African Journal of Biotechnology Vol. 10(80), pp. 18503-18512, 14 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2527
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Combined strategies for the improvement of heterologous expression of a His-tagged *Yarrowia lipolytica* lipase Lip2 in *Pichia pastoris*

Xiaofeng Wang, Xuguang Shen, Heyun Zhao, Yongchuan Sun, Tao Liu, Yun Liu, Li Xu and Yunjun Yan*

Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, P. R. China.

Accepted 28 October, 2011

Yarrowia lipolytica **lipase Lip2 (YlLip2) is an important biocatalyst for ester synthesis, biodiesel production and enantiomer resolution. The YlLip2 with an N-terminal histidine-tag (His_6-YlLip2) was successfully expressed in *Pichia pastoris*. Three different cultivation strategies had been compared for the production of His_6-YlLip2 by *P. pastoris* using a 10-l bioreactor. The results showed that His_6-YlLip2 activity and cell viability could be greatly improved by employing the combined strategies. Using a low salt medium (LSM) instead of the basal salt medium (BSM) and lowering the temperature from 28 to 25°C, the maximum His_6-YlLip2 activity and volumetric productivity were respectively increased by 55.3 and 79.8%. The production of His_6-YlLip2 and cell viability was further improved by combining sorbitol co-feeding with methanol. In this culture strategy, the maximum activity of His_6-YlLip2 reached 15,600 U/ml after 114 h of induction. The cell mortality decreased by 11.2% (while the control decreased about 27.6%) after 120 h methanol induction. The N-terminal histidine-tag brought convenience to purification. The molecular weight of His_6-YlLip2 was about 38 kDa. The pure His_6-YlLip2 presented a specific activity of 4,830 U/mg when olive oil was used as the substrate.**

Key words: *Pichia pastoris*, *Yarrowia lipolytica*, combined strategies, fed-batch culture, purification, lipase.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a class of enzymes, which catalyze the hydrolysis of triglycerides over an oil-water interface (Treichel et al., 2010). They play an important role in a number of industrial applications, such as detergent, food, fine chemistry, flavour, esters and amino acid derivatives, pharmaceuticals and energy industry (Hasan et al., 2006).

Yarrowia lipolytica could produce several types of lipases when the oils are present in the culture medium, including extracellular, cell-bound and intracellular lipases (Yu et al., 2007). YlLip2 is responsible for the most extracellular lipase activity (Pignede et al., 2000). YlLip2 gene *lip2* (GenBank Accession No. DQ831123) has 1005 bp, encoding 334 amino acid residues, where the first 33 amino acids act as a signal peptide (Yu et al., 2007). YlLip2 shows high activity in hydrolysis, esterification and transesterification reactions, which has been applied to ester synthesis (He et al., 2002), biodiesel synthesis (Deng et al., 2003) and enantiomer resolution (Cancino et al., 2008). So far, Pignede et al. (2000) and Fickers et al. (2005) have successfully achieved a fairly high-level homologous expression of YlLip2 in *Y. lipolytica* by lipid induction. However, the residual oleic acid used for

*Corresponding author. E-mail: yanyunjun@mail.hust.edu.cn. Tel/ Fax: 86-27-87792213.

Abbreviations: BSM, Basal salt medium; **LSM,** low salt medium; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PCR,** polymerase chain reaction; **Mut$^+$,** Methanol utilization-plus transformants; **YlLip2,** *Yarrowia lipolytica* lipase Lip2; **His6-YlLip2,** *Yarrowia lipolytica* lipase Lip2 with an N-terminal of histidine-tag; **PTM1,** Pichia trace metals.

inducing *Y. lipolytica* lipase secretion was bound tightly to partial YlLip2 (Aloulou et al., 2007), which was a disadvantage to the purification process of YlLip2. In contrast, heterologous expression of the *lip2* gene in *P. pastoris* could obtain even higher production (Yu et al., 2007), and markedly reduce production cost. Moreover, heterologous expression of multiple copies of the *lip2* gene in *P. pastoris* combined with a lower temperature induction strategy yielded a much higher protein expression level of YlLip2. When using a titrimetric method with olive oil as the substrate, the activity of recombinant YlLip2 reached 11,000 U/ml in a 5-l bioreactor after 160 h of culture (Yu et al., 2010). However, they also reported that the YlLip2 activity resulted in 42,900 U/ml by a pH-stat method. The methods of determination and definitions of unit activities of lipases vary widely in the current literature (Fickers et al., 2005; Surribas et al., 2007; Yu et al., 2010). It is quite difficult to compare lipase activities from different laboratories because these activities differ markedly according to the different assay methods and substrates employed.

The methylotrophic yeast *P. pastoris* is widely used for the production of various recombinant heterologous proteins (Macauley-Patrick et al., 2005). Until now, over 20 microbial lipase genes have been successfully expressed in this yeast. The productivity and the product quality could be considerably improved by careful control of the cultivation process (Jahic et al., 2006b). Lowering culture temperature in methanol induction phase could be a simple and potentially effective method to increase protein production (Jahic et al., 2006a). Previous studies had shown that low temperature induction resulted in higher cell viability, secreting less proteases, decreasing heterologous protein degradation, little folding stress but strengthened alcohol oxidase (AOX) expression (Dragosits et al., 2009; Surribas et al., 2007; Wang et al., 2009). High osmotic pressure of the standard basal salt medium (BSM) used for *P. pastoris* cultures was detrimental to the cell growth and viability. Meanwhile, the release of a lipid-like compound to the culture supernatant prevented efficiently downstream processing of the heterologous protein (Brady et al., 2001; Surribas et al., 2007). Lowering the salt concentration of BSM medium could reduce cell lysis and protease release, and increase expression level and recombinant proteins purity (Jahic et al., 2006b; Zhao et al., 2008). Sorbitol co-feeding with methanol for the production of recombinant proteins with *P. pastoris* strains could improve the yield, decrease the cell mortality and reduce the proteolytic degradation (Wang et al., 2010).

In this work, the recombinant YlLip2 with a 6×His-tag was functionally expressed in *P. patoris* in a 10-l scale biorector using combined fermentation strategies, and the protein was purified with a Ni-NTA affinity chromatography.

The aim of this work is to improve the His_6-YlLip2 production and decrease the production cost.

