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Research Article

Enhancing production of lipase MAS1 from marine *Streptomyces* sp. strain in *Pichia pastoris* by chaperones co-expression

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ABSTRACT

Background: A thermostable lipase MAS1 from marine *Streptomyces* sp. strain was considered as a potential biocatalyst for industrial application, but its production level was relatively low. Here, the effect of chaperones co-expression on the secretory expression of lipase MAS1 in *Pichia pastoris* was investigated.
Result: Co-expression of protein disulfide isomerase (PDI), HAC1 and immunoglobulin binding protein could increase the expression level of lipase MAS1, whereas co-expression of *Vitreoscilla* hemoglobin showed a negative effect to the lipase MAS1 production. Among them, PDI co-expression increased lipase MAS1 expression level by 1.7-fold compared to the control strain harboring only the MAS1 gene. Furthermore, optimizing production of lipase MAS1 with *Pichia pastoris* strain X-33/MAS1-PDI in a 30-L bioreactor were conducted. Lower induction temperature was found to have a benefit effect for lipase MAS1 production. Lipase activity at 24 and 22°C showed 1.7 and 2.1-fold to that at 30°C, respectively. Among the induction pH tested, the highest lipase activity was obtained at pH 6.0 with activity of 440 U/mL after 144 h fermentation.
Conclusion: Our work showed a good example for improving the production of recombinant enzymes in *Pichia pastoris* via chaperon co-expression and fermentation condition optimization.

1. Introduction

Enzymes play a key role in the "white biotechnology" due to their properties of high catalytic efficiency, substrate specificity, reaction under mild condition and environment-friendliness. Thus, they have found many applications in biotechnology industry, including detergents, cosmetics, foods, pharmaceuticals and flavor industry [1]. Currently, enzymes from microorganisms are attracting enormous attention because they are stable and can be obtained in bulk at low cost [2].

Although enzymes are great of value in industrial application, their yields are relative low via fermentation of original hosts. An alternative choice is production of recombinant enzymes in a heterologous host. Currently, several microorganism, including *Aspergillus oryzae*, *Aspergillus niger*, *Pichia pastoris (P. pastoris)*, *Saccharomyces cerevisiae* and *Escherichia coli*, have been developed for heterologous proteins production [3]. Among them, the methylotrophic yeast *P. pastoris* was considered as an excellent host for various proteins production, dues to its potential for high-level production, efficient secretion, high cell density growth and posttranslational modification. Several heterologous proteins, such as human serum albumin, have been reported to produce at grams per liter level by *P. pastoris* [4]. However, secretion level of some other proteins was in a relatively low level [5]. Therefore, strategies for elevating expression level of those proteins in *P. pastoris* are urgently needed.

Generally, over expression of recombinant proteins in eukaryotic cells, e.g., *P. pastoris*, might result in increasing production of unfold or misfolded proteins. The accumulation of those function-loss proteins in the endoplasmic reticulum (ER) could cause the activation of the unfolded protein response (UPR), which seriously affect the secretion and transportation of proteins [6]. Some cellular chaperones were found to have the functions for releasing the UPR stress to the cells. Protein disulfide isomerase (PDI) and immunoglobulin binding protein located in ER involve in formation of disulfide bond and refolding of protein [7], while HAC1 is a transcription factor for activating target genes coding for chaperones and foldases when UPR occurs [8]. Thus, co-expression of those chaperones with the target gene seems to be an effect strategy for enhancing the expression level of recombinant proteins [9,10].

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Table 1
Primers used for construction of chaperon expression vectors.

Gene name	Gene length (bp)	Primer name	Primer sequences[a]
*mas*1	780	MAS1-F	5′-CGGAATTCGCCACGCCAGCTGCTGAGGCTACTT-3’
(H0B8D4)		MAS1-R	5′-CCGGTACCGCCAATCACAGAAGCACAGGTTGTA-3’
pdi	1554	PDI-F:	5′-AAACAACTAATTATTCGAAGGATCCAAACGATGCAATTCAACTGGAAT-3’
(EU 805807)		PDI-R:	5′-TAATTCGCGGCCGCCCTAGGGAATTCTTAAAGCTCGTCGTGAGCGTC-3’
*hac*1	996	HAC1-F	5′-CGCGGATCCATGCCCGTAGATTCTTCTC-3’
(XM 002489994.1)		HAC1-R	5′-CGGAATTCCTATTCCTGGAAGAATACAAAGTC-3’
bip	2037	Bip-F	5′-CGCGGATCCATGCTGTCGTTAAAACCATCTT-3’
(XM 002490982)		Bip-R	5′-ATTGCGGCCGCCTACAACTCATCATGATCATA-3’
vhb	441	Gene synthesis	
(AY 278220)			

[a] The restriction sites in the primers were underline.

