J Ind Microbiol Biotechnol (2004) 31: 525–530
DOI 10.1007/s10295-004-0182-1

ORIGINAL PAPER

Li Gao · Jian-He Xu · Xin-Jun Li · Zuo-Zhen Liu

Optimization of *Serratia marcescens* lipase production for enantioselective hydrolysis of 3-phenylglycidic acid ester

Received: 12 March 2004 / Accepted: 13 September 2004 / Published online: 11 November 2004

Abstract Lipase production and cell growth of *Serratia marcescens* ECU1010 were optimized in shake flasks, with lipase production being enhanced 9.5-fold (4,780 U/l) compared with the initial activity (500 U/l). Optimal carbon and nitrogen sources were Tween-80 and peptone, and the optimal ratio of Tween-80 to peptone was 1:3. The optimized cultivation conditions were 25°C and pH 6.5. Lipase activity, particularly specific activity, could be improved by decreasing the cultivation temperature from 35 to 25°C. Enzyme stability was significantly improved by simple immobilization with synthetic adsorption resin no. 8244. After five reaction cycles, enzyme activity decreased only very slightly, while enantioselectivity of the preparation remained constant, and the ee_s (enantiomeric excess of the remaining substrate) achieved in all cases was higher than 97%. The resin-8244-lipase preparation can be used for efficient enantioselective hydrolysis of trans-3-(4′-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM], a key intermediate in the synthesis of Diltiazem.

Keywords *Serratia marcescens* ECU1010 · Lipase biosynthesis · Medium optimization · Enzyme immobilization · Synthetic resin

L. Gao · J.-H. Xu (✉)
Laboratory of Biocatalysis and Bioprocessing,
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology,
130 Meilong Road, Shanghai, 200237, China
E-mail: jianhexu@ecust.edu.cn
Fax: +86-21-64252250

X.-J. Li · Z.-Z. Liu
Laboratory of Bioseparation Engineering,
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology,
130 Meilong Road, Shanghai, 200237, China

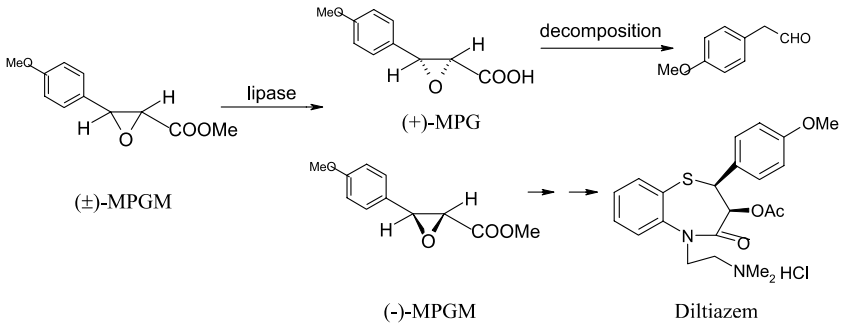
Introduction

Chiral 3-phenylglycidic acid ester compounds are high-value intermediates in the synthesis of enantiopure Diltiazem hydrochloride, which has been widely used in more than 100 countries as a coronary vasodilator [15]. Synthesis of Diltiazem hydrochloride via enantioselective hydrolysis of trans-3-(4′-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM] is a more efficient process than conventional chemical synthesis, and lipases play an important role because of their practical applications in industry for resolution of (±)-MPGM (Scheme 1). Both commercial lipases and a lipase from *Serratia marcescens* Sr 41 8000 have been applied in practice [6, 9, 13, 14, 17].

Lipases are produced by a widespread range of microorganisms, including bacteria, fungi and yeasts [16]. In our laboratory, an extracellular lipase produced by *S. marcescens* ECU1010 was shown to be a potentially useful biocatalyst for the kinetic resolution of (±)-MPGM. Thus, it was considered a promising enzyme for future application in chiral synthesis. However, systematic optimization of the enzyme is necessary for its practical utilization because initial yields were relatively low (500 U/l), and another disadvantage, poor enzyme stability, has to be overcome. Although numerous papers have been published on fermentation processes for lipase production, only a few apply to *S. marcescens* lipase [1, 7, 10, 19].

