J Ind Microbiol Biotechnol (2008) 35:189–195
DOI 10.1007/s10295-007-0283-8

ORIGINAL PAPER

Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter

Wei Zhao · Jinwen Wang · Riqiang Deng · Xunzhang Wang

Received: 27 July 2007 / Accepted: 21 November 2007 / Published online: 18 December 2007

Abstract The high-cell-density fermentation of *Candida rugosa* lipase in the constitutive *Pichia pastoris* expression system was scaled up from 5 to 800 l in series by optimizing the fermentation conditions at both lab scale and pilot scale. The exponential feeding combined with pH-stat strategy succeeded in small scale studies, while a two-stage fermentation strategy, which shifted at 48 h by fine tuning the culture temperature and pH, was assessed effective in pilot-scale fermentation. The two-stage strategy made an excellent balance between the expression of heterogeneous protein and the growth of host cells, controlling the fermentation at a relatively low cell growth rate for the constitutive yeast expression system to accumulate high-level product. A stable lipase activity of approximately 14,000 IU ml^{-1} and a cell wet weight of ca. 500 g l^{-1} at the 800-l scale were obtained. The efficient and convenient techniques suggested in this study might facilitate further scale-up for industrial lipase production.

Keywords Scale-up · *Candida rugosa* lipase · *Pichia pastoris* · GAP promoter · High-cell-density

Introduction

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerides at water/oil interfaces, which produce valuable materials for flavor, bio-diesel, pharmaceutical, and other industrial applications. Currently, the rapid developments of molecular biology techniques, as well as the availability of more reliable high-throughput-screening methods, have enhanced the utility of lipases.

Up to seven closely related lipases have been identified form *Candida rugosa*. However, each isozyme possesses different substrate specificity and thermal stability for biocatalysis, which might lead to divergent catalytic performances [5]. Cloning and expression of the major lipase gene (*lip*1) is the most acceptable approach for obtaining pure *C. rugosa* lipase (CRL) with optimized properties for commercial use [3, 6, 7].

In a previous study [1], an artificial *lip*1 gene, which consisted of 20 codons of preference in *Pichia pastoris*, was synthesized and cloned into an expression vector pGAPZαA. The gene was driven by the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter to avoid the accumulation of formaldehyde and hydrogen peroxide in the host. The secretion signal sequence in this vector, which encodes *Saccharomyces cerevisiae*α-factor prepro-peptide additionally, helps to secrete the functional recombinant protein into the medium.

By using the recombinant yeast containing this vector, fermentation was scaled up from lab scale to pilot scale. Shaking flask studies resulted in cheap and facile medium, and successful feeding strategies and fermentation parameters were developed for 800 l pilot-scale fermentation, which reached a high lipase activity of 14,000 IU ml^{-1}.

Materials and methods

Strains, plasmids and enzymes

Pichia pastoris X-33, expression vector pGAPαA, and Zeocin were from Invitrogen. Restriction enzymes, T4-DNA

W. Zhao · J. Wang · R. Deng · X. Wang (✉)
State Key Laboratory of Biocontrol,
School of Life Science, Sun Yat-sen (Zhong shan) University,
Guangzhou 510275, People's Republic of China
e-mail: WXZ@mail.sysu.edu.cn

ligase, and LA Taq DNA polymerase were from TaKaRa Biotechnology (Da lian) Co., Ltd and New England Biolabs (USA). PCR purification kit, DNA Gel-Extraction Kit, S.N.A.P.™ Plasmid Mini Kit were purchased from Omega (USA). All reagents were of analytical grade unless otherwise stated. Target gene sequencing primers 5′-TACTATTGCCAGCATTGCTGC-3′ and 5′-GCAAAT GGCATTCTGACATCC-3′, were synthesized by Shanghai Sangon biological engineering technology & service Co., Ltd.