Enhancement of microorganism hosts productivity can also be achieved by fermentation conditions optimization. Effects of carbon source, nitrogen source, dissolved oxygen (DO), induction temperature, pH and methanol concentration on the production of recombinant proteins have been extensively studied [11,12,13]. Among these fermentation factors, fermentation temperature and pH are key factors affecting the recombinant protein production and cell growth. Both cell death and target protein degradation could be decreased under low fermentation temperature [14]. Wang et al. [15] had reported that activity of recombinant polygalacturonate lyase in *P. pastoris* was found to be higher at low fermentation temperature than that at high fermentation temperature [15]. The pH value can affect the activity of recombinant proteins and cell growth by changing their environment charge. Charoenrat et al. [16] had reported that the proteolysis activity could be inhibited by pH control to increase the production of recombinant fungal endoglucanase in *P. pastoris* [16].

In our previous study, MAS1 lipase from marine *Streptomyces sp*. strain W007 was characterized as a thermostable enzyme which might have potentials for industrial application [17]. However, the yield of MAS1, constitutively expressed under control of glyceraldehyde-3-phosphate dehydrogenase gene promoter in *P. pastoris*, was found to be low. In here, to improve the expression level of MAS1 lipase in *P. pastoris,* the effect of co-expression of chaperones gene and optimization of fermentation conditions on lipase MAS1 production in *P. pastoris* in a 30-L bioreactor was investigated.

2. Material and methods

2.1. Strains, plasmids and materials

E. coli DH5α was used as cloning host. The plasmid pPICZαA and pPIC9K (Invitrogen, Carlsbad, CA, USA) were used as cloning vector. *P. pastoris* X-33 (Invitrogen, Carlsbad, CA, USA) strain was used for expression. 4-Nitrophenyl octanoate was purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were of analytical grade.

2.2. Construction of expression strain

The *mas*1 gene was amplified using primers MAS1-F and MAS1-R (Table 1) with a previously constructed vector PGAPαA-MAS1 as template [17]. The PCR products were double digested with *Eco*RI and *Kpn*I, and ligated into the same sites in pPICZαA containing an AOX1 promoter to generate vector pPICZαA-MAS1. For construction of chaperones expression vectors, all the chaperone encoding genes were cloned into pPIC9K vector (Fig. 1b). PDI, HAC1 and BIP gene were obtained by PCR amplification using *P. pastoris GS115* genomic DNA as template. PCR product of *hac*1 and *bip* gene were digested by *BamH*I and *Eco*RI or *Not* I and ligated into the same site of pPIC9K, while PDI gene was cloned in the pPIC9K using In-Fusion™ cloning kit (Takara, Dalian, China) according to the instruction from the manufacturer. The *Vitreoscilla* hemoglobin (VHB) gene was synthesis by GENEWIZ biotechnology company (Shuzhou, China) and cloned into pPIC9Kvector. All the constructions were confirmed by fully sequencing.

P. pastoris X-33 was transformed with 10 μg of *Pme*I-linearized pPICZαA-MAS1 vector by electroporation to get X-33/MAS1. Transformants were selected by YPD medium plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, and 2% (w/v) agar) containing 100 μg mL^{-1} Zeocin. For construction of MAS1 and chaperon co-expression strain, X-33/MAS1 strain as the parent strain was transformed with linearized pPIC9K vector containing each chaperones gene by electroporation to get X-33/MAS1-PDI, X-33/MAS1-BIP, X-33/MAS1-HAC1 and X-33/MAS1-VHB strains. Transformants were selected by YPD medium plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, and 2% (w/v) agar) containing 100 μg mL^{-1} G418. Transformants containing inserts of chaperones gene were confirmed by colony PCR with their specific primers.