In this paper, we investigated the effect of medium composition and cultivation temperature on cell growth and extracellular lipase accumulation of *S. marcescens* ECU1010, with the main goal of increasing enzyme production. Tween-80 was first selected as a single carbon source for *S. marcescens* extracellular lipase production. *S. marcescens* 532 S produces both extracellular lipase and extracellular protease [7]. We aimed to minimize extracellular protease and to increase extracellular lipase secretion by controlling cultivation temperature and pH. Moreover, the lipase was

Scheme 1 Lipase-catalyzed resolution of trans-3-(4′-methoxyphenyl)glycidic acid methyl ester [(±)- MPGM] in the synthesis of enantiopure Diltiazem



immobilized by simple adsorption from culture broth supernatant onto a synthetic resin (no. 8244) without any pretreatment. Enzyme stability was significantly improved, with a half-life (238 h at 30°C) much longer than that (127 h at 22°C) reported for *S. marcescens* Sr 41 8000 lipase immobilized on a hydrophilic membrane [14].

Materials and methods

Chemicals

(±)-MPGM was prepared from 4-anisaldehyde and methyl chloroacetate in the presence of sodium methoxide, and purified by recrystallization [5]. *p*-Nitrophenyl acetate (*p*NPA) was prepared using the method described by Wynn et al. [20]. A synthetic resin (GMA-EDMA copolymer, no. 8244) was kindly donated by Shanghai Huazhen Scientific and Technological Co., China. All other chemicals were obtained commercially and were of reagent grade.

Microorganism, medium and culture conditions

The strain of *S. marcescens* ECU1010 used in this work was a Gram-negative bacterium stored in our laboratory and maintained on agar slants in medium containing (per liter): peptone 5 g; yeast extract 5 g; KH_2PO_4 9.1 g; K_2HPO_4 5.7 g; $MgSO_4$ 0.5 g; NaCl 0.5 g; $FeSO_4{\cdot}7H_2O$ 0.01 g; agar 20 g. The pH of the medium was adjusted to 6.5 before autoclaving.

Seed cultures were prepared by inoculating 30 ml of the above medium without agar in a 250-ml flask with cells grown on an agar slant, with subsequent incubation for 12 h at 30°C with shaking (150 rpm). The flask culture experiments were performed at 30°C and 150 rpm in 250-ml flasks containing 30 ml medium, inoculated with 2.5% (v/v) of the seed culture. An initial medium, designed according to a reference medium [14], comprised (per liter): peptone 10 g; yeast extract 10 g; dextrin 10 g; $CaCl_2$ 0.1 g; KH_2PO_4 1.0 g; $MgSO_4$ 0.5 g; NaCl 0.5 g; $FeSO_4{\cdot}7H_2O$ 0.01 g; Tween-80 5.0 g. The composition of other media used are described in the corresponding figure or table legends.

To determine the optimal culture pH, the medium was adjusted to different pH values by varying the ratio of KH_2PO_4 to K_2HPO_4 (keeping the PO_4^{3-} concentration in the medium at 0.1 M). Under the culture conditions used, the pH was effectively maintained around the initial value.

The culture broth was centrifuged to obtain a clear supernatant, which was used for all assays.

Analytical methods and enzyme assay

Cell concentration was determined by measuring the optical density at 600 nm and calibrated to dry cell weight (DCW).

Extracellular lipase activity was measured spectrophotometrically using *p*NPA as substrate. Lipase or blank solution (100 μl) was added to 2.870 ml 100 mM potassium phosphate buffer (KPB, pH 7.0). After preincubation at 25°C for 3 min, the reaction was initiated by a quick mixing of the reaction mixture with 30 μl 100 mM *p*NPA solution in dimethylsulfoxide (DMSO) and the variation in absorbance at 405 nm was recorded. One unit of esterase activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute.

