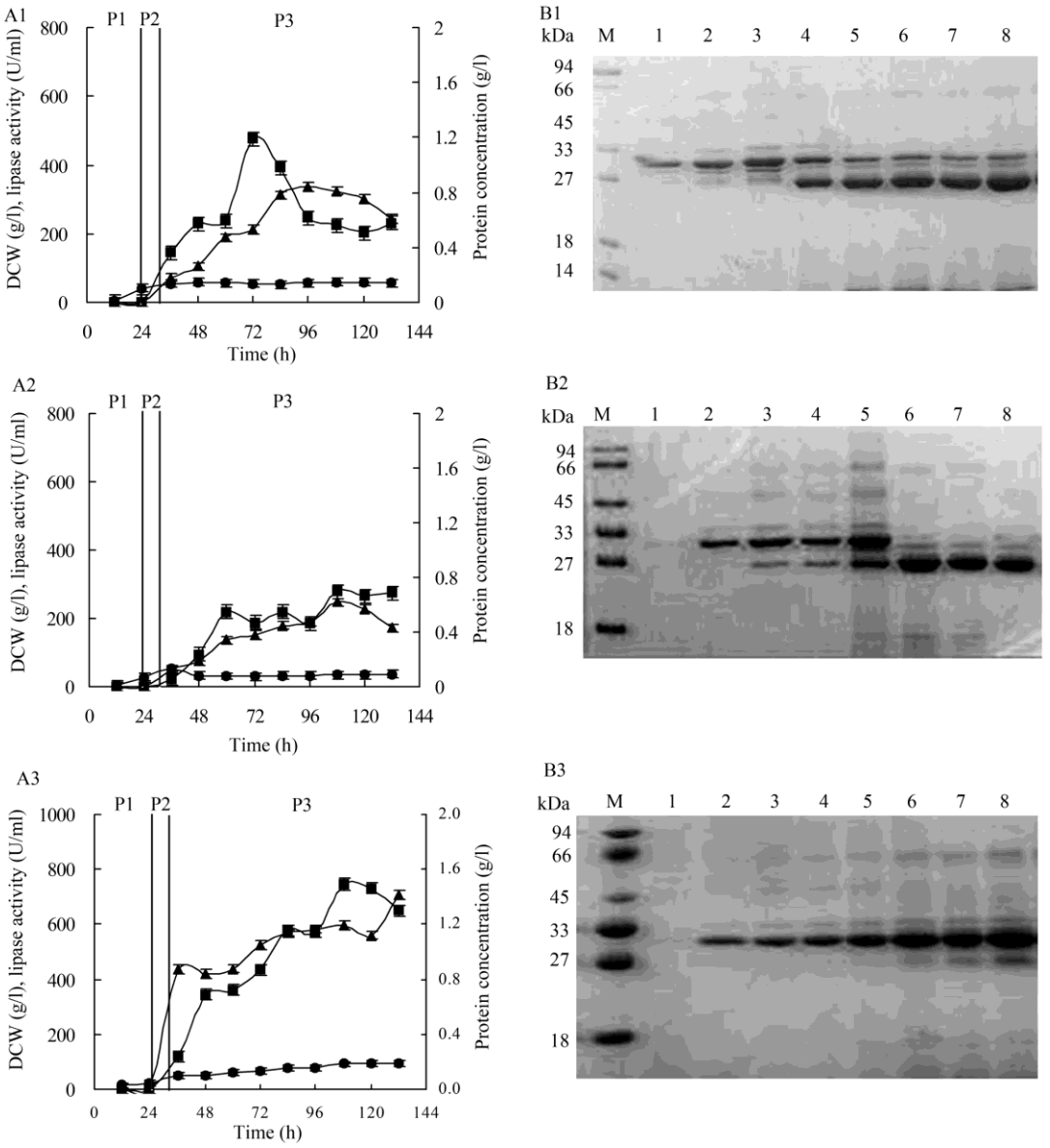

Fig. 3. (A) Typical time-course profiles of dry cell weight (●), lipase activity (■), and protein concentration (▲) in methanol fed-batch with different medium additives. (A1) 0.5% corn steep powder; (A2) 0.5% YNB; (A3) 0.5% casein. (B) SDS-PAGE analysis of culture supernatant with different medium additives. (B1) 0.5% corn steep powder; (B2) 0.5% YNB; (B3) 0.5% casein. Lane M: protein marker; Lane 1-8: Supernatant from methanol fed-batch culture, 36, 48, 60, 72, 84, 96, 108, 120 h. P1: Glycerol batch phase; P2: Glycerol fed-batch phase; P3: Methanol fed-batch phase. The glycerol feeding conditions were as given in Fig. 2. The methanol induction conditions were as given in Fig. 2 using on-line methanol feed strategies, the methanol concentration was kept at about 3 to 5 mL/L.