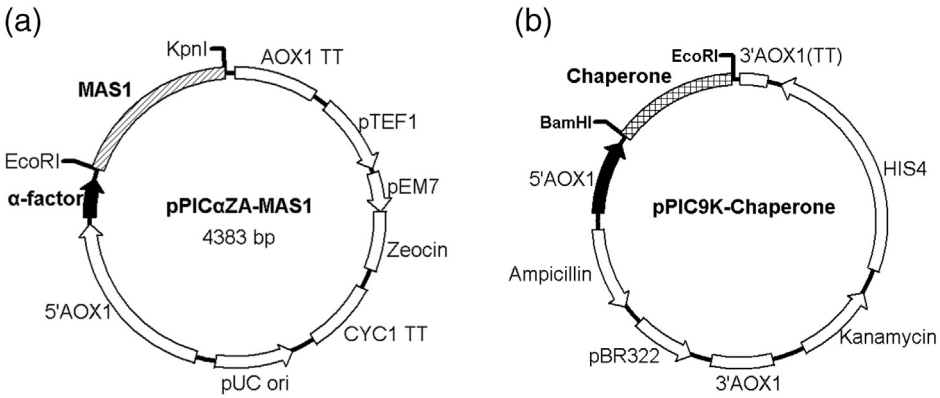

Fig. 1. Construction of expression vector. Physical maps of pPICαZA-MAS1 (a) and pPIC9K-Chaperone (b). The chaperones gene in pPIC9K included PDI, HACI, BIP and VGH.

2.3. Transformants screening and expression in shaking flask

Transformants with G418 resistance were picked from plate and inoculated into glass tubes containing 3 mL BMGY medium. The glass tubes harboring the cultures were incubated at 30°C with a shaking speed of 200 rpm for 24 h. Then the cell pellets were collected by centrifugation and transferred to a new glass tubes with 3 mL BMMY medium. Methanol was added to the culture with a final concentration of 1% (V/V) at every 24 h. After 72 h, the lipase activity of each transformants was measured using 4-nitrophenyl octanoate as substrate under the optimal condition. Transformants harboring each chaperon with highest activity were selected for cultivating in shaking flask.

Colony that showed high lipase activity with initial cultivation in glass tubes were inoculated in a 500 mL flask containing 100 mL BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glycerol, 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% w/v biotin) in a rotary shaker at 30°C and 200 rpm. The cells were harvested by centrifugation at an optical cell density of OD_{600} (2.0–6.0), and the cell pellet was suspended with BMMY medium (containing 1% methanol v/v) to OD of 1.0 with the final culture volume of 50 mL. Methanol was added at final concentration of 1% (v/v) at 24 h interval for 5 d continuously, and the activities of lipase in the supernatant of fermentation broth were measured and the OD of cell in the culture was monitored.

2.4. Fermentation scale-up

The fermentation inoculums was prepared by cultivating the cells at 30°C with shaking at 200 rpm for 18–24 h in a 500 mL shaking flask containing 100 mL YPD medium. And then, 10% (v/v) of the culture was inoculated into the 30-L bioreactor (Baoxing Co., Shanghai, China). The initial OD_{600} of the culture in bioreactor was about 0.5. The X-33/MAS1 and X-33/MAS1-PDI strain were both grown in a 30-L bioreactor containing 7 L basal salt medium made of 0.47 g/L $CaSO_4 \times 2H_2O$, 9.1 g/L K_2SO_4, 7.5 g/L $MgSO_4 \times 7H_2O$, 6.2 g/L KOH, 13.35 mL/L H_3PO_4 (85%), 20.0 g/L glycerol and 1.5 mL Pichia trace solution (PTM1) (Guangzhou Chemical Reagent Factory, Guangzhou, China). One liter PTM1 consists of 6 g $CuSO_4 \times 5H_2O$, 0.08 g NaI, 3 g $MnSO_4 \times H_2O$, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 0.02 g H_3BO_3, 0.2 g $Na_2MoO_4 \times 2H_2O$, 65 g $FeSO_4 \times 7H_2O$, 0.2 g biotin and 30 mL 6 N H_2SO_4. The pH of the medium was adjusted and controlled at 5.0 using NH_4OH (28%) and H_3PO_4 (10%). The methanol or glycerol feeding to the fermentor was controlled by a pump. Temperature and agitation rate were maintained at 30°C and 600 rpm, respectively. DO concentration was constantly maintained between 20% and 60% saturation. Once glycerol was depleted from culture broth, which was indicated by a sharp increase in DO, the glycerol fed-batch phase was started with feeding 50% (v/v) glycerol until the biomass was reached about 180 g/L (wet cell weigh). After half an hour, the methanol fed-batch phase was carried out, and the culture was supplied with 100% (v/v) methanol to induce lipase expression. Samples were taken at regular intervals to monitor the biomass, lipase activity and total protein concentration.

2.5. Optimization of the induction temperature and pH

To investigate the effects of temperature and pH on the production of lipase MAS1 from X-33/MAS1-PDI strain, the induction temperature and pH were optimized in a 30-L bioreactor. At the glycerol fed-batch phase, the condition was maintained at 30°C, pH 5.0 for the cell growth. Then, during the methanol fed-phase, the temperature was set in the range of 30°C to 22°C, and then the pH was controlled at pH 4.0–8.0, respectively. The lipase activity, protein concentration, cell density and viability were monitored at 24 h interval.