Lipase immobilization was accomplished by adding 2 g resin-8244 to 10 ml culture supernatant. The mixture was shaken for 2 h at 30°C and 160 rpm. The enzyme-loaded resin was then filtered and washed thoroughly with 200 mM Tris-HCl buffer (pH 8.0). The immobilized enzyme was stored at 4°C prior to use.

The activity of the immobilized lipase was measured in a toluene:buffer (1:1) two-phase system using (±)-MPGM as substrate [13]. To 5 ml 0.2 M Tris-HCl buffer (pH 8.0) containing the immobilized lipase, was added 5 ml toluene solution of 100 mM (±)-MPGM. The reaction in the two-phase system was carried out at 30°C and 160 rpm in 100-ml flasks stoppered with tight plugs. The concentrations of optical isomers were determined by HPLC with a chiral column (Chiralcel OJ, 25×ϕ 4.6 cm, Daicel Chemical Industries, Tokyo, Japan), with elution by *n*-hexane:isopropanol (60: 40, v/v; 0.8 ml/min) and detection at 254 nm. Concentrations of residual (+)- and (−)-MPGM were calculated from areas of the two enantiomer peaks (at 13.5 and 15.7 min, respectively) using a calibration curve. The enantiose-

lectivity was expressed as the E value, calculated from the enantiomeric excess of the remaining ester (ee_s) and the conversion degree (c) based on an equation reported by Chen et al. [4]: $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$.

Stability experiments

The pH stability was examined by adjusting the culture supernatant to the specified pH, incubating at 30°C for 4 h and then measuring the residual enzyme activity spectrophotometrically under standard conditions as described above. The activity measured immediately before incubation was defined as 100%.

To examine the effect of temperature on stability of the enzyme, culture supernatant was held for 24 h at 25, 30 or 35°C, before measuring residual activity.

Results and discussion

Lipase activity of *S. marcescens* ECU1010 on an initial medium

The extracellular lipase produced by *S. marcescens* ECU1010 was first examined by cultivation of the bacterium in an initial medium as described in Materials and methods. The initial medium was designed with reference to the literature [14], and gave an extracellular lipase production of merely 500 U/l. This prompted us to optimize medium composition as well as culture conditions to improve production of bacterial lipase.

Effect of carbon source on lipase production and cell growth

A range of different carbon sources, mainly carbohydrates, alcohols, organic acids and lipids, was screened for their capacity to support cell growth and lipase production of *S. marcescens* ECU1010. Ammonium sulfate was used as a nitrogen source to avoid interference of carbon compounds in organic nitrogen sources (Table 1).

Table 1 Effect of carbon source on lipase production and cell growth. The medium comprised (g/l): $(NH_4)_2SO_4$ (5), KH_2PO_4 (9.1), K_2HPO_4 (5.7), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), and 5.0 g of each carbon source (pH 6.5). The cultures were carried out in 250-ml flasks containing 30 ml medium, shaken for 24 h at 30°C and 150 rpm. Values are the average of two independent cultures ± SD

Carbon source (5 g/l)	Dry cell weight (g/l)	Lipase activity (U/l)
Tween-80	1.0 ± 0.1	1,210 ± 11
Olive oil	3.8 ± 0.2	750 ± 61
Oleic acid	2.8 ± 0.2	370 ± 58
Glucose	1.7 ± 0.1	333 ± 33
Dextrin	0.4 ± 0.1	315 ± 102
Glycerol	2.0 ± 0.1	313 ± 33

On the basis of the activity assay, it was concluded that better growth, but lower activity, was obtained on media supplemented with glucose, oleic acid, glycerol and olive oil. As indicated in Table 1, low biomass and low enzyme activity were obtained with dextrin, whereas a non-ionic surfactant, Tween-80, supported cell growth with moderate biomass (1.0 g/l) but yielding the maximum lipase activity (1,210 U/l).