In order to evaluate whether the use of nitrogen-enriched media could inhibit protease activity and increase the ROL activity, batch fermentation was also performed using corn steep, yeast nitrogen base without amino acids (YNB), or casein as nitrogen sources. As observed in Fig. 3, adding 0.5% YNB or corn steep considerably reduced ROL production and cell growth. Conversely, inclusion of 0.5% casein not only increased cell growth but also increased ROL accumulation, reaching a maximum value

at the end of the induction of 837.3 U/mL and 1.6 g/L protein, which are 1.8-fold and 2.0-fold higher, respectively, than in the corresponding culture containing 0.5% corn steep. SDS-PAGE analysis of samples indicated that the ROL was not degraded until 60 h into the induction phase (Fig. 3C). This can be compared with samples from a fed-batch fermentation containing 0.5% corn steep, which showed the 27 kDa degradation product at 12 h. The result could be due to caseins preventing ROL degradation from serine protease (Patrick *et al.* 2005; Salamin *et al.* 2010). Previous reports disclosed that the major store of proteolytic activity in yeasts is located within the lumen of the vacuolar compartment (Jones 1991). Casein may inhibit protease activity by providing competing enzyme substrates (Todde *et al.* 2009). Hence, addition of casein is an effective means for reducing proteolytic degradation and increasing lipase production. To determine the appropriate/optimal concentration of casein, we compared the effect of adding 0.25% (w/v), 0.5%, or 0.75% casein in the induction phase. The result showed that 0.5% casein is the most favorable to ROL expression (data not shown).

Previous studies showed that both cell death and degradation of target proteins were reduced at lower temperatures. Wang *et al.* found that polygalacturonate lyase (PGL) activity exhibited a 2.9-fold increase when the induction temperature was lowered from 30 °C to 22 °C, which resulted in higher cell viability, significant improvement of PGL stability, and a reduced activity of released host proteases. Figure 4 A3 shows that cell growth almost ceased throughout the whole process at 22 °C. Compared to cultivation at 22 °C, the DCW reached 124.9 and 137.2 g/L by increasing the temperature to 26 °C (Fig. 4 A2) and 30 °C (Fig. 4 A1), respectively. In all cases, induction at lower temperature led to a large increase in ROL production. The maximum ROL activity at 30 °C reached 370 U/mL. The shift to 26 °C and 22 °C resulted in a 1.6–fold (583.4 U/mL) and 1.9–fold (698.5 U/mL) increase in the maximum ROL activity (Fig. 4 A1-3). However, the protein concentration was almost the same at the different induction temperatures. This indicates that the relative amount of active ROL increased. In order to evaluate whether the low induction temperature could decrease proteolysis, recombinant ROLs induced at different temperatures were analyzed by SDS-PAGE (Fig. 4 B1-3). A decrease in the induction phase temperature of the *P. pastoris* culture from 30 °C to 22 °C led to great increase in the production of a recombinant ROL of 32 kDa instead of ROL of 27 kDa, indicating that the reduction in proteolysis at low induction temperature improved the integrity and quantity of the recombinant ROL. This result could be due to a reduction in proteolysis at lower temperatures, a decrease in protease activity, or a decrease in protease production (Jahic *et al.* 2003).

The pH of the culture broth can affect both the protease activity and the stability of expressed proteins, and it must therefore be monitored and maintained within an optimum range. While the highest levels of ROL activity were detected at pH 6.0, induction media with pH values of 5.0 were the least conducive to ROL expression (Fig. 5). Serine and aspartic proteases, both secreted by *P. pastoris*, are activated at low pH (Ohya *et al*. 2002; Idiris *et al*. 2010), which can explain why the highest protein concentration and the lowest levels of ROL activity were observed at pH 5.0. The relative activity of ROL (U/mg) increased with increasing pH, peaking at pH 6.0, even though the pH values did not support the highest levels of cell growth and protein concentration upon induction. These results suggest that induction medium pH values of 6.0 to 6.5 were most conducive to ROL production, despite the significant differences in cell growth rates.

