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ORIGINAL PAPER

Overexpression and characterization in *Bacillus subtilis* of a positionally nonspecific lipase from *Proteus vulgaris*

Yaping Lu · Qian Lin · Jin Wang · Yufan Wu · Wuyundalai Bao · Fengxia Lv · Zhaoxin Lu

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Abstract A *Proteus vulgaris* strain named T6 which produced lipase (PVL) with nonpositional specificity had been isolated in our laboratory. To produce the lipase in large quantities, we cloned its gene, which had an opening reading frame of 864 base pairs and encoded a deduced 287-amino-acid protein. The *PVL* gene was inserted into the *Escherichia coli* expression vector pET-DsbA, and active lipase was expressed in *E. coli* BL21 cells. The secretive expression of *PVL* gene in *Bacillus subtilis* was examined. Three vectors, i.e., pMM1525 (xylose-inducible), pMMP43 (constitutive vector, derivative of pMM1525), and pHPQ (sucrose-inducible, constructed based on pHB201), were used to produce lipase in *B. subtilis*. Recombinant *B. subtilis* WB800 cells harboring the pHPQ-PVL plasmid could synthesize and secrete the PVL protein in high yield. The lipase activity reached 356.8 U/mL after induction with sucrose for 72 h in shake-flask culture, representing a 12-fold increase over the native lipase activity in *P. vulgaris*. The characteristics of the heterologously expressed lipase were identical to those of the native one.

Y. Lu · J. Wang · Y. Wu
College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

W. Bao · F. Lv · Z. Lu (✉)
College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, People's Republic of China
e-mail: fmb@njau.edu.cn

Q. Lin
Department of Chemistry and Biology, Yulin Normal College, Yulin 537000, Guangxi, People's Republic of China

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Introduction

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triglycerides at the interface between the aqueous and lipid phases [1]. Microbial lipases are a class of useful enzymes that have practical and potential applications in the detergent, food-processing, bioenergy, organic synthesis, and pharmaceutical industries [2, 3]. Several bacterial lipases have been purified and biochemically characterized, and their corresponding genes have been cloned and sequenced [4–7].

The application of alkaline lipases in the detergent and leather-processing industries has gained great interest in recent times [2]. A lipase that does not exhibit any positional specificity may markedly improve the efficiency of hydrolysis and transesterification. In addition, high activity and high productivity are two most desirable properties of lipases for industrial applications. We had previously isolated and maintained a *Proteus vulgaris* strain that produced an alkaline non-position-specific lipase (data not shown). The aim of this study is to overproduce the *P. vulgaris* lipase in a heterologous host.

Molecular cloning and overexpression of lipase genes in *Escherichia coli* have been reported by several researchers in recent times [8–10]. However, in most of these studies, the overexpressed lipases formed inactive inclusion bodies [11–13]. In our initial experiments, inclusion bodies were formed when the *PVL* gene was expressed in *E. coli* expression system under the control of the T7 promoter

cultured at 30°C. When the culture temperature was reduced, the amount of inclusion bodies decreased and the enzyme activity was enhanced.

We investigated the *Bacillus subtilis* expression system for secretive expression of the *P. vulgaris* lipase. To the best of our knowledge, there are few reports on expression of lipase genes in *B. subtilis*. Jørgensen et al. [14] reported expression of *Pseudomonas cepacia* lipase under the control of the alpha-amylase gene promoter, with productivity ranging from 30 to 40 U/mL in this system. Expression of the *P. vulgaris* lipase in *B. subtilis* has not yet been reported. In this study, the *PVL* gene was inserted into two vectors (pMMP43 and pHPQ) that have been newly constructed in our laboratory, and the resulting plasmids were transformed into *B. subtilis* to obtain high yields of secreted lipase.

Materials and methods

Bacterial strains and plasmids

Proteus vulgaris T6 which produced lipase was isolated from soil samples and kept in our laboratory. T6 was cultured in LB at 30°C, and culture supernatant was used for lipase assay. *E. coli* BL 21 [F^- *omp*T *hsd*S_B ($r_B^- m_B^-$)*gal dcm*(DE3)pLysS] and *B. subtilis* WB800 [*nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg*] [15] were used as expression hosts. The *E. coli* expression vector pET-DsbA was a product of Qinbaosheng Biotechnology Co. Ltd. (Shenzhen, China). Plasmid pMM1525 [16] was purchased from MoBiTec (Germany), and pHB201 [17] was kindly provided by the Bacillus Genetic Stock Center (BGSC).