MATERIALS AND METHODS

Strain and plasmid

P. pastoris GS115 (*his4*), the pPIC9K and pPICZαA vectors were purchased from Invitrogen (Carlsbad, CA, USA). Professor Ma from Institute of Microbiology, the Chinese Academy of Science, presented us the *Y. lipolytica* CGMCC 2.1405 strain, which was deposited in China General Microbiological Culture Collection Center (CGMCC). *Escherichia coli* Top10F' (Invitrogen, USA) was used as a host for DNA manipulation.

Plasmid construction and transformation

The mature lipase gene *lip2* without 99 bp sequence encoding a signal peptide was amplified from the genomic DNA of *Y. lipolytica*. For the facility of downstream purification, a sequence coding for a histidine tag was introduced before the triplet encoding the first Val residue of the wild-type enzyme. The amplification primers containing the restriction sites for EcoR? and Not? was designed as: Lip2F (5'-GACGAATTCCATCATCATCATCATCATGTGTACA-CCTCTACCGA-3'), Lip2R (5'-TATAGCGGCCGCTTAGATACCAC-AGAC ACCCTCGG-3'). The fragments carrying a 6×His-tag and *lip2* gene was obtained by the double digestion with EcoR*I* and *NotI*, then they were ligated into pPIC9K and pPICZαA vectors linearized with the same enzymes, resulting in pPIC9K-*lip2*H (Figure 1A) and pPICZA-*lip2*H (Figure 1B). Finally, the recombinant expressing vectors pPIC9K-*lip2*H and pPICZA-*lip2*H were used to transform *E. coli* Top10F'. Positive clones were selected and sequenced to confirm their correct sequences. *P. pastoris* GS115 was respectively transformed with 10 µg of *Bst*X*I*- linearized pPICZA-*lip2*H and pPICZαA vectors by electroporation according to Invitrogen's recommendations. Transformed colonies were denoted as GS/ZA-lip2H and GS/pPICZαA. Screening and isolation of *P. pastoris* Mut^+ transformants were followed by *Pichia* expression kit manual. Positive clones were initially selected by YPDS medium plates (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, 1 M sorbitol, and 20 g/l agar) containing 100 µg/ml Zeocin. Then, multiple-copy transformants were further screened by YPDS resistance plates containing Zeocin at a final concentration of 0.5, 1 and 2 mg/ml. The high Zeocin resistance clones were screened by BMMY-rhodamine B-olive oil medium plates containing 0.008% (w/v) rhodamine B and 1% (v/v) emulsified olive oil. Thirty randomly picked clones on the BMMY-rhodamine B-olive oil medium plates were selected according to the size of the halos that formed around the colonies, and then checked their lipase activities by shaking flask fermentation.

A clone of GS/ZA-lip2H exhibiting the maximum lipase activity in shaking flask was chosen as a host for the transformation of *Sal*I-linearized pPIC9K-*lip2*H vector, and the resultant strain was denoted as GS/9KZA-*lip2*H. The *Sal*I-linearized pPIC9K vector was transformed into GS/ZA-lip2Hto generate GS/ZA*lip2*H-pPIC9K. Both GS/pPICZαA and GS/ZA*lip2*H-pPIC9K were used as control strains during the experiments. Screening and isolation of Mut^+ phenotype and multiple-copy transformants were followed by *Pichia* expression kit manual. The high G418 resistance clones were screened by BMMY-rhodamine B-olive oil medium plates as described above. Transformed colonies were confirmed by PCR and sequencing. Thirty randomly picked clones of GS/9KZA-*lip2*H were selected to check their lipase activities by shaking flask fermentation. A clone of GS/9KZA-*lip2*H exhibiting the maximum lipase activity was chosen as the host for fed-batch culture.

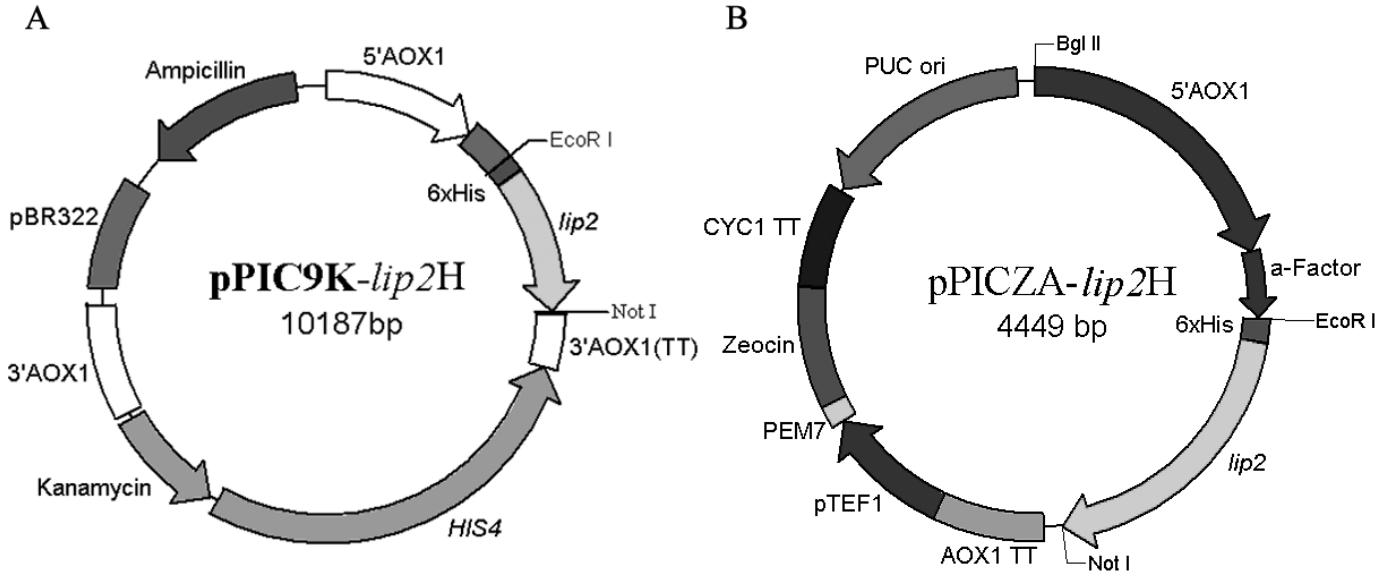

Figure 1. Schematic diagram of the *Pichia* expression plasmids pPIC9K-*lip2*H (A) and pPICZA-*lip2*H (B).The plasmid pPIC9K-*lip2*H and pPICZA-*lip2*H were created by inserting *Y. lipolytica lip2* gene into pPIC9K and pPICZαA, respectively.