Supplementation with Tween-80, olive oil, or oleic acid, yielded enzyme activities of 1,210, 640, and 330 U/l, respectively. Tween-80 was better than olive oil and oleic acid, which was similar to observations of *Acinetobacter radioresistens* lipase production [12]. A possible reason is that high oleic acid concentration could repress enzyme production. Lipase is known to have higher activity at an oil-water interface than in homogeneous solution—a phenomenon known as interfacial activation—thus the hydrolysis rate of water-miscible Tween-80 was slower than that of water-immiscible olive oil by *S. marcescens* lipase. Using Tween-80 (polyoxyethylene sorbitan monooleate) as sole carbon source, the oleic acid did not accumulate to a concentration high enough to repress enzyme production [2, 12]. Tween-80 provided oleic acid for cell growth through a mode of controlled release (via enzymatic hydrolysis) and showed no significant repression of enzyme production, so it was used as the sole carbon source in subsequent cultures.

Effect of nitrogen source on lipase production and cell growth

Different nitrogen sources (either organic or mineral) were tested at a fixed concentration (5 g/l) of Tween-80. Both the cell growth and enzyme activity were greatly affected (Table 2). The bacterium did not grow in the medium containing $NaNO_3$. Among other nitrogen sources, the biomass varied from 1.0 g/l on $(NH_4)_2SO_4$ to 1.8 g/l on yeast extract; while the enzyme activity increased from 750 u/l on yeast extract to 1,770 u/l on peptone. Good cell growth but low enzyme activity was obtained with yeast extract, so it was omitted in the

Table 2 Effect of nitrogen source on lipase production and cell growth. The culture medium comprised (g/l): Tween-80 (5.0), KH_2PO_4 (9.1), K_2HPO_4 (5.7), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), and 5.0 g of each nitrogen source (pH 6.5). The cultures were carried out in 250-ml flasks containing 30 ml medium and shaken for 24 h at 30°C and 150 rpm. Values are the average of two independent cultures ± SD

Nitrogen source (5 g/l)	Dry cell weight (g/l)	Lipase activity (u/l)
Peptone	1.6 ± 0.1	1,770 ± 77
$(NH_4)_2SO_4$	1.0 ± 0.1	1,210 ± 11
Yeast extract (YE)	1.8 ± 0.1	750 ± 77
$NaNO_3$	No growth	–
$(NH_4)_2SO_4$ + peptone (1:1)	1.2 ± 0.1	1,570 ± 21
Peptone + YE (1:1)	1.6 ± 0.0	1,260 ± 135
$(NH_4)_2SO_4$ + YE (1:1)	1.3 ± 0.1	990 ± 80

subsequent cultures. A possible mechanism may be that yeast extract is a complex nitrogen source and thus requires the cells to secrete more protease for its enzymatic degradation before utilization. This would result in less production and more degradation of the extracellular lipase. Peptone gave the highest activity of 1,770 u/l, but the specific activity was not the highest as compared to that on a mixed nitrogen source [$(NH_4)_2SO_4$ + peptone]. Since *S. marcescens* produces an extracellular lipase and the total enzyme activity is more important for practical application, so peptone was selected as the optimal nitrogen source due to its larger capacity of supporting the lipase production.

Effect of pH

The stability of the crude enzyme in the culture medium was shown to decrease with either increasing or decreasing pH values. The enzyme was relatively stable at pH 6.5–7.0, with 90% (at pH 6.5) or 92% (at pH 7.0) of the initial activity remaining after 4 h at 30°C. The enzyme activity decreased more sharply at high pH than at low pH, the residual activity being 79% at pH 5.0, but only 57% at pH 9.0, and 28% at pH 9.5. Purified *S. marcescens* lipase has been reported to be relatively stable at pH 5–9 [18], so there may be two mechanisms behind the effect of pH on the activity of the crude enzyme in the culture medium, especially at pH > 7.0. The first may be ascribed to stability of the enzyme itself, and the second to degradation of the lipase protein by proteases, which may be more active at pH > 7.0. Proteases are known to be secreted or released from dead cells.

On the basis of the above stability assay, medium at a pH of 6.5–7.0 was considered as suitable for enzyme accumulation. This hypothesis was confirmed by experiments in shake flask cultures (Fig. 1). Variation of the cultivation pH between 5 and 8 caused much larger differences in enzyme activity (250–1,640 U/l) than in biomass (1.2–1.5 g/l).