Construction of expression plasmid pGAPαA-*lip*1

DNA manipulation was performed according to the standard procedures [13]. The gene encoding *C. rugosa* lipase sequence was divided into four fragments of circa 400 bp each, which were separately synthesized by using the mutually priming long oligonucleotides in a PCR. After digestion and ligation, the obtained 1635 bp *lip*1 gene and the pBluescript II SK(+) cloning vector were digested with *Xho*I/*Xba*I and ligated with T4-DNA ligase at 16°C for 12 h. The resulting plasmid (pBluescript II SK(+)-*lip*1) was transformed into *Escherichia coli* DH5αby using chemical transformation and selected on Luria–Bertani (LB) plates [1.0% (w/v) tryptone, 0.5% (w/v)yeast extract, 0.5% (w/v) NaCl, 1.0% (w/v) agarose, pH 7.5] containing 25 μg/ml Zeocin. Transformants were selected and screened by direct PCR using the above primers. Positive transformants were grown overnight in 50 ml liquid LB medium containing 25 μg ml-1 Zeocin, and the recombinant plasmids were isolated with a Plasmid Mini Kit (Omega). The cloned *lip*1 gene was confirmed by sequencing to prove that no mutation occurred during the PCR. PBluescript II SK(+)-*lip*1 was isolated from *E. coli* DH5αand then the *lip*1 gene was cut off from pBluescript II SK(+)-*lip*1. Gene fragments were resolved by 1% agarose gel electrophoresis. Gel slices containing the expected size band were excised and extracted with DNA Gel-Extraction Kit from Omega (USA). The 1,635-bp *lip*1 gene was inserted between the *Xho*I and *Xba*I sites of the expression vector pGAPαA, which already contained the wild type α-signal sequence, producing the construct pGAPαA- *lip*1 (Fig. 1).

Transformation of Pichia pastoris

The constructs (5–10 μg) were linearized with *Avr* II (NEB) and transformed into *P. pastoris* wild-type strain X-33 by electroporation at 25 μF, 200 Ω, and1500 V using a Bio-Rad GenePulser. Transformants were incubated at 30°C on YPDS (1.0% yeast extract, 2% peptone, 2% dextrose, and 1 mol l^{-1} sorbitol) plates.

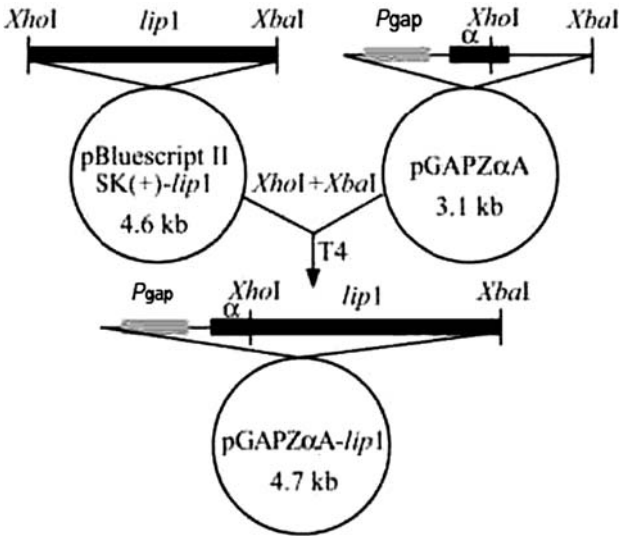

Fig. 1 Construction of the recombinant plasmid pGAPZαA-*lip*1. *lip*1 gene was cut off from pBluescript II SK(+)-*lip*1 and then inserted between the *Xho* I and *Xba* I sites of pGAPZαA vector, following α factor. α,α singal sequence ; T4, T4 DNA ligase; Pgap, gap promoter

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide gel on a vertical mini gel apparatus (Bio-Rad) at 150 V for 1 h [13]. Molecular weight marker was purchased from TaKaRa. Proteins were stained with Coomassies Brilliant Blue G-250 (Tiangen Biotech).

Enzyme assay

Lipase activity was routinely measured using olive oil as substrate. Five percent (v/v) olive oil was emulsified in distilled water with 2% (w/v) gum arabic as stabilizer using a homogenizer for 10 min at maximum speed at 4°C. Before measuring, substrate solution was heated to 37°C and adjusted to pH 7.2. After addition of 5–20 μl enzyme solution, the activity was measured with a pH-stat titrator (METROHM). Liberated fatty acid was titrated automatically with 0.05 mol l^{-1} NaOH to maintain pH at 7.2. One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 μmol fatty acid per minute under assay conditions.