Shake flask cultivations

The growth and induction media were BMGY (10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate buffer, pH 6.0, 13.4 g/l yeast nitrogen base without amino acids, 400 µg/l biotin, 10 g/l glycerol) and BMMY(10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate buffer, pH 6.0, 13.4 g/l yeast nitrogen base without amino acids, 400 µg/l biotin, 5 ml/l methanol), respectively. A single colony of selected recombinant was inoculated into an Erlenmeyer flask containing 50 ml BMGY and was grown at 30°C. After 24 h, cells were harvested by centrifugation and resuspended in another flask containing 50 ml BMMY. Methanol (1.0%, v/v) was added to the Erlenmeyer flask every 24 h for five days. The positive strains of GS/ZA-lip2H were grown in BMGY and BMMY mediums containing 0.004% (w/v) histidine. The effects of various induction temperatures (20, 22, 25, 28 and 30°C) and pH values (4.5, 5.0, 6.0, 6.5, 7.0 and 7.5) on His_6-YlLip2 production were tested by using a single-factor experiment. The activity of lipase was measured at 72, 96 and 120 h, respectively.

Fermentor cultivations

Inoculum was produced in a 500 ml shake flask with 75 ml BMGY medium. Cells were grown for 16 to 18 h at 30°C on a shaker of 220 rpm, which resulted in an OD_{600} between 4 and 6. Then, 10% (v/v) of the inoculum were inoculated into a 10-l fermentor (BIOTECH-10JGZ, Baoxing Co., shanghai, China) with 4 l BSM or LSM. The standard BSM for *P. pastoris* containing per litre: 26.7 ml, 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, 40.0 g glycerol and 4.35 ml of PTM1 solution, which has an initial conductivity of about 40 mS cm^{-1}. The PTM1 solution contained per litre: $CuSO_4 \cdot 5H_2O$, 6.0 g; NaI, 0.08 g; $MnSO_4 \cdot H_2O$, 3.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.3 g; and H_2SO_4 concentrated, 5 ml. Low salt medium (LSM) medium has an initial conductivity of about 20 mS cm^{-1}, containing per litre (Brady et al., 2001): $(NaPO_3)_6$, 10.83 g; $CaSO_4 \cdot 2H_2O$, 0.38 g; K_2SO_4, 7.58 g, $MgSO_4 \cdot 7H_2O$, 6.22 g, KOH, 1.72 g, glycerol, 40 g and 4.35 ml of PTM1 solution. The $(NaPO_3)_6$ and PTM1 solutions were added sterile filtered after bioreactor sterilization. To prevent salt deficiency, a salt feed solution was pumped into the culture broth to keep a constant conductivity around 8 mS cm^{-1}. This feed solution contained per litre (Surribas et al., 2007): $CaSO_4 \cdot 2H_2O$, 2.63 g; 85% phosphoric acid, 10 ml; K_2SO_4, 45.5 g; $MgSO_4 \cdot 7H_2O$, 37.3 g; KOH, 10.3 g and $(NaPO_3)_6$, 65 g.

Temperature was set at 30°C at glycerol batch and fed-batch phase, and then decreased to 28°C at the beginning of the methanol induction phase unless stated otherwise in some operational strategies. The pH was controlled at 6.0 by addition of 28% (*w/w*) ammonium hydroxide. The dissolved oxygen (DO) level was maintained over 25% of air saturation by a cascaded control of agitation rate at 500 to 800 rpm and airflow rate at 150 to 400 l/h. Foaming was controlled by the automatic addition of antifoam (Dowfax DF103, USA). Once glycerol was exhausted from culture, as indicated by an increase of DO level, the transition phase was started, which consisted in a 6 h glycerol exponential phase at a growth rate of 0.16 h^{-1}. The fed-batch feeding medium was pumped into the fermentor according to a predetermined protocol. The composition of the fed-batch feeding medium: 12 ml PTM1 solution was added to 1 L of glycerol (50%, v/v); 12 ml PTM1 solution was added to 1 L of pure methanol.

Culture process setting in methanol induction phase

After depletion of the glycerol, methanol was fed to start the induction phase. Three different induction strategies were employed in His_6-YlLip2 expression phase, denoted strategy 1, 2 and 3. In strategy 1, using BSM medium and the temperature was maintained at 28°C in methanol induction phase. In strategy 2, using LSM as the medium and the temperature was maintained at 25°C in methanol induction phase. In strategy 1 and 2, methanol limited fed-batch was conducted by following a standard method as *Pichia* fermentation process guidelines described (Invitrogen). In strategy 3, 50% sorbitol (w/v, containing 12 ml/L of a PTM1 solution) and methanol mixed in the ratio of 1:1 (*v/v*) as the fed-batch medium, and the other conditions were the same as the strategy 2.

Biomass analysis

Cell density was expressed as grams dry cell weight (DCW) per liter broth, which was obtained by centrifuging 10 ml samples in a pre-weighted sample tube at 5,000 *g* for 15 min, and then the pellet was dried at 105°C to constant weight.

Lipase activity assay and protein concentration

The lipase activity in the culture filtrate was determined by titrimetry method (Kojima and Shimizu, 2003). The reaction mixture was added in 50 ml Erlenmeyer flask, consisting of 4 ml of substrate (25% olive oil emulsified with 2% polyvinyl alcohol solution), 5 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, and 1 ml of proper dilute enzyme solution. Then, the reaction mixture was incubated at 40°C for 10 min under agitation conditions. The reaction was stopped by addition of 15 ml of cold acetone/ethanol (1/1, v/v). The amount of fatty acid liberated was determined by titration with 50 mM NaOH using phenolphthalein as an indicator. One enzyme unit (U) was defined as the activity that liberated 1 µmol of free fatty acid per minute under the assay conditions. Protein content was determined spectrophotometrically according to the Bradford method using bovine serum albumin (BSA) as standard (Braford, 1976).