At pH 6.5, the maximum biomass, enzyme activity and specific activity obtained were 1.5 g/l, 1,640 U/l and 1,120 U/g, respectively. The enzyme activity decreased to 810 U/l at pH 5.0 or 250 U/l at pH 8.0, indicating that this strain of bacterium prefers a slightly acidic pH for lipase production, and these conditions were also suitable for the stabilization and accumulation of extracellular lipase. Thus, the optimal pH for lipase production, which is considered to be the cumulative effect of lipase synthesis and deactivation or degradation, was 6.5.

Effect of carbon/nitrogen ratio on lipase production and cell growth

Based on the above results, Tween-80 and peptone were selected as carbon and nitrogen sources, respectively. To optimize the ratio of Tween-80 to peptone, different concentrations of peptone were tested at a given concentration of Tween-80 (1.5 g/l). The results are shown in Fig. 2.

Both the final biomass and enzyme activity increased when peptone concentration increased, but the trends were quite different, and the specific activity was not constant. At a constant Tween-80 concentration, an increase in peptone concentration resulted in a nearly linear growth of biomass (0.082–0.873 g/l), indicating that peptone was a limiting growth factor under the conditions used. On the other hand, the enzyme activity significantly increased from 88 to 1,040 U/l when peptone concentration was increased from 0.5 to 4.5 g/l.

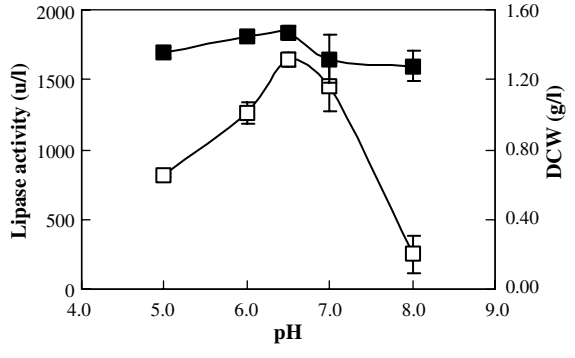


Fig. 1 Effect of medium pH on lipase production and cell growth. The medium comprised (g/l): Tween-80 (5), peptone (5), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01). The medium was adjusted to different pH values by adjusting the ratio of KH_2PO_4 to K_2HPO_4 (keeping the PO_4^{3-} concentration in the medium at 0.1 M). The cultures were carried out in 250-ml flasks containing 30 ml medium and shaken for 24 h at 30°C and 150 rpm. Values shown are the average from two independent cultures ± SD. *Open squares* Lipase activity, *filled squares* dry cell weight

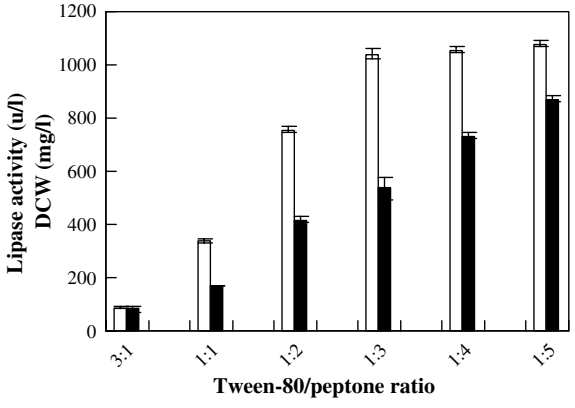


Fig. 2 Effect of carbon/nitrogen ratio on lipase production and cell growth. The medium comprised (g/l): Tween-80 (1.5), KH_2PO_4 (9.1), K_2HPO_4 (5.7), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01); pH 6.5. The peptone concentrations used were 0.5, 1.5, 3.0, 4.5, 6.0 and 7.5 g/l. Cultures were carried out in 250-ml flasks containing 30 ml medium and shaken for 24 h at 30°C and 150 rpm. Values shown are the average from two independent cultures ± SD. *Open squares* Lipase activity, *filled squares* dry cell weight

An increase in specific activity from 1,010 to 1,990 U/g was also observed as the peptone concentration increased from 0.5 to 1.5 g/l. Between 1.5 and 4.5 g/l the specific activity was no longer sensitive to peptone concentration; however, the specific activity of the enzyme decreased sharply from 1,940 to 1,240 U/g as the peptone concentration was further increased from 4.5 to 7.5 g/l, although the decrease in specific activity was compensated by an increase in biomass, resulting in a slight increase in total enzyme activity (1,040–1,080 U/l). Therefore, the optimal ratio of Tween-80 to peptone is 1:3.