The *P43* promoter, a strong promoter of *B. subtilis*, was amplified with the primers P43-F (CTA*GCTAGC*TGAT AGGTGGTATGTTTTCGC) and P43-R (CC*TGTACA*G TGTACATTCCTCTCTTACC) using *B. subtilis* 168 genome DNA as template. pMM1525 was double-digested with *Nhe*I/*Bsr*GI to cut its promoter region and ligated with *P43* promoter to generate pMMP43.

pHPQ was a derivative of pHB201. The region containing P59 and cat86::lacZ in pHB201 was substituted with the sucrose-inducible promoter from levansucrase gene (*sacB*) and the *sacB* signal sequence [18]. The *degQ* gene directed by the *P43* promoter was inserted upstream of the *sacB* promoter. *P43* promoter, *degQ* gene, the promoter and signal peptide sequence of *sacB* from *B. subtilis* 168 were amplified and ligated by splicing by overlapping extension polymerase chain reaction (SOE-PCR) [19] with the primers presented in Table 1.

Cloning of the lipase gene from *P. vulgaris*

The following primer pair was designed to amplify the open reading frame of *PVL* gene based on the U33845 [6] sequence in GenBank: PVL-F (atgtcaactacatatccaat) and PVL-R (ttacagctttttacttgctaaga). The PCR-amplified product was gel-purified, ligated into the pMD19-T vector (Takara, Ltd.), and sequenced using a 3730 sequencer (Jinsite Co. Ltd., Nanjing, China). Both the nucleotide sequence and the deduced protein sequence were analyzed using the NCBI website. The *P. vulgaris* lipase gene was inserted into the pET-DsbA, pMM1525, pMMP43, and pHPQ plasmids.

Lipase assay

Lipase activity was determined by the method of Lee [5]. Crude enzyme solution (10 μL) was added to 2.99 mL phosphate-buffered saline (PBS) buffer (pH 7.4) containing 30 μM *p*-nitrophenyl butyrate (pNPB) as substrate. The reaction was carried out in the spectrophotometer cell followed by spectrophotometry at 410 nm. Lipase activity was calculated according to the standard curve of *p*-nitrophenol measured in the same condition. All assays were performed in triplicate. One unit of lipase activity was defined as the amount of enzyme required to produce 1 μmol *p*-nitrophenol per minute.

Gene expression in *E. coli*

LB medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl was used as both seed medium and growth medium. The recombinant plasmid pET-PVL was

Table 1 Primers used for SOE-PCR of *P43* promoter, *degQ* gene, the promoter and signal peptide sequence of *sacB*

Primer	Sequence
P1	5′ agcttactttattgttttgccattttaaaaggttcaaataaaatatttttcataaaat 3′
P2	5′ cgattttatgaaaaatattttatttgaacctttaaaatggcaaaacaataaagta 3′
P3	5′ atcgataaaaaagcccgctcattaggcgggctgctcacgaaattttcattgcataattgtatttatcg 3′
P4	5′ cattataggtaagagaggaatgtacacatggaaaagaaacttgaag aagtaaaacaattg 3′
P5	5′ caattgttttacttcttcaagtttcttttccatgtgtacattcctctcttacctataatg 3′
P6	5′ caggtatatgtgatgggttaaaaaggatcagatcttgataggtggt atgttttcgcttg 3′
P7	5′ caagcgaaaacataccacctatcaagatctgatccttttttaacccatcacatatacctg 3′
P8	5′ atccgctcgaggcaaacgcttgagttgcgcctcctg 3′

transformed into *E. coli* BL21 cells [20], and a single colony was inoculated into LB medium, followed by overnight shaking at 90 rpm under 30°C. One milliliter of this preculture fluid was inoculated into 50 mL LB medium supplemented with 1.5% glucose and 200 μg/mL ampicillin. The mixture was incubated by rotary shaking at 37°C until the cells reached the exponential growth phase. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to induce enzyme production at 15°C. Cells were harvested by centrifugation and disrupted ultrasonically. After centrifugation, the resulting supernatant was used as the enzyme solution.