Measurement of cell viability

The measurement of cell viability was performed by methylene blue dye exclusion technique as described by Sinha et al. (2005). Fermentation samples were taken at regular intervals. A suitably diluted cell suspension (approximate OD_{600} = 20 to 30) was mixed with an equal volume of methylene blue dye solution (0.1 g/l of methylene blue, 20 g/l of trisodium citrate) for 1 min and then mounted on a hemocytometer to count the percentage of live cells in the total population. Cells which took up methylene blue and appeared deep blue were considered as dead compared to live cells, which appeared translucent. At least 300 cells were counted for each aliquot.

SDS–PAGE and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide gel on a vertical mini gel apparatus (Bio-Rad, USA) at 120 V for 1.5 h. Molecular weight marker was purchased from Fermentas (Canada) and TaKaRa (Japan). Proteins were stained with Coomassies Brilliant Blue R-250 (Amresco, USA). For Western blot analysis, proteins were transferred onto nitrocellulose membranes and immuno-logically detected with a mouse monoclonal IgG2b anti-His antibodies (Tiangen, China) as described by the manufacturer.

His_6-YILip2 purification

The cell culture medium (100 ml) was obtained by a refrigerated centrifuge (KUBOTA 7700, Japan) at 4°C and 8,000 *g* for 15 min. The supernatant was concentrated and interchanged with 25 mM Tris–HCl buffer (pH 7.5) by ultrafiltration using a 10 kDa membrane (Millipore, USA). Then, the 10 ml crude sample was loaded into a Ni-NTA Superflow Columns (Qiagen, CA, USA) containing 1.5 ml of Ni-NTA resin and washed with NTA buffer. 0.1% of Triton X-100 in NTA buffer was added to maintain the stability of the protein. The lipase was eluted with a linear gradient of 0 to 500 mM imidazole in the NTA-0 buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 10% glycerol).

RESULTS AND DISCUSSION

Selection of lipase producing clones

Thirty randomly picked clones of GS/ZA-lip2H were selected to check the lipase production. In shaking flask, the lipase activity of His_6-YILip2 reached its highest value after 96 h of methanol cultivation (data not shown). A clone was denoted as GS/ZA-lip2H 9#, exhibiting the maximum lipase activity of 950 U/ml. This clone was chosen as the host for the transformation of *SalI*-linearized pPIC9K-*lip2*H vector. Thirty randomly picked clones of GS/9KZA-*lip2*H were also selected to measure the lipase production in shaking flask. The maximum lipase activity reached 1,350 U/ml after 96 h culture by a clone of GS/9KZA-*lip2*H 26#. Yu et al. (2007) reported that the lipase YILip2 with a C-terminal of histidine-tag showed low activity (different clones varied between 0 and 57 U/ml) and were rather unstable. Nevertheless, YILip2 with an N-terminal of histidine-tag showed much higher activity than that of YILip2 with a C-terminal of histidine-tag, and were fairly stable in the fermentation supernatant. The reason is probably that the C-terminal is closer to the catalytic triad of YILip2 than N-terminal, which may disturb the correct folding of the lipase and the contact of lipase and substrate when the histidine-tag is added. In consideration of easy purification of the recombinant lipase, a clone of GS/9KZA-*lip2*H 26# exhibiting the maximum lipase activity in shaking flask was chosen for bioreactor cultivations.

Effect of induction temperature on heterologous His_6-YILip2 production

Temperature is one of the most important parameters for optimization of heterologous proteins expressed in *P. pastoris* (Dragosits et al., 2009). To evaluate the effect of induction temperature on His_6-YILip2 production by *P. pastoris* in shaking flask, the temperature in the range of 20 to 30°C was employed at pH 6.0. After 96 h methanol induction, the maximum enzyme production was observed in 25°C (Figure 2). The production of His_6-YILip2 reduced significantly when incubating at 20°C and 30°C, but the temperature had a little effect on biomass. The possible explanation is that the increase of central carbon metabolism fluxes to the production of His_6-YILip2 and the decrease of folding stress to recombinant *P. pastoris* cells at 25°C. Until now, several authors have reported that the production could be improved by lowering the methanol induction temperature in *P. pastoris* bioreactor cultures (Dragosits et al., 2009; Jahic et al., 2003; Surribas et al., 2007; Wang et al., 2009). Furthermore, the lower cell lysis and proteolytic activity were usually obtained by lowering the methanol induction temperature.

Yu et al. (2010) also reported that expression of this lipase was enhanced at 25°C. However, the mechanisms

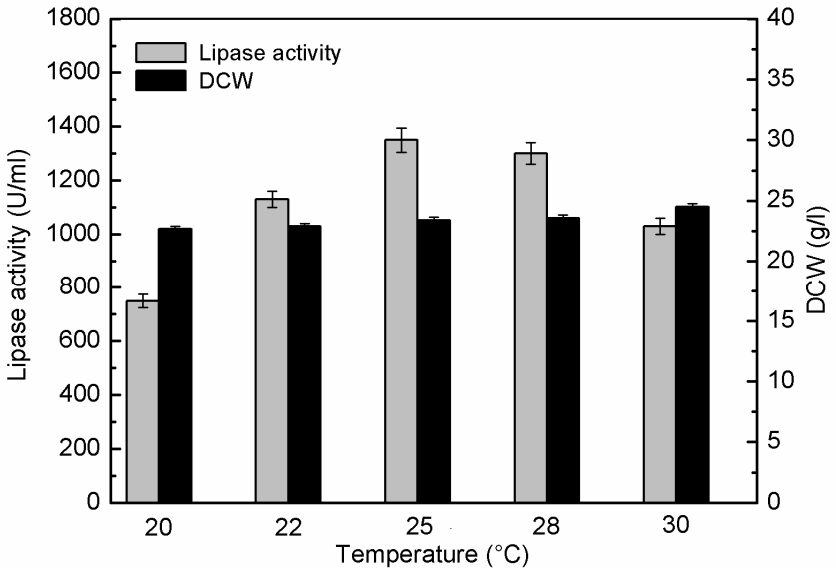

Figure 2. Effect of temperature on cell growth and lipase activity in shake flask cultures after 96 h of incubation at temperatures varying from 20 to 30°C. Data are expressed as means ± S.D. of triplicate determinations.

behind the effect of induction temperature on cell growth and recombinant protein expression may be more complex. Dragosits et al. (2009) reported that the proteolytic activity did not change upon a temperature shift from 30 to 20°C. Inna et al. (1999) found that the temperature did not have any significant effect on the production of recombinant hookworm anticoagulant peptide by *P. pastoris*. Many important cellular processes, including the central carbon metabolism, protein folding and stress response are affected by changing the induction temperature (Dragosits et al., 2009).