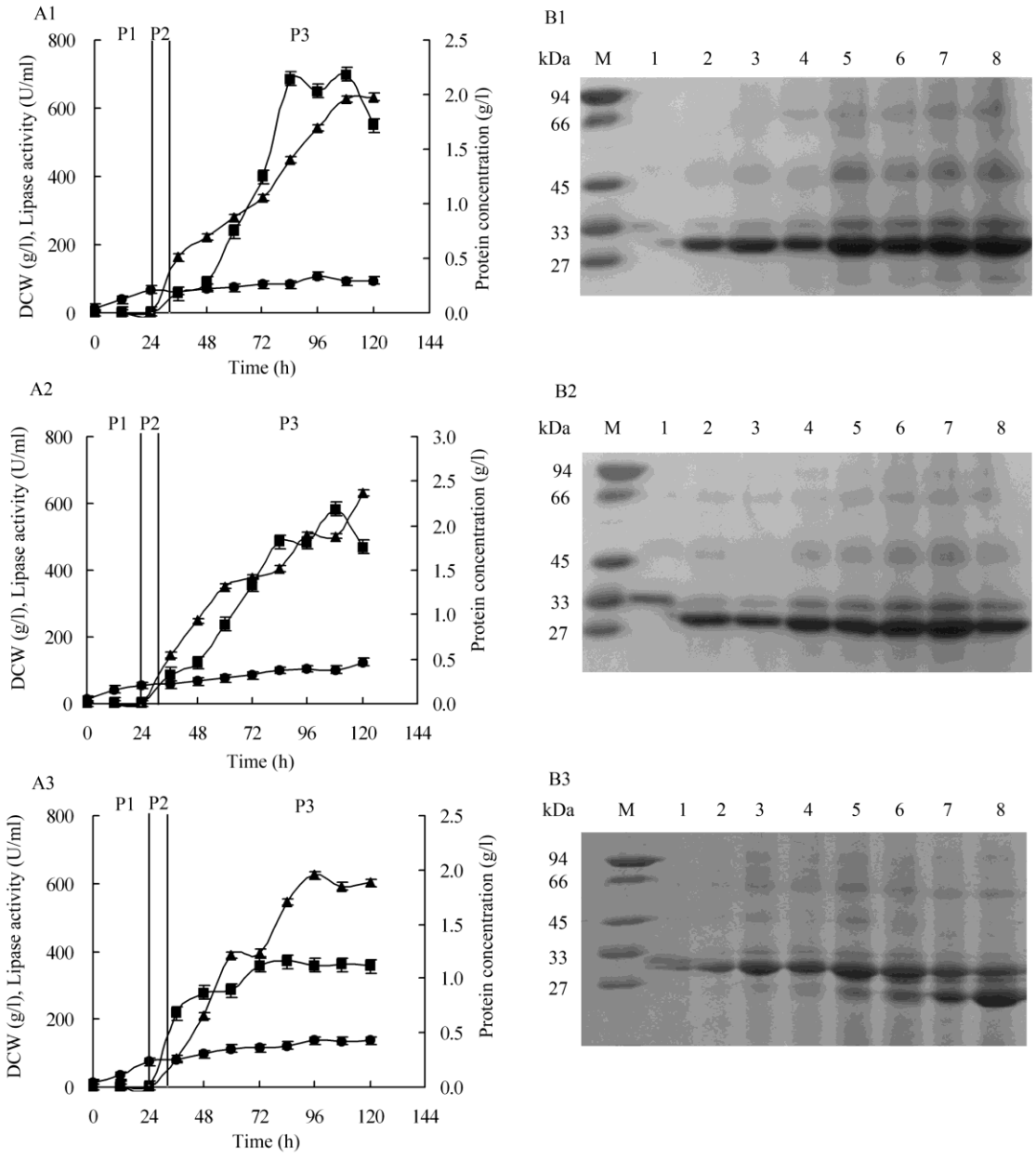

Fig. 4. (A) A typical time-course profiles of dry cell weight (●), lipase activity (■), and protein concentration (▲) in methanol fed-batch at different induce temperature. (A1) 22 °C; (A2) 26 °C; (A3) 30 °C. (B) SDS-PAGE analysis of culture supernatant taken at different induction temperature. (B1) 22 °C; (B2) 26 °C; (B3) 30 °C. Lane M: protein marker; Lane 1-8: Supernatant from fed-batch culture, 36, 48, 60, 72, 84, 96, 108, 120 h. P1: Glycerol batch phase; P2: Glycerol fed-batch phase; P3: Methanol fed-batch phase. The glycerol fed-batch phase was initiated at 24 h. The glycerol feeding conditions were as given in Fig. 2. The methanol induction conditions were as given in Fig. 3 at different temperature.

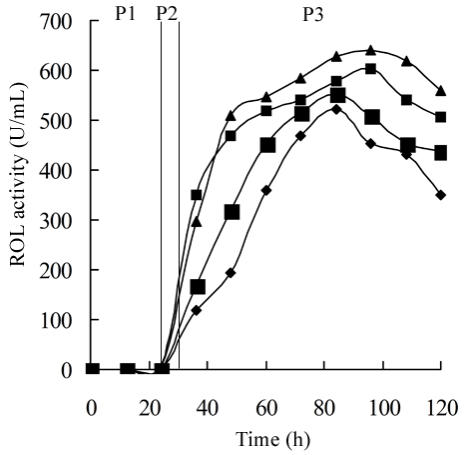

Fig. 5. Effect of induction medium pH on ROL production. (◆) pH 5.0; (■) pH 5.5; (▲) pH 6.0; (■) pH 6.5. P1: Glycerol batch phase; P2: Glycerol fed-batch phase; P3: Methanol fed-batch phase. The glycerol fed-batch phase was initiated at 24 h. The glycerol feeding conditions were as given in Fig. 2. The methanol induction conditions were as given in Fig. 3 at different pH.