2.6. Analysis of lipase activity and total protein concentration

The activity of lipase MAS1 was determined by colorimetric techniques using 4-nitrophenyl octanoate as substrate. The reaction mixture was consisted of 80 μL 100 mM phosphate buffer of pH 8.0, 10 μL proper dilute enzyme solution and 10 μL substrate dissolved in ethanol (10 mM). The reaction was incubated at 25 °C for 5 min, and terminated by adding 100 μL of 1% SDS. The absorbance of the reaction mixture was measured at 405 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute. The protein content was determined according to the Bradford method using BSA as standard.

2.7. Analysis of biomass and cell viability

Dry cell weight (DCW) of the cell suspension was determined by centrifugation of 10 mL cell broth in a pre-weighed centrifuge tube at $8000 \times g$ for 10 min, followed by washing twice with distilled water and drying to constant weight at 105°C in an oven. The cell viability measurement was performed using methylene blue dye exclusion technique as described by Sinha et al. [14].

3. Results and discussion

3.1. Effect of chaperons co-expression on the production of lipase MAS1

To enhance the expression yield of MAS1 lipase in *P. pastoris*, co-expression of chaperones with MAS1 lipase was conducted. Chaperones genes encoding PDI, HAC1, BIP and VHB were cloned in the expression vector pPIC9K (Fig. 1b) and were separately transformed into the *P. pastoris* strain X-33/MAS1 following by antibiotic G418 selection. Initially, at least 40 colonies of each chaperon co-expression strains were randomly selected, cultivated and their activities were tested using 4-nitrophenyl octanoate as substrate. For each chaperon co-expression strains, most of colonies tested showed similar lipase activity, but were slight higher than that of MAS1 expressing strains. Despite the fact that all the transformants contained the genes of lipase *mas1* and chaperons confirmed by PCR, a few transformants without any activity were also found. One possible reason is that the integration of chaperon genes by the second electroporation disrupted the existing expression cassette of the mas1 gene in the genomic DNA of the host resulted in gene expression deficiency. The transformants with highest activity were cultivated in shaking flasks.

After 120 h induction with methanol in shaking flasks, lipase activity of each X-33/MAS1 transformants having each chaperon was measured. Highest activity was found in the broth supernatant of X-33/MAS1-PDI, which was 1.7-fold to that of X-33/MAS1 (10 U/mL) (Fig. 2). PDI located in the ER involves in the formation of disulfide bonds in folding proteins and the rearrangement of incorrect disulfide [7]. And lipase MAS1 was predicted to have two pairs of disulfide bonds by the DiANNA webserver (http://clavius.bc.edu/~clotelab/DiANNA/) [18]. Therefore, PDI might help in formation of disulfide bonds of lipase MAS1 in ER resulting in improving the production of recombinant lipase MAS1. Co-expression of HAC1 and BIP had slightly improved the expression level of lipase MAS1. BIP and HAC1 are chaperones that can stabilize the mis-folded proteins or induce production of foldase. Increase of those chaperones content in cell resulted in enhancing the production of soluble recombinant proteins. VHB, a bacterial hemoglobin produced by *Vitreoscilla* under hypoxic conditions [19], has been reported to improve the growth and productivity of heterogous host [20,21]. However, co-expression of VGB had a negative effect to the lipase MAS1 production. One reason can be explained for that oxygen deficiency in fermentation broth was not a bottleneck for lipase MAS1 production in *P. pastoris* with shaking flask cultivation, while expression of VGB have a

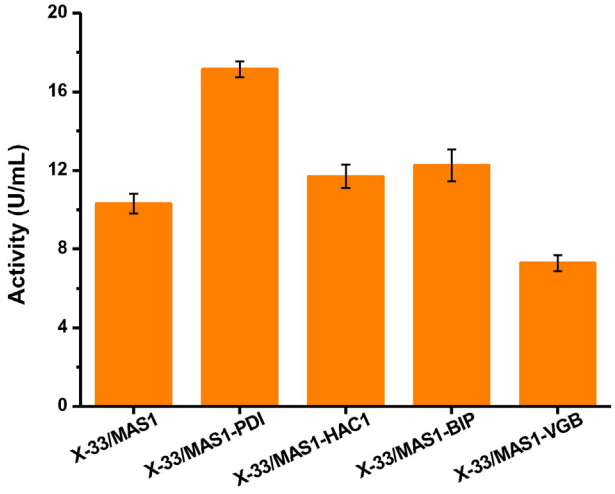

Fig. 2. Effect of co-expression of chaperons on the production of lipase MAS1.