Cell density measurement

After centrifugation (Centrifuge AG 22331, Eppendorf, Hamburg, Germany) at 6,000 g for 5 min and washing with distillated water, the pellet of 10 ml cell suspension sample was weighed (wet cell weight WCW).

Cultivation

The shaking flask culture was grown in 50 ml medium in a rotary shaker for 72 h at 29°C using 500 ml flasks containing YPD culture medium (1.0% yeast extract, 2% peptone, 2% glucose), and a trace element solution.

Fed-batch fermentation

The culture medium for bench-scale and pilot-scale fermentation contained 4 g l^{-1} KH_2PO_4, 4 g l^{-1} $(NH_4)_2SO_4$, 0.38 g l^{-1} $CaCl_2$, 18.2 g l^{-1} K_2SO_4, 9.4 g l^{-1} $MgSO_4{\cdot}7H_2O$, 40 g l^{-1} glucose, and 1 ml l^{-1} trace element solution. The trace element solution consisted of 2.50 g l^{-1} $MnSO_4{\cdot}H_2O$, 54.17 g l^{-1} $FeSO_4{\cdot}7H_2O$, 16.67 g l^{-1} $ZnCl_2{\cdot}2$ H_2O, 0.17 g l^{-1} $Na_2MoO_4{\cdot}2H_2O$. Glucose and MgSO4·7H2O were sterilized separately. The feeding medium was 600 g l^{-1} glucose solution with 2 g l^{-1} $(NH_4)_2PO_4$. All these materials were industrial grade and purchased from Chinese market.

The 500 ml flasks, a 5-l bioreactor, and a 30-l bioreactor containing culture medium were used for the first, second, and third stage seed cultures, respectively.

Fermentation studies were conducted in a 5 l B. Braun Biostat, a 30 l B. Braun Biostat, and an 800 l commercial bioreactor. All bioreactors were equipped with controls for temperature, pH, agitation speed, and aeration rate. In fed-batch cultivations the feeding medium was sterilized separately in a reservoir vessel and fed into the bioreactor using a peristaltic pump or compressed air in discrete pulses or exponential flow based on the feeding strategy selected. The pH was controlled from 5.5 to 6.5 through automatic addition of 30% NH_4OH and the agitation speed was automatically feedback-controlled based on DO at a set point of 30%.

The initial working volumes for the 5, 30, and 800 l fermentors were 1.8, 10, and 280 l respectively. The proportion of inoculation was 10% (v/v) of the initial working volume. The exponential feeding was started at the end of a batch phase triggered by the increase of DO above set point of 50%. Four of the relevant growth rates for each feeding profile from $\mu = 0.10\ h^{-1}$ to $\mu = 0.25\ h^{-1}$ was fed into the equation $F = 0.081\ \mu X_0 V_0 e^{\mu t}$, where X_0 stands for the initial cell concentration and V_0 stands for the initial working volume, and a computer coupled peristaltic pump was used to pump condensed glucose in the feed stream. The glucose concentration was controlled no more than 40 g l^{-1} in all experiments. Samples of 10 ml were removed from the broth every 6 h during fermentation. Cell growth was measured by weighing the wet cell weight after centrifuging.

Results

Screening of steady recombinant yeast strains

More than 1,200 positive clones were initially obtained through screening of recombinant yeasts on YPD Zeocin plates (100 μg ml^{-1}). To obtain steady recombinant *P. pastoris*, 129 big colonies were selected and transferred into YPD liquid medium with 150 μg ml^{-1} Zeocin. The survived strains were re-screened on plates containing 200 μg ml^{-1} Zeocin. After 50 generations, three biggest colonies were collected and cultured in a 5 l fermentor.

PCR analysis using the primers mentioned in the methods demonstrated that *lip*1 gene was successfully integrated into *P. pastoris*. SDS-PAGE of supernatant indicated that the integrated gene was expressed effectively (Fig. 2).