Effect of induction pH on heterologous His_6-YILip2 production

The effects of different medium initial pH values ranging from 4.5 to 7.5 at 25°C on cell biomass and the production of His_6-YILip2 were examined by shaking flask culture (Figure 3). Different initial pH values had no conspicuous effect on cell growth at 5 to 7.5 but His_6-YILip2 production was higher at pH 6.0 to 7.5. It was shown that extreme low pH value had a negative effect on cell density and YILip2 production (Figure 3). The maximum His_6-YILip2 activity of 1,390 U/ml was obtained at pH 6.5 after 96 h of cultivation, but the maximum biomass reached at 24.4 g/l at pH 6.0. *P. pastoris* can grow across a relatively broad pH range (3.0 to 7.0) that does not affect the growth significantly (Macauley-Patrick et al., 2005). Although neutral proteases were inhibited when lowered the pH of the fermentation medium in methanol induction phase, different pH values were found to be optimal from the point of view of a recombinant protein's stability. YILip2 could remain 100% activity after 15 h incubation at 25°C at pH 5 to 8 (Yu et al., 2007). The residual activity of YILip2 decreased rapidly when pH was beyond this range. That is why the maximum His_6-YILip2 activity was achieved at a high initial pH range.

Effects of different fermentation strategies on His6-YILip2 production

The high cell density fermentation process is usually affected by several factors, including the fermentation medium, pH, temperature, DO level and methanol fed-batch strategies. However, only a few systematic investigations have reported the impact of multiplicate operational strategies on the expression of lipase in *P. pastoris* (Surribas et al., 2007).

The time courses of DCW and His6-YILip2 production during the different cultivation strategies are shown in Figure 4. The maximum His6-YILip2 activity reached 7,600 U/ml after 132 h methanol induction under the induction condition of strategy 1 (Figure 4A). Although BSM medium as mentioned above is the most commonly used medium for the high cell density culture of *P. pastoris*, it may not be optimal for the production of every heterologous protein, and cause some serious problems, such as precipitation, high osmotic pressure and unbalanced composition (Ghosalkar et al., 2008). Therefore, it is important to use a low ionic strength and suitable medium for recombinant protein production process in *P. pastoris*. When LSM medium was used as the initial fermentation medium and lowing the temperature to 25°C in the methanol induction phase

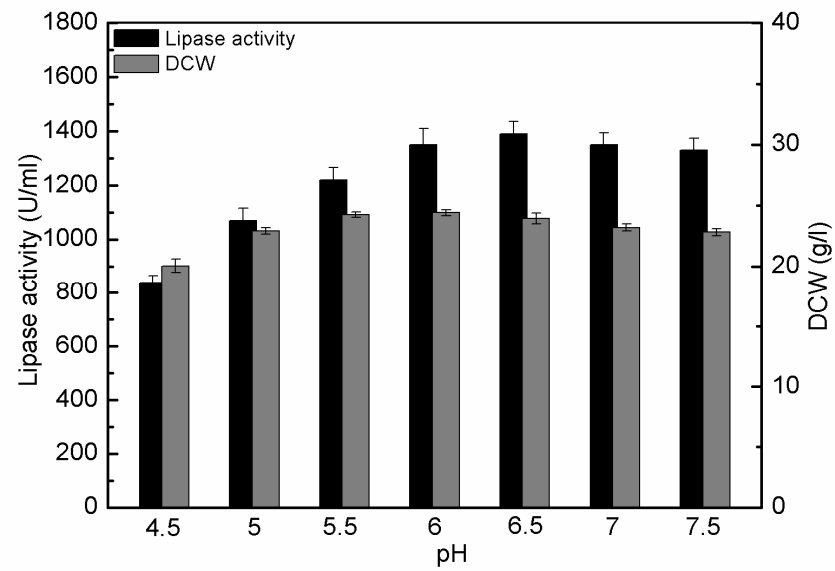

Figure 3. Effect of pH on cell growth and lipase activity in shake flask cultures after 96 h of incubation at pH values varying from 4.5 to 7.5. Data are expressed as means ± S.D. of triplicate determinations.

(strategy 2), the maximum His6-YlLip2 activity reached up to 11,800 U/ml after 114 h methanol induction (Figure 4B). The maximum His6-YlLip2 activity and volumetric productivity were respectively increased by 55.3 and 79.8% compared with those of strategy 1 (Table 1), indicating that the LSM and low temperature had a significant impact on His6-YlLip2 production.

Sorbitol is a non-repressing carbon source with respect to AOX1 promoter (Ramon et al., 2007). Sorbitol as co-carbon source added together with methanol is an effective strategy to increase cell density and process productivity, as well as to shorten the induction time (Arnau et al., 2010). To further improve His6-YlLip2 productivity, a combined strategy using sorbitol co-feeding with methanol based on the strategy 2 was employed in methanol induction phase. The maximum His6-YlLip2 activity reached 15,600 U/ml after 114 h of methanol induction in strategy 3 (Figure 4C). Compared with strategy 1, the maximum His6-YlLip2 activity, lipase yield on biomass ($Y_{lipase/X}$) and volumetric productivity of strategy 3 were enhanced 2.05, 2.04 and 2.38 folds (Table 1), respectively. These results suggested that the combined cultivation strategies used in methanol induction phase was an efficient method for His6-YlLip2 production.