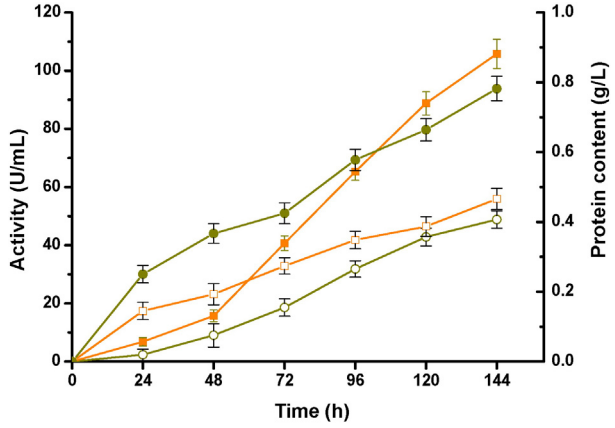

Fig. 3. Comparison of lipase activity (square) and total protein concentration (circle) between X33/MAS1 (open symbols) and X33/MAS1-PDI (filled symbols) strains in a 30-L bioreactor.

competitive effect for lipase MAS1 production. Therefore, the X-33/MAS1-PDI strain was selected for subsequent investigation in 30-L bioreactor since it displayed the highest activity among the strains with chaperon co-expression.

3.2. Promoting production of lipase MAS1 by PDI co-expression in a 30-L bioreactor

To investigate the effect of PDI on promoting secretion of MAS1 in fed-batch fermentation, X33/MAS1 and X33/MAS1-PDI strains were grown in a 30-L bioreactor. In the initial study, induction pH and temperature were set at pH 5.0 and 30°C, respectively. As shown in Fig. 3, the lipase activity in fermentation broth of X33/MAS1 and X33/MAS1-PDI strains were about 48 U/mL and 110 U/mL after 144 h fermentation, respectively. The total protein concentration of X33/MAS1-PDI (0.782 g/L) in fermentation broth was higher than that of X33/MAS1 (0.466 g/L). The lipase activity and total protein concentration in broth of X33/MAS1-PDI cells were about 2.3 and 1.7-fold to that of X33/MAS1 strain. The biomass had no obvious differences between X33/MAS1 and X33/MAS1-PDI strains (data no shown), indicating that PDI co-expression did not inhibit the growth of cell. However, in a previously study, Liu et al. [22] had found that co-expression of PDI had a negative effect to the growth of cell resulting to decrease the expression level of recombinant α-glucosidase [22].

3.3. Effect of temperature on the production of lipase MAS1

Temperature plays a key role in fermentation process that affects the cell growth and protein production. To investigate the effect of induction temperatures on production of MAS1, induction temperatures ranging from 22 to 30°C were tested. Meanwhile, the lipase activity and total protein concentration in fermentation broth were monitored. As shown in Fig. 4a, MAS1 activities in broth supernatant at lower induction temperature were higher than that at higher temperature. Lipase activity at 24 and 22°C were 185 and 220 U/mL showing 1.7 and 2.1-fold to that at 30°C, respectively. The total protein concentration in fermentation broth at lower induction temperature (22, 24 and 26°C) is also higher than that at 28°C and 30°C (Fig. 4a). These finding agreed with previous reports that lower fermentation temperature have a positive effect for protein production [23]. It was found that induction at high temperature may have a slightly detrimental effect on biomass at induction phase. The cell density of cell was 150 g/L at 30°C, which was 18% lower than that of 24°C (Fig. 4b). Furthermore, the cell viability under the temperature at 30 and 28°C was below 40%, while it was higher than 80% at 22 and 24°C (Fig. 4b), indicating that higher induction temperature has a detrimental effect to yeast cell.