Condition optimization for flask culture

Results of carbon source optimization showed that 40 g l^{-1} glucose was the best choice for both cell growth and lipase

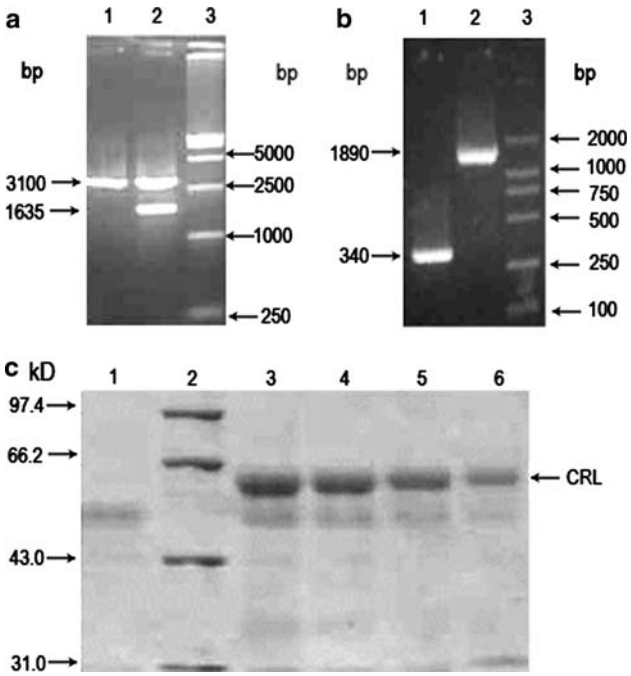

Fig. 2 Identification of integrated plasmid and expressed target protein. **a** Characterization of pGAPZαA-*lip*1 by *Xho*I/*Xba*I digestion. *Lane 1* pGAPZαA-lip1 digested by *Xho*I/*Xba*I; *lane 2*, pGAPZαA by *Xho*I/*Xba*I; *lane 3*, DNA marker; **b** X-33 transformed with expression vectors. *Lane 1*, X-33 transformed with pGAPZαA; *lane 2*, X-33 transformed with pGAPZαA-*lip*1; *lane 3*, DNA marker. **c** SDS-PAGE of supernatants from 5 l scale *Pichia pastoris* X-33 fermentation. *Lane 1*, 72 h after transformation with pGAPZαA; *lane 2*, protein marker; *lane 3–6*, post transformation with pGAPZαA-lip1 (PGL) at 72, 60, 48, and 36 h, respectively

production. Lipase activity achieved an average level of 102.5 IU ml^{-1} after 72 h cultivation by additionally adding 1.2% (W/V) $NaMO_4$, 1.0% (W/V) $ZnCl_2$, 1.0% (W/V) $FeSO_4$, and 1.2% (W/V) $MnSO_4$. The optimal temperature and pH for lipase formation and for cell growth were different, lipase activity being favored at pH 6.5 and 26°C, while cell growth being benefited at pH 5.5 and 30°C.

Five-liter bench-scale studies

Using the optimal conditions for flask culture, 5-l scale fermentation reached a maximum lipase activity of 653.94 IU ml^{-1} and a final wet cell weight (WCW) of 74.1 g l^{-1} at 60 h (Fig. 3). Subsequently, fed-batch fermentation using the substrate-stat feeding strategy (Fig. 4) and other strategies, i.e. the specific growth rate-stat (μ-stat) strategy and the μ-stat combined with pH-stat strategy for use in larger scale fermentations, were further investigated at 5-l scale to overcome the disadvantage of lacking on-line probes in large-scale fermentations.

The μ-stat strategy was exponentially fed following the equation described in Materials and methods, feeding being initiated at X_0 = 73 g l^{-1} (WCW), V_0 = 1.8 l to maintain the specific growth rate of 0.15 h^{-1} (approximately 60% μ_{max}). The biomass and the lipase activity obtained after 72 h cultivation were 433 g l^{-1} (WCW) and 2,255 IU ml^{-1}, respectively (Fig. 5).

The combined strategy, which simultaneously controls the specific cell growth rate and culture pH, resulted in the highest lipase activity (Fig. 6).