Effect of temperature

The stability of crude enzyme in the culture medium decreased with increasing temperature. After incubation for 12 h at 35, 30 or 25°C, residual activities were 46, 61 and 75%, respectively, and, after 24 h, residual activity dropped further to 39, 47 and 64%, respectively. It has been reported that purified *S. marcescens* lipase is relatively stable at temperatures up to 50°C [18]. Thus, the action of proteases may be responsible for the remarkable difference in thermal stability of the crude enzyme between 25 and 35°C. It was therefore considered that a lower cultivation temperature might favor accumulation of the active lipase protein secreted by *S. marcescens* cells in shake-flask culture. As expected, the detected activity of the extracellular lipase was significantly improved when the culture temperature was lowered from 30 to 25°C (Fig. 3), although it decreased if the temperature was further lowered to 20°C (data not shown). Figure 3 shows that this strain of bacterium produces and accumulates extracellular lipase optimally at 25°C, with the highest enzyme activity reaching as much as 4,780 U/l. Both enzyme activity and biomass were much higher at 25°C as compared to 30 and 35°C. Moreover, the specific activity (930 U/g) at 25°C was also the highest, while those at 30 and 35°C were only 840 and 760 U/g. The optimal temperature for growth and lipase production was 25°C, as also reported for *Cryptococcus* sp. S-2 extracellular lipase [11]. It has been reported that production of heterologous laccase expressed by *Saccharomyces cerevisiae* cultivated in shake-flasks was improved by lowering the temperature to approximately 19°C [3], and a decrease in the cultivation temperature of *Pichia pastoris* also improved recombinant laccase activity, particularly specific activity [8]. The reason for this effect of fermentation temperature on activity, especially specific activity of the crude enzyme in the culture medium, may be ascribed to the poor stability of the extracellular lipase, the release of more proteases from dead cells, and higher protease reaction rate at higher temperatures. Thus, cultivation at 25°C results in less degradation of the secreted lipase compared to that at 30 and 35°C [8].