3.4. Effect of pH values on the production of lipase MAS1

The effect of induction pH on lipase production within the range of pH 4.0–8.0 in a 30-L bioreactor with induction temperature at 24°C

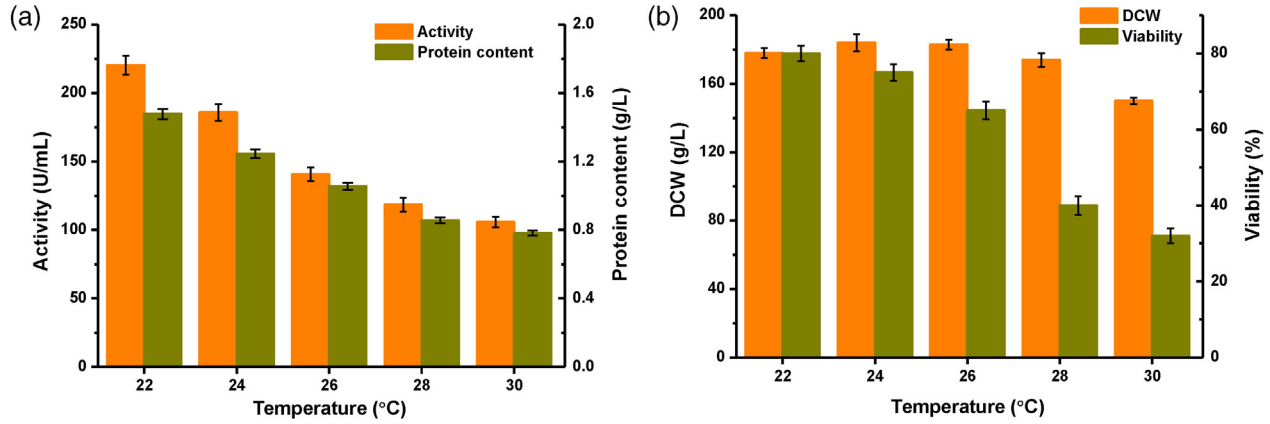

Fig. 4. Effect of induction temperatures on the production of lipase MAS1. Analysis of lipase MAS1 activity (a), protein content (a), cell density (b) and viability (b) of *P. pastoris* X-33/MAS1-PDI were conducted in a 30-L bioreactor after 144 h fermentation.

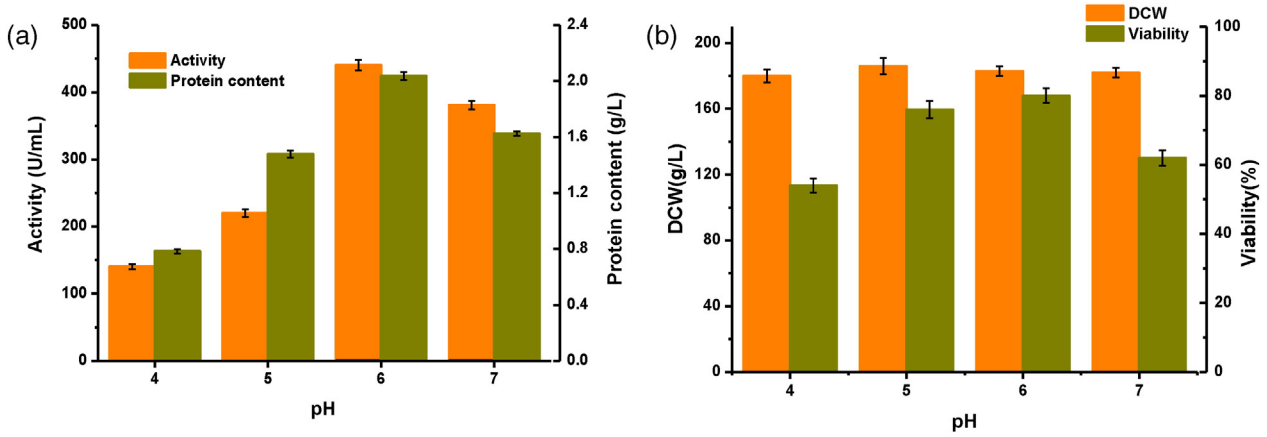

Fig. 5. Effect of induction pH on the production of lipase MAS1. Analysis of lipase MAS1 activity (a), protein content (a), cell density (b) and viability (b) of *P. pastoris* X-33/MAS1-PDI were conducted in a 30-L bioreactor after 144 h fermentation.

was studied. As shown in Fig. 5a, the highest lipase activity was obtained at pH 6.0 with 440 U/mL after 144 h fermentation, which was 2 and 3-fold to that at pH 5 and 4, respectively. The protein concentration at pH 6.0 was 2.0 g/L, which was higher than that at other induction pH (Fig. 5a). Fig. 5b shows that the cell growth profiles under various pH values seem similar, and their cell density after 144 h fermentation was above 180 g/L. It should mention that cell growth sharply declined at pH 8.0 with a cell density of 120 g/L at the end of fermentation (data not shown), and the lipase activity has not been detected. The yeast cell showed more robust at pH 6.0 (82% cell viabilities) than that at other induction pH (Fig. 5b).