Investigation on the relationship among μ, WCW, and lipase activity led to an interesting result that a relatively low μ (0.15) induced a high lipase activity peak, although the maximum μ value (0.25) brought to maximum cell density and lipase activity (Table 1). This result suggested that high lipase activity could be obtained at relatively low cell growth rate, which was the base for μ-stat strategy combining the shift of pH and temperature at 48 h, or a two-stage shift strategy, used in 30-l and 800-l scale fermentation in this study.

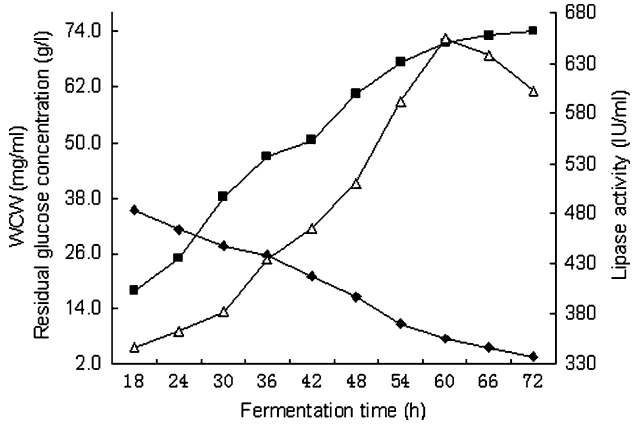

Fig. 3 Time course of batch fermentation in a 5 l bench-scale fermentor containing 3.5 l fermentation medium under shaking flask conditions at 29 ± 1°C, pH 5.5 and 250 rpm. (*filled square*) wet cell weight (WCW) (*open triangle*) lipase activity (*filled diamond*) residual glucose concentration

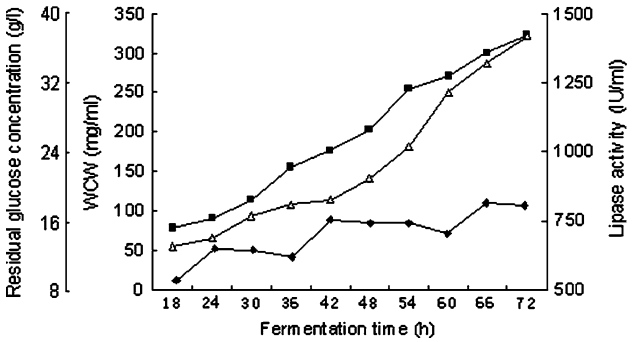

Fig. 4 Time course of substrate-stat fed-batch fermentation in 5 l fermentor with 1.8 l initial medium and 1.7 l feeding volume cultivating at 29 ± 1°C, pH 5.5, and 250 rpm. *Filled square* wet cell weight (WCW); *open triangle* lipase activity; *filled diamond* residual glucose concentration

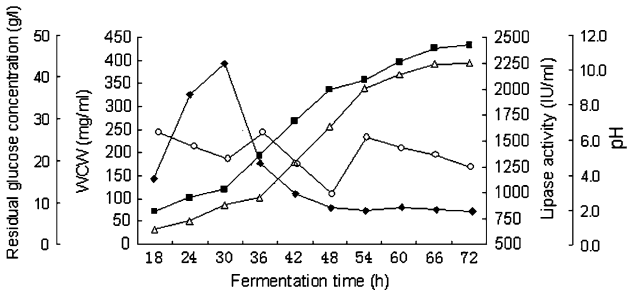

Fig. 5 Time course of μ-stat feeding fed-batch fermentation in 5 l fermentor with 1.8 l initial medium and 1.7 l feeding volume cultivating at 29 ± 1°C, 250 rpm. Aqueous ammonia was fed every 12 h. *Filled square* wet cell weight (WCW); *open triangle* lipase activity; *filled diamond* residual glucose concentration; *open circle* pH in the broth