Effects of different fermentation strategies on cell viability

Metabolic and environmental stress situations can lead to a decrease in culture viability, which can result in lower productivity or cell lysis (Surribas et al., 2007). Therefore, it is important to assess cell viability during culture. The methylene blue dye exclusion technique was employed to investigate the effect of the different induction temperature on cell viability. After 120 h methanol induction, the cell viability remained below 73% of the culture with strategy 1, and respectively above 80 and 88% in the cultivation with strategy 2 and 3 (Figure 5). Thus, the data indicate that the lower temperature and low salt mediums are an advantage of cell viability. Moreover, an even low level of cell death was observed according to combined appropriate sorbitol co-feeding strategy. This explains why His6-YlLip2 volumetric productivity could be enhanced 2.38-fold compared with that of the strategy 1. A possible cause was mixed feeds of sorbitol and methanol could alleviate methanol toxicity and reduce the metabolic burden, which affects the secretion and the energetic state of cells caused by the overexpression of heterologous proteins (Ramon et al., 2007).

His-tagged YlLip2 purification

For many commercial bioprocesses, the recovery and purification of the target product are economically essential, because downstream processing may constitute up to 50% of the total manufacturing cost (Surribas et al., 2007). Purification of protein was facilitated by the presence of six histidine residues at the N-terminus of the lipases. Moreover, it was convenient to a specific surface immobilization of proteins on nickel-chelating materials.

The rising activity during the fermentation of strategy 3 was shown by SDS–PAGE (Figure 6A). The recombinant

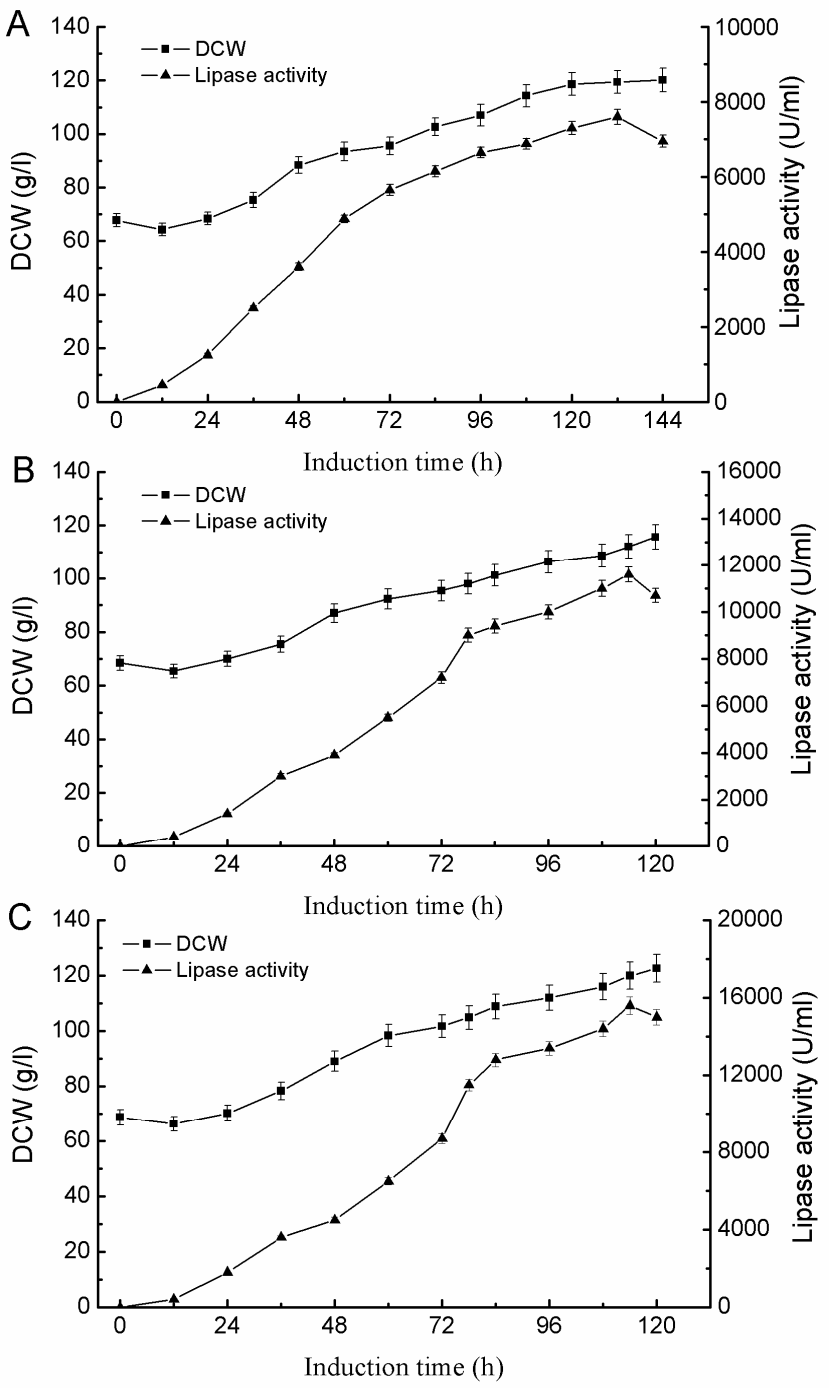

Figure 4. Time courses of DCW and His_6-YlLip2 activity during the cultivation strategy 1 (A), 2 (B) and 3 (C). In strategy 1, using BSM medium and maintaining the induction temperature at 28℃. In strategy 2, using LSM medium and maintaining the temperature at 25℃. In strategy 1 and 2, using methanol limited fed-batch strategy. In strategy 3, mixed feeds of 50% sorbitol (w/v, containing 12 ml of a PTM1 solution) and methanol in the ratio of 1:1 (v/v), and the other conditions were the same as strategy 2. Data are expressed as means ± S.D. of triplicate determinations.

His_6-YlLip2 had a relative high purity in the supernatant of culture. The result of SDS–PAGE analysis of the purified lipase showed a single band (Figure 6B) and was verified by Western blotting analysis (Figure 6C). The molecular

Table 1 Comparison of His_6-YlLip2 production by *P. pastoris* fermentation using different operational strategies.

Produced substances	Culture Strategy		
	1	2	3
Lipase activity (U/ml) [a]	7600	11800	15600
$Y_{lipase/X}$ (U/g) [a]	63,652	105,263	130,000
Volumetric productivity (U l^{-1} h^{-1}) [a]	57,576	103,508	136,842
Total protein concentration (g/l) [a]	2.38	3.48	4.06
Protein specific activity (U/mg) [a]	3193	3391	3842

[a] Calculated at the maximal mean lipolytic activity reached.