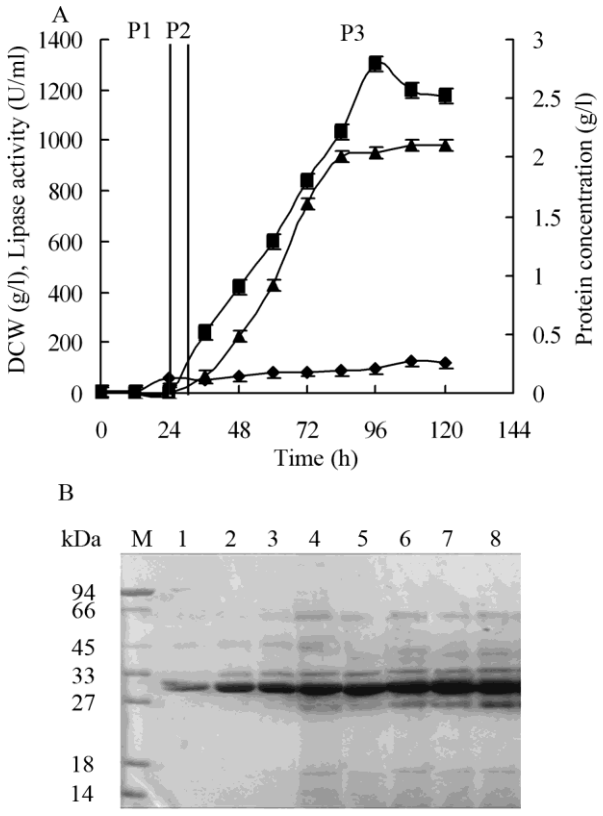

Fig. 6. (A) Typical time-course profiles of dry cell weight (◆), lipase activity (■), and protein concentration (▲) in methanol fed-batch with optimal conditions (The glycerol fed-batch phase was initiated at 24 h. The glycerol feeding conditions were as given in Fig. 2. Methanol induction conditions were as given in Fig. 3, and the cultivation was kept at 22 °C and pH 6.0 by adding 0.5% casein.). (B) SDS-PAGE analysis of supernatant from fed-batch culture with optimal conditions. Lane M: protein marker; Lane 1-8: Supernatant from fed-batch culture, 36, 48, 60, 72, 84, 96, 108, 120 h. P1: Glycerol batch phase; P2: Glycerol fed-batch phase; P3: Methanol fed-batch phase.

Optimization of Conditions in the Fermenter

Fermenter conditions were optimized based on the ROL expression results obtained in fed-batch cultures of *P. pastoris*. Through comprehensive analyses of fed-batch operational strategies and strategies to reduce proteolysis, we chose to use combined glycerol feeding strategies and on-line methanol feeding strategies, culture at 30 °C, and induction at 22 °C, a pH of 6.0 with addition of 0.5% (w/v) casein. These conditions reduced the proteolysis of secreted ROL and gave a maximum biomass of 96 g DCW/L, a protein content of 2.0 g/L, and a ROL activity of 1302.2 U/mL (Fig. 6). This result strongly suggests that the efficiency of recombinant protein expression is directly dependent on feeding strategies, a lower induction temperature, and the addition of casein.

CONCLUSIONS

The *P. pastoris* expression system is being used extensively for heterologous protein production, primarily due to the ease with which it can be propagated and its inherent ability to secrete large amounts of protein. For production of ROL in a Mut^S strain of *P. pastoris*, we identified several problems, such as early oxygen limitation and the protein degradation, which required changes from the standard approach to overcome these disadvantages.

The DO-stat method with tightly regulated glycerol feed resulted in increased cell biomass and the shortest time to reach a high cell density. We also demonstrated that in the *P. pastoris* system, the on-line methanol control method, which improved the expression rate of the recombinant lipase, is more effective than DO-stat for lipase production in the induction phase.

However, a rapid ROL decay was observed in these values. To the best of our knowledge, a systematic study of operational strategies to reduce proteolytic degradation has not been reported. Three remedial measures have been tried as ways to circumvent proteolytic degradation of the recombinant protein. Medium composition, pre-induction osmotic stress, and temperature were found to have the greatest influence on ROL production. The high yield and high enzyme activity would thus validate our method as an economical and feasible method for lipase production in *P. pastoris*.

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