The time course change of lipase activity, biomass and DO values during the fermentation under in 30-L bioreactor were monitored. The profiles of each parameter were shown in Fig. 6. DO values is an important parameter reflecting the metabolic rate of the culture during the fermentation procedure. The consumption of all glycerol in the culture resulted in rapidly raising the DO value after stop feeding the glycerol, and then methanol can be fed into the culture to induce the expression of MAS1 lipase. Although the biomass at 168 h was higher than that at 144 h, the activity in culture had slight increase with time extension.

In conclusion, the expression level of lipase MAS1 has been significantly elevated by chaperones co-expression and fermentation condition optimization. Besides VGB, co-expression of chaperones (PDI, BIP and HAC1) could promote the lipase MAS1 production. Lower induction temperature and pH 6.0 have the benefit effect on lipase MAS1 production in a 30-L bioreactor. After optimization, the expression level of lipase MAS1 have increased to 442 U/mL, which were roughly 44-fold to that of shaking flask cultivation (10 U/mL). Our work not only provided enough amount of lipase MAS1 for subsequent application research but also gave some clues for improving heterologous proteins production in *P. pastoris*.

Conflict of interest

The authors have declared no conflicts of interest.

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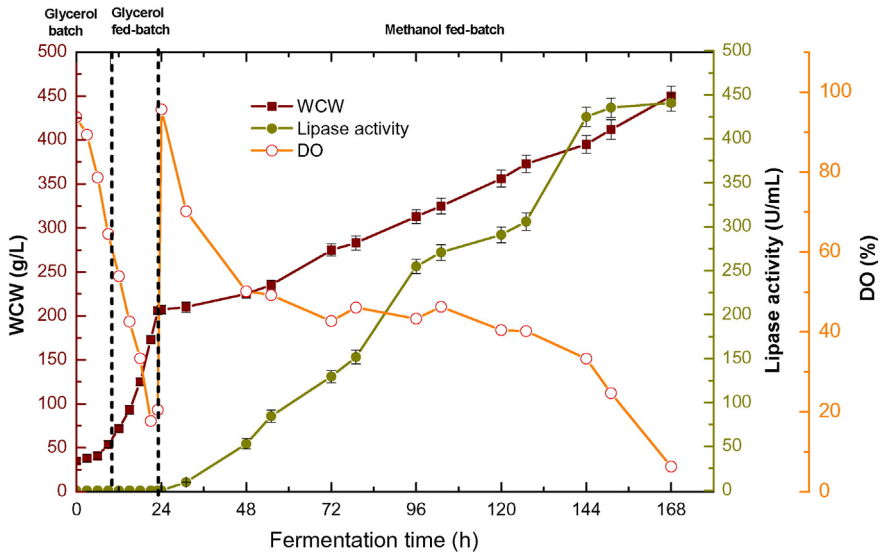

Fig. 6. Time course of lipase activity, biomass and DO values during the fermentation in the bioreactor. Error bars show standard deviations (SD).

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References

[1] Kirk O, Borchert TV, Fuglsang CC. Industrial enzyme applications. Curr Opin Biotechnol 2002;13:345–51. http://dx.doi.org/10.1016/S0958-1669(02)00328-2.

[2] Joseph B, Ramteke PW, Thomas G. Cold active microbial lipases: Some hot issues and recent developments. Biotechnol Adv 2008;26:457–70. http://dx.doi.org/10.1016/j.biotechadv.2008.05.003.

[3] Yin JC, Li GX, Ren XF, Herrler G. Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. J Biotechnol 2007;127:335–47. http://dx.doi.org/10.1016/j.jbiotec.2006.07.012.

[4] Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Ohi H, et al. High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. J Biosci Bioeng 2000;89:55–61. http://dx.doi.org/10.1016/S1389-1723(00)88050-0.

[5] Cregg JM, Cereghino JL, Shi JY, Higgins DR. Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol 2000;16:23–52. http://dx.doi.org/10.1385/Mb:16:1:23.

[6] Atkin JD, Farg MA, Walker AK, McLean C, Tomas D, Horne MK. Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. Neurobiol Dis 2008;30:400–7. http://dx.doi.org/10.1016/j.nbd.2008.02.009.

[7] Damasceno LM, Huang CJ, Batt CA. Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 2012;93:31–9. http://dx.doi.org/10.1007/s00253-011-3654-z.