Gene expression in *B. subtilis*

The plasmids pMM1525, pMM1525-PVL, pMMP43, pMMP43-PVL, pHPQ, and pHPQ-PVL were electrotransformed [21] into *B. subtilis* WB800. The recombinant *B. subtilis* cells were cultured at 37°C in MSR medium (2.5% yeast extract, 1.5% tryptone, and 0.3% K_2HPO_4) containing tetracycline (for pMM1525-PVL and pMMP43-PVL) or erythromycin (for pHPQ-PVL). Xylose or sucrose was added after the cells reached the exponential growth phase to induce lipase production. The mixture was incubated with rotary shaking for 80 h, and culture supernatant was collected at different time points after induction. The expressed lipase from *PVL* gene in WB800 was calculated as lipase activity in WB800 with *PVL* gene minus lipase activity in WB800 without *PVL* gene. The growth curve of recombinant strain WB800 [pHPQ-PVL] was measured according to the absorbance value at 600 nm with cultivation temperature of 37°C in MSR medium.

Characterization of native and recombinant lipase from *B. subtilis*

The optimum temperature for the enzymes was determined by measuring the lipase activity at different temperatures using pNPB as substrate. The activity assay was performed in PBS (pH 7.4). The effect of temperature on lipase stability was determined by measuring residual activity after incubation for 12 h at different temperatures.

The optimum pH value was determined by measuring the lipase activity in different buffers in a pH range of 6–11 (Na_2HPO_4–KH_2PO_4 buffers in pH range 6–9 and Na_2CO_3–$NaHCO_3$ buffers in pH range 9–11). The pH stability of the lipase was measured by incubating the enzyme in the above-mentioned buffers in the pH range 6–11 for 12 h and subsequently measuring residual activity. All assays were performed in triplicate.

The positional specificity of the lipase with respect to triacylglycerols was analyzed by using thin-layer chromatography (TLC). Two milliliters of crude lipase was added to PBS buffer (pH 7.4) containing 100 mM triolein, and the reaction mixture was shaken at 180 rpm at 37°C for 2 h. The reaction products were extracted with the same volume of *n*-hexane for 1 h, and 10 μL of the products in *n*-hexane was analyzed by TLC. The silica gel plate (silica gel G, 10 cm × 20 cm, Qingdao Haiyang, China) was developed in a mixture of petroleum ether, ether, and acetic acid (80:20:0.5). 1,2-Diolein, 1,3-diolein, and triolein from Sigma were used as standards. Lipozyme was included in the experiment as a 1,3-specific lipase control [22]. Spots were visualized by iodine vapor.

Results and discussion

Cloning of the *P. vulgaris* lipase gene

An approximately 0.86-kb band was obtained by PCR using T6 genome DNA as the template. The DNA sequence encoding the mature lipase of 287 amino acids was an open reading frame of 864 bp, which was deposited to GenBank with accession number FJ643627. No signal peptide sequence was detected at the N-terminal. The nucleotide sequence of this enzyme exhibited approximately 90% identity to that of U33845, and there was a 13-amino-acid difference between these two lipases. The deduced protein sequence indicated the presence of three residues (Ser79, Asp232, and His254) in the active site of the enzyme, which formed the catalytic triad [23].

Expression of the *P. vulgaris* lipase gene in *E. coli*

The functions of the cloned *PVL* gene were examined in *E. coli*. Lipase activity was detected in BL21 [pET-PVL] cells 1 h after IPTG addition. The activity increased with time, suggesting that soluble active lipase was successfully produced in the BL21 cells. Maximum activity was observed after induction for 15 h at 15°C.

Construction of plasmid pHPQ

P43 promoter, *degQ* gene, the promoter and signal peptide sequence of levansucrase gene (*sacB*) from *B. subtilis* 168 were amplified and ligated with a fragment containing *E. coli trpA* terminator by SOE-PCR (Fig. 1). To remove *P59* promoter and *cat::lacZα* region, pHB201 was digested with *Hin*dIII and *Cla*I, and ligated with two complementary oligonucleotides P1 and P2 (Table 1), which recovered the deleted replication origin of pUC in pHB201. The resulting plasmid was designated pHB-hc. The fragment T_{trpA}-degQ-P43-P_{sacB}-SP_{sacB} was digested with *Cla*I and *Xho*I, and then inserted into pHB-hc to generate the expression vector pHPQ (Fig. 2).