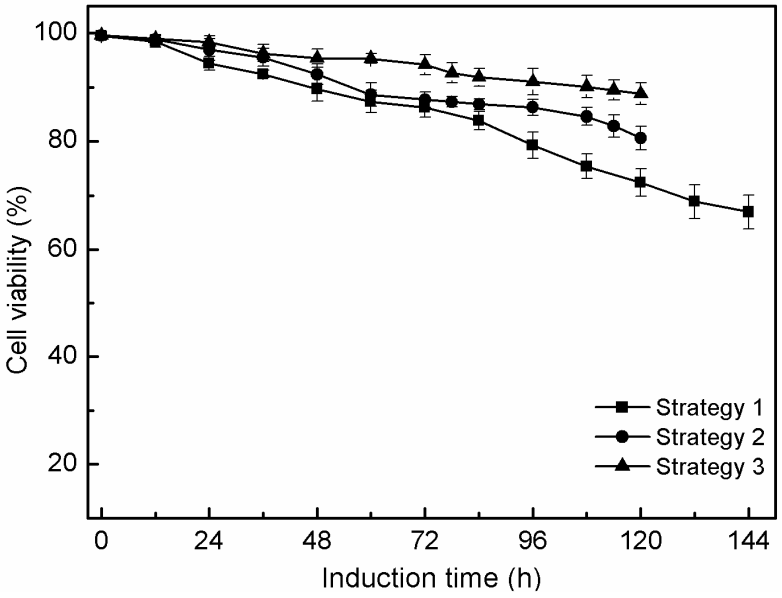

Figure 5. Time courses of cell viability profiles of *P. pastoris* measured during the cultivation. Data are expressed as means ± S.D. of triplicate determinations.

weight of His_6-YlLip2 was about 38 kDa, similar to the mature enzyme from *Y. lipolytica*. The pure His_6-YlLip2 produced with strategy 3 presents a specific activity of 4,830 U/mg when olive oil was used as the substrate.

Due to the heterogeneous characters of lipase, there are a lot of different techniques of the enzyme assay. Therefore, the determination method and definition unit of lipases are very confused in the current literature (Pignede et al., 2000; Fickers et al., 2005; Yu et al., 2007). So, it is hard to compare lipase activities from different authors because lipase activities differ markedly with different activity assay methods they employed. Yu et al. (2007) reported that the specific activity of the purified YlLip2 was 19,880 U/mg with a pH-stat method. However, we employed the classical olive oil titrimetry method for enzyme activity assay in order to compare our results with those of other researchers easily. In a recent paper, Yu et al. (2010) had compared the above two methods in determination of lipase activity. They reported that the value of lipase activity was about 4-folds higher when using the pH-stat method instead of traditional titrimetry method. The specific activity of purified His_6-YlLip2 was slightly lower than that of the free YlLip2 when compared with the same assay method. This observation might be due to the N-terminus histidine-tag that possibly had a negative effect on the three-dimensional structure of YlLip2. Nevertheless, the purification and immobilization of His_6-YlLip2 was very simple, which suggested that this lipase can be produced in large-scale and used as an industry biocatalyst.

Conclusions

In summary, the His_6-YlLip2 was successfully expressed

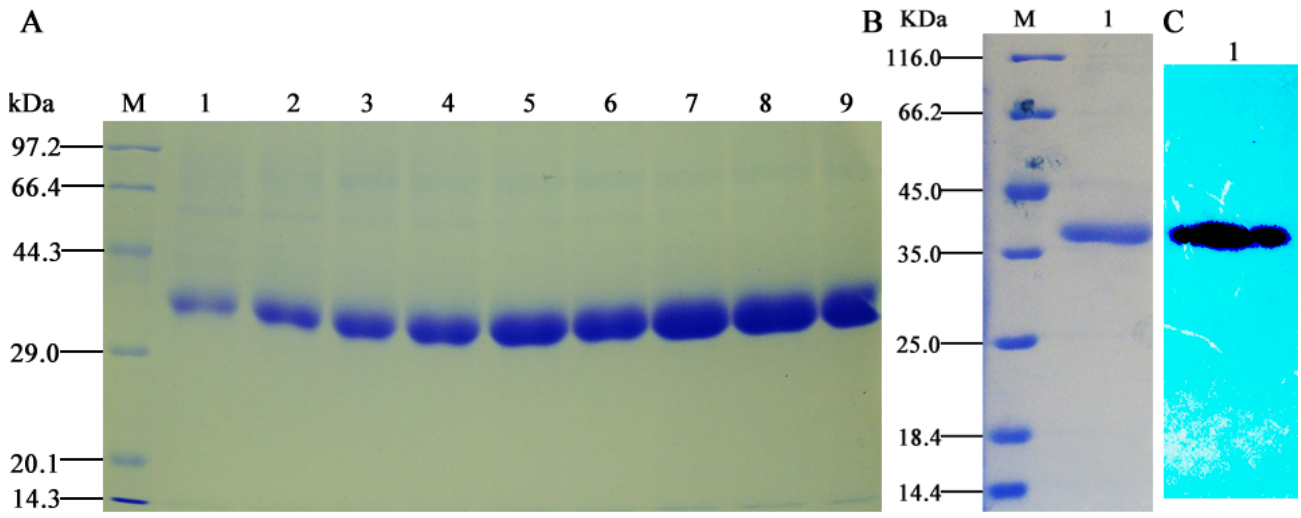

Figure 6. SDS–PAGE and Western blot analysis of the recombinant His_6-YlLip2 expressed in *P. pastoris*. (A) SDS–PAGE (12%) of culture supernatant during the fermentation using strategy 3. Lane M, low molecular weight marker (TaKaRa); lanes 1–9, 10 µL of culture supernatant of 24, 36, 48, 60, 84, 96, 108, 114 and 120 h, respectively. (B) SDS–PAGE (12%) analysis of purified His_6-YlLip2. Lane M, protein molecular weight marker (Fermentas); lane 1, 5 µL purified His_6-YlLip2; (C) Western blotting analysis for detection of purified His_6-YlLip2 using anti-His antibody. Lane 1, purified His_6-YlLip2.

in the *P. pastoris* expression system. It was found that different induction temperatures have significant effect on His_6-YlLip2 production in shaking flask. The maximum His_6-YlLip2 activity was achieved at a high initial pH range (6.0 to 7.5). The combined strategy was an efficient method, which was beneficial to both cell viability and the His_6-YlLip2 production. Moreover, the recombinant His_6-YlLip2 was easy to be purified and showed relatively high specific activity. Therefore, the above results suggest that using combined strategies in the methanol induction phase of recombinant *P. pastoris* might have a good perspective for improving other industry enzymes productions.