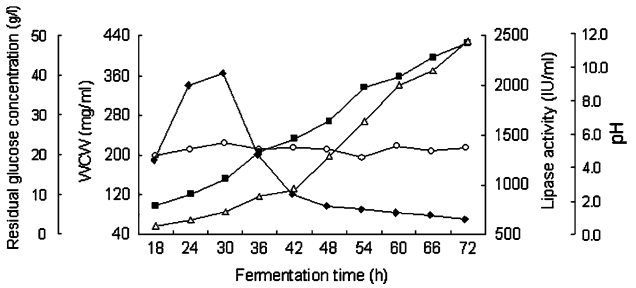

Fig. 6 Time course of μ-stat and pH-stat combination fed-batch fermentation in 5 l fermentor with 1.8 l initial medium and 1.7 l feeding volume cultivating at 29 ± 1°C, 250 rpm. PH was controlled at 5.5 by feeding aqueous ammonia. *Filled square* wet cell weight (WCW); *open traingle* lipase activity; *filled diamond* residual glucose concentration; *open circle* pH in the broth

Table 1 Wet cell weight, lipase activity, and kinetic parameters at different specific cell growth rate

μ (1/h)	WCW (mg/ml)	Lipase activity (IU/ml)	$Y_{x/s}$ (g/g)	$Y_{p/s(IU/g)}$	$Y_{p/x\ (IU/g)}$	m (g/g h)	$q_{lipase\ (IU/g\ h)}$
0.10	353	2,060	0.3334	1,945	5,840	0.0040	81.1
0.15	376	2,273	0.3571	2,159	6,046	0.0061	84.0
0.20	421	2,215	0.3846	2,023	5,260	0.0084	73.1
0.25	447	2,302	0.4005	2,060	5,150	0.0110	71.5

[a] All cultivations were carried out at 30°C,the maximum specific cell growth rate was 0.247, and the fermentation time was 72 h

Thirty liter pilot-scale studies

A two-stage shift strategy was adopted at the thirty-liter scale fermentation to achieve high productivity. The fermentation was μ-stat controlled with a pH and temperature shift at 48 h, pH5.5 and 30°C being maintained at the first stage for cell growth, while pH6.5 and 26°C at the secondary stage for lipase production. The shift time of 48 h has been chosen based on a couple of experiments (Table 2). This strategy resulted in a marginal increase in lipase activity, reaching 7,663 IU ml^{-1} averagely with a cell wet weight of ca. 500 g l^{-1} (Fig. 7), and was adopted in the following 800-l scale fermentation.

Eight hundred liter pilot-scale studies

The two-stage shift strategy used in 30-l scale fermentation was also adopted in 800-l scale fed-batch fermentation, which resulted in a stable lipase activity of approximately 14,000 IU ml^{-1} and a cell wet weight of ca. 500 g l^{-1} (Fig. 8), exhibiting a successful performance of the strategy for 800-l pilot-scale fermentation.

Discussion

Fermentation environment is an important factor impacting successful scale-up. High cell density fermentation conditions deviate far from the natural growth conditions [12] and cause environmental stresses. Environmental stresses, e.g. high osmolarity, low pH, and high temperature, may be in turn exerted on host cells [2, 14], which may either directly or indirectly affect the foreign protein expression in host cells [4, 8, 12]. On the other hand, high-level expression of foreign proteins also limits other metabolic reactions by competing for substrates, energy or co-factors, and induces stress reactions in host cells [12]. Other culture conditions such as substrate concentration and dissolved oxygen level also have great effects on cell growth and product formation [14, 16].

At large scale, high-cell-density fermentation faces two essential problems: the oxygen limitation and the environment stress. Either of them may lead to decreased productivity, decreased viability or increased cell lyses [11, 12]. In this study, we tried using a relatively low cell growth

Table 2 Shift time study for two-stage temperature and pH control

Shift time (h)	WCW (mg/ml)	Lipase activity (IU/ml)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (IU/g)
36	346	5,360	0.294	4,554
48	433	7,581	0.357	6,250
60	465	7,248	0.398	6,205

[a] The fermentation were conducted in a 30-l fermentor for 72 h, at 29 ± 1°C and pH 5.5 at the first stage and 26 ± 1°C and pH 6.5 at the second stage