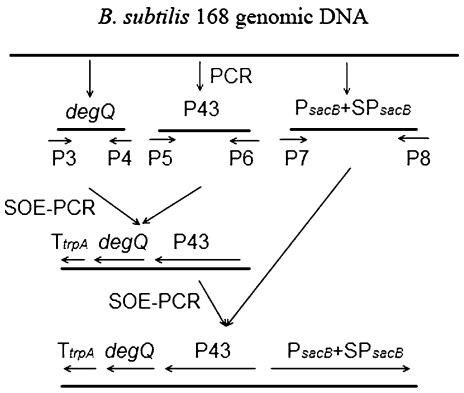

Fig. 1 Assembly of fragment T_{trpA}-degQ-P43-P_{sacB}-SP_{sacB} by SOE-PCR

Expression of the *P. vulgaris* lipase gene in *B. subtilis*

To extend the applications of this lipase in the detergent and leather-processing industries, high-level expression and secretion of the *PVL* gene was attempted in *B. subtilis*. This organism is a promising expression system that is safe and permits secretion of large amounts of proteins. However, there are no universal high-performance vectors that can be used for expression in *B. subtilis*. Expression of lipase genes in *B. subtilis* has rarely been reported. Kim et al. [24] reported that the lipase B26 gene expressed as inclusion bodies in *E. coli* was successfully expressed using a *Bacillus* expression system, with a relative low productivity of approximately 8 U/mL.

The first vector we used in *B. subtilis* was pMM1525, which had a xylose operon as the regulatory element and the signal peptide sequence of *lipA* from *Bacillus megaterium*. Lipase activity could be detected 24 h after xylose addition into culture supernatant, and the maximum activity was observed after induction for approximately 56 h. The results suggested that the *B. megaterium* plasmid could be used as an expression vector in *B. subtilis*. However, the expression efficiency of pMM1525-PVL was not high (40 U/mL). One possible explanation is that *B. subtilis* could not absorb or utilize xylose effectively. Moreover, we found that pMM1525 was not stable during the cloning and expression processes, which would limit its utility and application.

To improve the expression efficiency, we substituted the xylose promoter with the *B. subtilis P43* promoter, which led to the construction of the pMMP43 vector. The recombinant WB800 [pMMP43-PVL] cells were plated on LB medium containing 0.001% Rhodamine B and 1% emulsified olive oil, on which clear orange zones could be seen [25]. The colonies that showed large hydrolysis zones were collected for liquid culture. Lipase activity was detected after fermentation for 8 h in MSR medium, and the activity peaked at 48 h. The maximum activity was twofold that of pMM1525-PVL.

pHB201, a shuttle plasmid formed from the *B. subtilis* cryptic plasmid pTA1060 and *E. coli* plasmid pUC19, is comparatively stable during cell passage and fermentation. pHB201 was altered by substituting the region containing the *P59* promoter and cat86::lacZ with the promoter and signal sequence of *sacB*. Upstream of this, the *P43-degQ* expression cassette was inserted to enhance the efficiency of *sacB* promoter. The lipase gene was inserted at the *Xho*I site to form pHPQ-PVL. WB800 [pHPQ-PVL] was cultured in MSR medium, and sucrose was added as an inducer. In the absence of inducer, trace lipase was detected in the culture supernatant from the recombinant WB800 cells. In contrast, lipase activity was detected after 6 h of sucrose induction in culture supernatant. The activity of WB800 [pHPQ-PVL] was calculated by subtracting the lipase activity of WB800 [pHPQ] cultured under the same condition, which was at a low level (about 25 U/mL at 72 h). The enzyme activity in the culture supernatant continually increased until 72 h after induction, with maximum activity of 356.8 U/mL. The intracellular lipase activity was also determined, which was much smaller than the extracellular activity (Fig. 3). Figure 3 shows the pattern of strain growth and lipase formation in *B. subtilis*, along with the time-course profile of *PVL* expression in *E. coli* and *P. vulgaris*. Secretion of PVL began at the late logarithmic phase, and increased linearly during the stationary phase in *B. subtilis*.