ACKNOWLEDGEMENTS

This work was funded by National High Technology Research and Development Program of China (863 Program) (Nos, 2009AA03Z232, 2010AA101501, 2011AA02A204), Program for New Century Excellent Talents in University (NCET-07-0336), and Natural Science Foundation of Hubei Province (2009CDA046).

REFERENCES

Aloulou A, Rodriguez JA, Puccinelli D, Mouz N, Leclaire J, Leblond Y, Carriere F (2007). Purification and biochemical characterization of the LIP2 lipase from *Yarrowia lipolytica*. Biochim. Biophys. Anta. 1771(2): 228-237.

Arnau C, Ramon R, Casas C, Valero F (2010). Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. Enzyme Microb. Tech. 46(6): 494-500.

Braford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Brady CP, Shimp RL, Miles AR, Whitmore M, Stowers AW (2001). High-level production and purification of P30P2MSP1(19), an important vaccine antigen for malaria, expressed in the methylotropic yeast *Pichia pastoris*. Protein Expres. Purif. 23(3): 468-475.

Cancino M, Bauchart P, Sandoval G, Nicaud J, Andr I, Dossat V, Marty A (2008). A variant of *Yarrowia lipolytica* lipase with improved activity and enantioselectivity for resolution of 2-bromo-arylacetic acid esters. Tetrahedron: Asymmetry, 19(13): 1608-1612.

Deng L, Tan T, Wang F, Xu X (2003). Enzymatic production of fatty acid alkyl esters with a lipase preparation from *Candida sp*. 99-125. Eur. J. Lipid Sci. Tech. 105(12): 728-734.

Dragosits M, Stadlmann J, Albiol J, Baumann K, Maurer M, Gasser B, Sauer M, Altmann F, Ferrer P, Mattanovich D (2009). The effect of temperature on the proteome of recombinant *Pichia pastoris*. J. Proteome Res. 8(3): 1380-1392.

Fickers P, Fudalej F, Nicaud JM, Destain J, Thonart P (2005). Selection of new over-producing derivatives for the improvement of extracellular lipase production by the non-conventional yeast *Yarrowia lipolytica*. J. Biotechnol. 115(4): 379-386.

Ghosalkar A, Sahai V, Srivastava A (2008). Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass production. Bioresour. Technol. 99(16): 7906-7910.

Hasan F, Shah AA, Hameed A (2006). Industrial applications of microbial lipases. Enzyme Microb. Technol. 39(2): 235-251.

He X, Chen B, Tan T (2002). Enzymatic synthesis of 2-ethylhexyl esters of fatty acids by immobilized lipase from *Candida sp*. 99-125. J. Mol. Catal. B: Enzyme, 18(4-6): 333-339.

Inan M, Chiruvolu V, Eskridge KM, et al (1999). Optimization of temperature-glycerol-pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microb. Tech. 24(7): 438-445.

Jahic M, Knoblechner J, Charoenrat T, Enfors SO, Veide A (2006a). Interfacing *Pichia pastoris* cultivation with expanded bed adsorption. Biotechnol. Bioeng. 93(6): 1040-1049.

Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO (2006b). Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. Biotechnol. Prog. 22(6): 1465-1473.

Jahic M, Wallberg F, Bollok M, Garcia P, Enfors SO (2003). Temperature limited fed-batch technique for control of proteolysis in

Pichia pastoris bioreactor cultures. Microb. Cell Fact. 2(1): p. 6.
Kojima Y, Shimizu S (2003). Purification and characterization of the lipase from *Pseudomonas fluorescens* HU380. J. Biosci. Bioeng. 96(3): 219-226.
Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005). Heterologous protein production using the *Pichia pastoris* expression system. Yeast, 22(4): 249-270.
Pignede G, Wang HJ, Fudalej F, Seman M, Gaillardin C, Nicaud JM (2000). Autocloning and amplification of LIP2 in *Yarrowia lipolytica*. Appl. Environ. Microbiol. 66(8): 3283-3289.
Ramon R, Ferrer P, Valero F (2007). Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. J. Biotechnol. 130(1): 39-46.
Sinha J, Plantz BA, Inan M, Meagher MM (2005). Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-τ. Biotechnol. Bioeng. 89(1): 102-112.
Surribas A, Stahn R, Montesinos JL, Enfors SO, Valero F, Jahic M (2007). Production of a *Rhizopus oryzae* lipase from *Pichia pastoris* using alternative operational strategies. J. Biotechnol. 130(3): 291-299.
Treichel H, de Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV (2010). A Review on Microbial Lipases Production. Food Bioprocess Technol. 3(2): 182-196.
Wang Y, Wang Z, Xu Q, Du G, Hua Z, Liu L, Li J, Chen J (2009). Lowering induction temperature for enhanced production of polygalacturonate lyase in recombinant *Pichia pastoris*. Proc. Biochem. 44(9): 949-954.
Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, Chen J (2010). Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation. Bioresour. Technol. 101(4): 1318-1323.
Yu M, Lange S, Richter S, Tan T, Schmid RD (2007). High-level expression of extracellular lipase Lip2 from *Yarrowia lipolytica* in *Pichia pastoris* and its purification and characterization. Protein Expr. Purif. 53(2): 255-263.
Yu M, Wen S, Tan T (2010). Enhancing production of *Yarrowia lipolytica* lipase Lip2 in *Pichia pastoris*. Eng. Life Sci. 10(5): 1-7.
Zhao H, Xue C, Wang Y, Yao X, Liu Z (2008). Increasing the cell viability and heterologous protein expression of *Pichia pastoris* mutant deficient in *PMR1* gene by culture condition optimization. Appl. Microbiol. Biot. 81(2): 235-241.