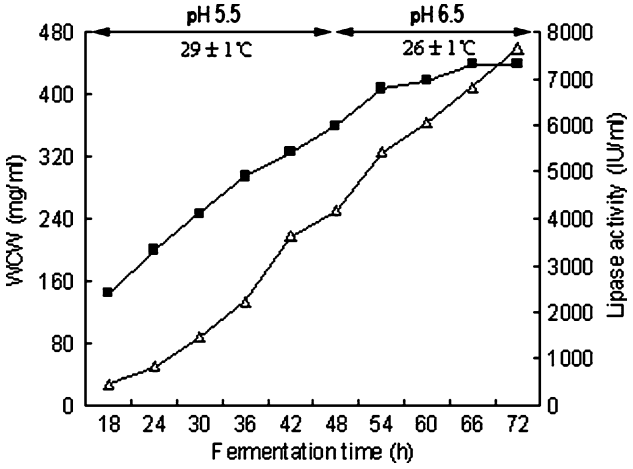

Fig. 7 Time course of combined μ-stat and pH control fed-batch fermentation with a culture pH and temperature shift from 5.5 and 29 ± 1 to 6.5 and 26 ± 1°C after 48 h in 30-l fermentor with 11 l initial medium and 10 l feeding volume. *Filled square* wet cell weight (WCW); *open triangle* lipase activity

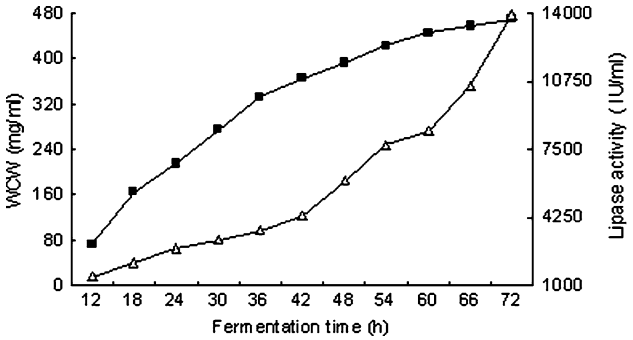

Fig. 8 Time course of 800-l pilot-scale fed-batch fermentation with 300 l initial medium and 300 l feeding volume. Culture pH and temperature were shifted from 5.5 and 29 ± 1 to 6.5 and 26 ± 1°C after 48 h. *Filled square* wet cell weight (WCW); *open triangle* lipase activity

rate and constant culture conditions to avoid these problems.

Our strategy was originally based on the result from this study that a relatively low cell growth rate was advantageous to high lipase activity formation (Fig. 6; Table 1), which is most desirable. And a major objective of high cell density fed-batch culture in this study was to maximize the lipase activity and at the same time decrease cost. Relatively low cell growth rate brings benefits to the increase of productivity and process repeatability, since microorganisms are prone to adapt to the dynamic environment at low growth rate [3, 9, 10 and 15].

The following reasons also made us preferring relative low cell growth rate rather than high one in the pilot-scale fed-batch fermentation.

At low growth rate, dissolved oxygen can be maintained at a high level, i.e. above 30 even 50%, using compressed air during the whole process, which avoids μ decrease during exponential growth. In addition, low growth rate facilitates feeding, avoiding too low concentration of substrate. And low constant growth rate can be maintained for a sufficient length of time to accumulate products.

Anabolic energy for product synthesis could be saved at relatively low cell growth rate. Result showed that the maintenance energy (m) consumption increased from 0.0040 to 0.0110 $g\ g^{-1}\ h^{-1}$ when growth rate rose from 0.10 to 0.25 h^{-1} (Table 1), which illuminated that cells might pay more for products and increased biomass than they required at high growth rate.

On the other hand, high growth rate would bring to low viability because of glucose limitation and oxygen absence. Low viability would result in cell lyses and overestimate of product formation. Cell lyses would bring to a significant release of host cell proteins including protease into the medium [7], contaminating the supernatant and degrading products. Since viability-lost cells stopped participating in bio-reactions, high-growth-rate culture would conduce to high biomass accumulation rather than product formation.

In this study, constant pH condition in fermentor was studied and trace elements were optimized to balance osmotic stress and cell requirements, which brought to increase in both cell growth and lipase production (Fig. 5). Further studies divided the whole fermentation into two stages, which resulted in a marginal increase of lipase activity, suggesting that the constitutive expression system could be enhanced by relatively low cell growth rate, different from the inducible expression system that can be enhanced by accumulating cells at high cell growth rate. The result also indicated that suitable specific cell growth rate and constant culture conditions played an important role in the scale-up process using constitutive expression system.