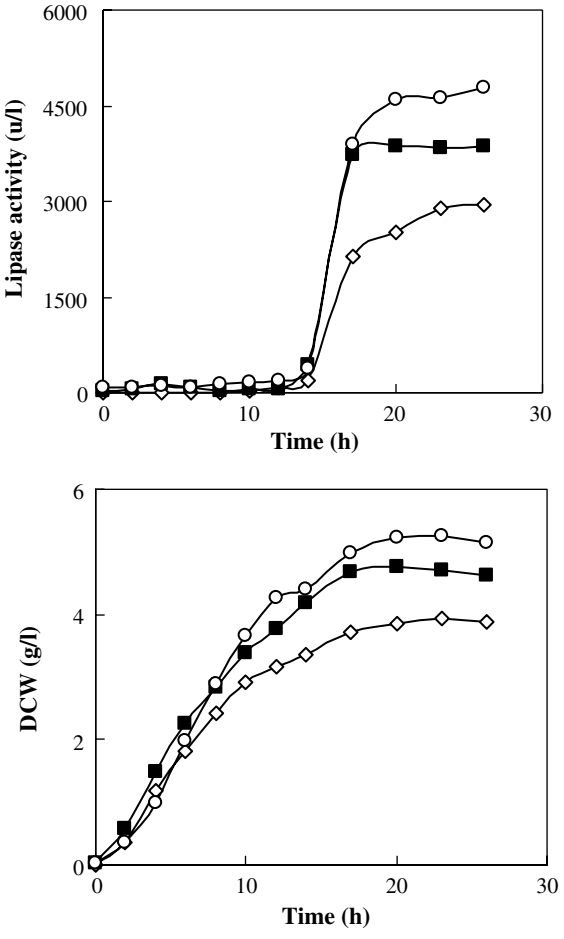


Fig. 3 Time-courses of lipase production and cell growth at different temperatures. The medium comprised (g/l): Tween-80 (10), peptone (30). KH_2PO_4 (9.1), K_2HPO_4 (5.7), $MgSO_4$ (0.5), NaCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01); pH 6.5. Cultures were carried out in 250-ml flasks containing 30 ml medium and shaken for 24 h at 150 rpm. Values shown are the average from two independent cultures. *Open diamonds* 35°C, *filled squares* 30°C, *open circles* 25°C

Immobilization and reuse of enzyme

The above results show that both the pH stability and thermal stability of the crude enzyme in culture supernatant were very poor. For this reason, the extracellular lipase was simply immobilized by direct adsorption from cultivation supernatant, without any pretreatment, onto a synthetic resin (no. 8244). Through this simple treatment, five cycles of hydrolysis of the racemic compound (±)-MPGM could be performed in a toluene-water two-phase system using the resin-8244-immobilized enzyme prepared from 10 ml culture supernatant (Fig. 4). After five reaction cycles, the residual enzyme activity was still above 80%, the enantioselectivity of the preparation remained constant, and the ee_s in all cases was higher than 97%, giving an *E*-value of 85 ± 10, which indicates a high enantioselectivity of the lipase toward the (+)-MPGM. As calculated from Fig. 4, the half-life of enzyme activity

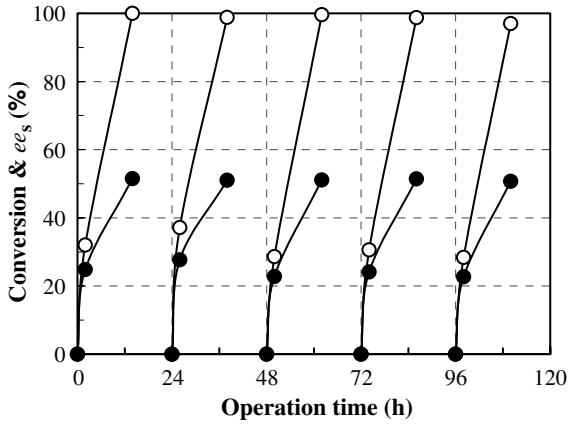


Fig. 4 Cycles of hydrolysis of compound (±)-MPGM in a toluene-water two-phase system catalyzed by resin-8244-immobilized enzyme prepared from 10 ml culture supernatant. Experiments were performed using a substrate concentration of 100 mM in 5 ml toluene solution and 5 ml 200 mM Tris-HCl buffer (pH 8.0). The reactions were carried out at 30°C and 160 rpm in 100-ml flasks equipped with tight plugs. *Filled circles* Conversion, *open circles* ee_s

at 30°C was 238 h, which is much longer than that reported for *S. marcescens* Sr 41 8000 lipase immobilized on a hydrophilic membrane (127 h at 22°C) [14].

Conclusions

The extracellular lipase produced by *S. marcescens* ECU1010 was demonstrated to be promising for the production of enantiopure 3-phenylglycidate, a key intermediate in the synthesis of Diltiazem hydrochloride. However, the relatively low yield and poor stability of the enzyme in the culture supernatant restricted its practical application on a large scale. Production of the lipase by *S. marcescens* was influenced by several factors, including the carbon source, nitrogen source, medium pH, cultivation temperature and protease activity. In this work, enzyme production was markedly enhanced by optimization of culture medium composition, pH and temperature. A simple immobilization treatment significantly improved enzyme stability. The immobilized enzyme was an efficient biocatalyst for asymmetric hydrolysis of (±)-MPGM. Further work is needed to optimize the immobilization process and the hydrolysis reaction system, and to set up a bioreactor for large-scale preparation.