[8] Guerfal M, Ryckaert S, Jacobs PP, Ameloot P, Van Craenenbroeck K, Derycke R, et al. The HAC1 gene from *Pichia pastoris*: Characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. Microb Cell Fact 2010;9:49. http://dx.doi.org/10.1186/1475-2859-9-49.

[9] Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol Bioeng 2004;85:367–75. http://dx.doi.org/10.1002/bit.10904.

[10] Sha C, Yu XW, Lin NX, Zhang M, Xu Y. Enhancement of lipase r27RCL production in *Pichia pastoris* by regulating gene dosage and co-expression with chaperone protein disulfide isomerase. Enzyme Microb Technol 2013;53:438–53. http://dx.doi.org/10.1016/j.enzmictec.2013.09.009.

[11] Fickers P, Nicaud JM, Gaillardin C, Destain J, Thonart P. Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. J Appl Microbiol 2004;96:742–9. http://dx.doi.org/10.1111/j.1365-2672.2004.02190.x.

[12] Zhang JX, Liu L, Li JH, Du GC, Chen J. Enhanced glucosamine production by *Aspergillus* sp BCRC 31742 based on the time-variant kinetics analysis of dissolved oxygen level. Bioresour Technol 2012;111:507–11. http://dx.doi.org/10.1016/j.biortech.2012.02.063.

[13] Kar T, Delvigne F, Masson M, Destain J, Thonart P. Investigation of the effect of different extracellular factors on the lipase production by *Yarrowia lipolityca* on the basis of a scale-down approach. J Ind Microbiol Biotechnol 2008;35:1053–9. http://dx.doi.org/10.1007/s10295-008-0382-1.

[14] Sinha J, Plantz BA, Inan M, Meagher MM. Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-t. Biotechnol Bioeng 2005;89:102–12. http://dx.doi.org/10.1002/bit.20318.

[15] Wang Y, Wang ZH, Xu QL, Du GC, Hua ZZ, Liu LM, et al. Lowering induction temperature for enhanced production of polygalacturonate lyase in recombinant *Pichia pastoris*. Process Biochem 2009;44:949–54. http://dx.doi.org/10.1016/j.procbio.2009.04.019.

[16] Charoenrat T, Khumruaengsri N, Promdonkoy P, Rattanaphan N, Eurwilaichitr L, Tanapongpipat S, et al. Improvement of recombinant endoglucanase produced in *Pichia pastoris* KM71 through the use of synthetic medium for inoculum and pH control of proteolysis. J Biosci Bioeng 2013;116:193–8. http://dx.doi.org/10.1016/j.jbiosc.2013.02.020.

[17] Yuan D, Lan D, Xin R, Yang B, Wang Y. Screening and characterization of a thermostable lipase from marine *Streptomyces* sp. strain W007. Biotechnol Appl Biochem 2016;63:41–50. http://dx.doi.org/10.1002/bab.1338.

[18] Ferre F, Clote P. DiANNA: A web server for disulfide connectivity prediction. Nucleic Acids Res 2005;33:230–2. http://dx.doi.org/10.1093/nar/gki412.

[19] Park KW, Kim KJ, Howard AJ, Stark BC, Webster DA. *Vitreoscilla* hemoglobin binds to subunit I of cytochrome *bo* ubiquinol oxidases. J Biol Chem 2002;277:33334–7. http://dx.doi.org/10.1074/jbc.M203820200.

[20] Wu JM, Wang SY, Fu WC. Lower temperature cultures enlarge the effects of *Vitreoscilla* hemoglobin expression on recombinant *Pichia pastoris*. Int J Mol Sci 2012;13:13212–26. http://dx.doi.org/10.3390/ijms131013212.

[21] Wu H, Li J, Dong D, Liu T, Zhang T, Zhang D, et al. Heterologous coexpression of *Vitreoscilla* hemoglobin and *Bacillus megaterium* glucanase in *Streptomyces lydicus* A02 enhanced its production of antifungal metabolites. Enzyme Microb Technol 2015;81:80–7. http://dx.doi.org/10.1016/j.enzmictec.2015.08.003.

[22] Liu X, Wu D, Wu J, Chen J. Optimization of the production of *Aspergillus niger* α-glucosidase expressed in *Pichia pastoris*. World J Microbiol Biotechnol 2013;29:533–40. http://dx.doi.org/10.1007/s11274-012-1207-y.

[23] Anasontzis GE, Pena MS, Spadiut O, Brumer H, Olsson L. Effects of temperature and glycerol and methanol-feeding profiles on the production of recombinant galactose oxidase in *Pichia pastoris*. Biotechnol Prog 2014;30:728–35. http://dx.doi.org/10.1002/btpr.1878.