During the experiments, a substantial increase in lipase activity was observed when going from 30 to 800 l scale, which is still a puzzle for us. It is probable that proteolytic degradation played an important role in the lipase activity increase in large-scale fermentation. The μ-stat strategy combining a pH and temperature shift at 48 h was used in the large-scale fermentation, which made excellent balance between the expression of target protein and cell growth. Controlling a relatively low cell growth rate would greatly decrease cell lyses, lowering protease activity in the 800-l fermentor.

Acknowledgments This work was funded by the Science and Technology Foundation of Guangzhou under Grant 2003Z3-E0161. The authors thank Jiangmen Sugarcane chemical factory (Group) Co., Ltd for providing fermentation instruments and technical assistant.

References

1. Bei JL, Wang JW, Wang XZ, Long QX, Yang L, Deng YY (2003) High expression of LIP1 in *Pichia pastoris*. ACTA Biochimi et Biophys Sin 35:366–370
2. Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell 12:323–337
3. Chang SW, Shieh CJ, Lee GC, Akoh CC, Shaw JF (2006) Optimized growth kinetics of *Pichia pastoris* and recombinant *Candida rugosa* LIP1 production by RSM. J Mol Microbiol Biotechnol 11:28–40
4. Enfors S-O, Jahic M, Rozkov A, Xu B, Hecker M, Ju¨rgen B, Kru¨ger E, Schweder T, Hamer G, O'Beirne D, Noisommit-Rizzi N, Reuss M, Boone L, Hewitt C, McFarlane C, Nienow A, Kovacs T, Tra¨ga°rdh C, Fuchs L, Revstedt J, Friberg PC, Hjertager B, Blomsten G, Skogman H, Hjort S, Hoeks F, Lin H-Y, Neubauer P, Lans Rvd, Luyben K, Vrabel P, A M (2001) Physiological responses to mixing in large scale bioreactors. J Biotechnol 85:175–185
5. Foss DL, Bennaars AM, Pennell CA, Moody MD, Murtaugh MP (2003) Differentiation of porcine dendritic cells by granulocyte–macrophage colony-stimulating factor expressed in *Pichia pastoris*. Vet Immunol Immunopathol 91:205–215
6. Gasser B, Maurer M, Gach J, Kunert R, Mattanovich D (2006) Engineering of *Pichia pastoris* for improved production of antibody fragments. Biotechnol Bioeng 94:353–361
7. Hohenblum H, Borth N, Mattanovich D (2003) Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry. J Biotechnol 102:281–290
8. Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO (2006) Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. Biotechnol Prog 22:1465–1473
9. Jenzsch M, Gnoth S, Beck M, Kleinschmidt M, Simutis R, L¨ubbert A (2006) Open-loop control of the biomass concentration within the growth phase of recombinant protein production processes. J Biotechnol 127:84–94
10. Jenzsch M, Gnoth S, Kleinschmidt M, Simutis R, bbert AL (2006) Improving the batch-to-batch reproducibility in microbial cultures during recombinant protein production by guiding the process along a predefined total biomass profile. Bioprocess Biosyst Eng 29:315–321

11. Marc C. d'Anjou, Daugulis JA (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol Bioeng 72:1–11
12. Mattanovich D, Gasser B, Hohenblum H, Sauer M (2004) Stress in recombinant protein producing yeasts. J Biotechnol 113:121–135
13. Sambrook J, Russell DW (2001) Molecular cloning : a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
14. Schmidt FR (2005) Optimization and scale up of industrial fermentation processes. Appl Microbiol Biotechnol 68:425–435
15. Soons ZI, Voogt JA, van Straten G, van Boxtel AJ (2006) Constant specific growth rate in fed-batch cultivation of *Bordetella pertussis* using adaptive control. J Biotechnol 125:252–268
16. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM (2005) Maximization of production of secreted recombinant proteins in *Pichia pastoris* fed-batch fermentation. Biotechnol Prog 21:386–393