Acknowledgements This work was financially supported by the National Natural Science Foundation of China (grant nos. 20272013 and 203900506), the Ministry of Education, P.R. China (a program for supporting excellent young teachers) and the Science and Technology Commission of Shanghai Municipal Government (grant no. 02QMF1412).

References

1. Bashkatova NA, Severina LO (1979) Effect of culturing conditions on lipase biosynthesis in *Serratia marcescens*. Mikrobiologiya 48:826–832
2. Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM, Turkenburg JP, Bjorking F, Huge-Jensen B, Patakar SA, Thim L (1991) A model for interfacial activation in lipase from the structure of a fungal lipase-inhibitor complex. Nature 351:491–494
3. Cassland P, Jönsson LJ (1999) Characterization of a gene encoding *Trametes versicolor* laccase A and improved heterologeous expression in *Saccharomyces* by decreased cultivation temperature. Appl Microbiol Biotechnol 52:393–400
4. Chen CS, Fujimoto Y, Girdaukas G, Sih CJ (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. J Am Chem Soc 104:7294–7299
5. Crotti P, Terretti M, Macchia F, Stoppioni A (1986) Ring-opening reaction of cis- and trans-2,3-bis(4-methoxybenzyl)oxirane: competition between assistance by and migration of an aryl group. J Org Chem 51:2759–2766
6. Dodds DR, Lopez JL (1993) Enzymatic hydrolysis of glycidate esters in the presence of bisulfite anion. US Patent 5274300
7. Henriette C, Zinebi S, Aumaitre MF, Petitdemange E, Petitdemange H (1993) Protease and lipase production by a strain of *Serratia marcescens* (532 S). J Ind Microbiol 12:129–135
8. Hong F, Meinader NQ, Jönsson LJ (2002) Fermentation strategies for improved heterologeous expression of laccase in *Pichia pastoris*. Biotechnol Bioeng 79:438–449
9. Hulshof LA, Roskan JH (1989) Phenylglycidate stereoisomers, conversion products thereof with e.g. 2-nitrothiophenol and preparation of diltiazem. European Patent Application 343714
10. Jaeger E, Winkler UK (1979) Methyl ester of hyaluronate is unable to stimulate exolipase formation by *Serratia marcescens*. J Bacteriol 139:1065–1067
11. Kamini NR, Fujii T, Kurosu T, Iefuji H (2000) Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2. Process Biochem 36:317–324
12. Li CY, Cheng CY, Chen TL (2001) Production of *Acinetobacter radioresistens* lipase using Tween-80 as the carbon source. Enzyme Microb Technol 29:258–263
13. Matsumae H, Furui M, Shibatani T (1993) Lipase-catalyzed asymmetric hydrolysis of 3-phenylglycidic acid ester, the key intermediate in synthesis of Diltiazem hydrochloride. J Ferment Bioeng 75:93–98
14. Matsumae H, Furui M, Shibatani T, Tosa T (1994) Production of optically active 3-phenylglycidic acid ester by the *S. marcescens* lipase on a hollow-fiber membrane reactor. J Ferment Bioeng 78:59–63
15. Nagao T, Sato M, Nakajima H, Kiyomoto A (1972) Studies on a new 1,5-benzothiazepine derivative (CRD-401). II. Vasodilator actions. Jpn J Pharmacol 22:1–10
16. Rapp P, Backhaus S (1992) Formation of extracellular lipases by filamentous fungi, yeasts and bacteria. Enzyme Microb Technol 14:938–943
17. Shibatani T, Nakamichi K (1990) Method for preparing optically active 3-phenylglycidic acid esters. European Patent Application 362556
18. Shibatani T, Matsumae H, Akatsuka H (1991) Esterase from *Serratia* and process for preparing the same. European Patent Application 446771
19. Ulrich KW, Martina S (1979) Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J Bacteriol 138:663–670
20. Wynn J E, Caldwell ML, Robinson JR, Beamer R L, Baugueess CT (1982) Synthesis and bioevaluation of a series of fatty acid esters of *p*-(N, N-bis(2-chloroethyl)amino)phenol. J Pharm Sci 72:772–776