The expressed PVL activity in WB800 was approximately 12-fold higher than that from the supernatant of *P. vulgaris*, which was about 27 U/mL. An expressed protein band from WB800 [pHPQ-PVL] was displayed in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was absent in WB800 [pHPQ] (Fig. 4). The intracellular lipase of WB800 harboring pHPQ-PVL was also measured, and the maximum activity was 40 U/mL. Overexpression of *PVL* gene in WB800 [pHPQ-PVL] showed that pHPQ was an efficient vector for lipase secretion.

Characterization of the recombinant lipase from *B. subtilis*

The effects of temperature and pH on the activity of the native lipase and recombinant lipase expressed in WB800 [pHPQ-PVL] were examined. Both lipases showed maximum activity at 50°C and were very stable when the temperature was below 50°C. The optimum pH of the

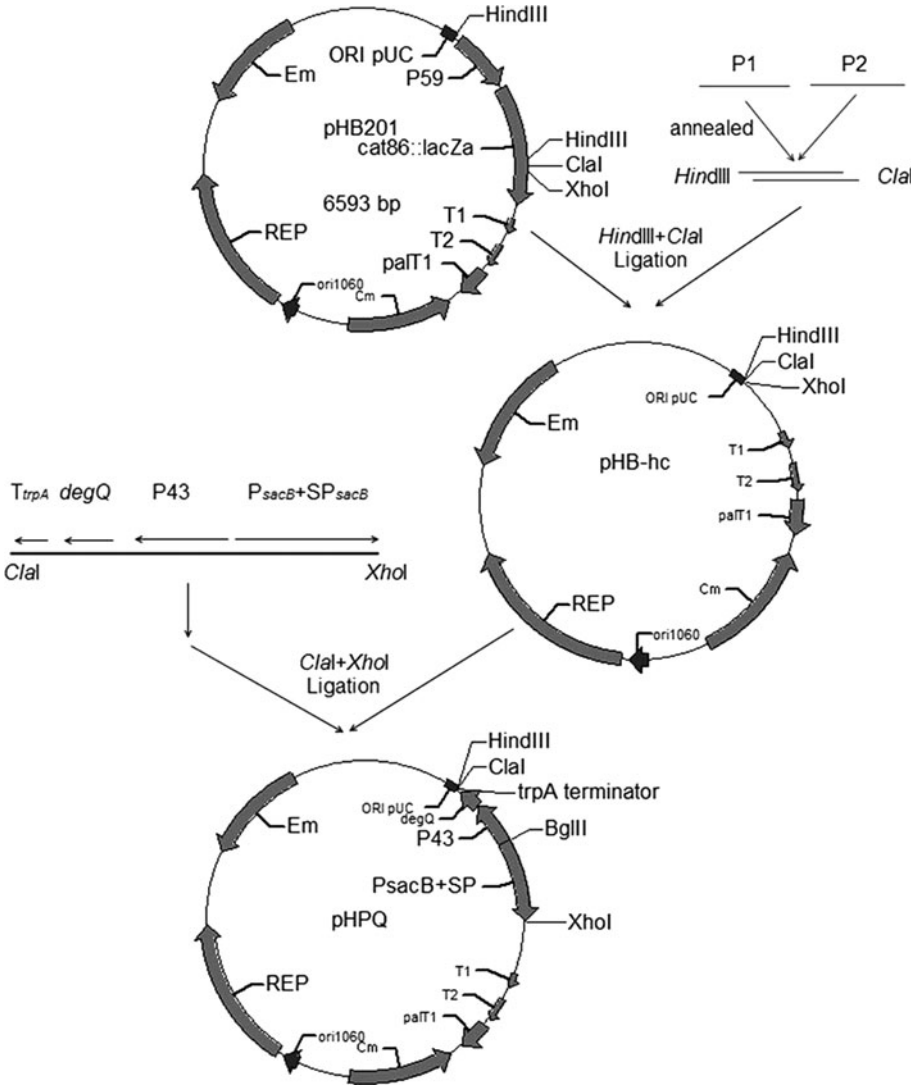

Fig. 2 Construction of pHPQ based on pHB201. The fragment T_{trpA}-*degQ*-P43-P_{sacB}-SP_{sacB} was inserted into pHB201 digested with *ClaI*/*Hind*III to generate pHPQ

lipases was 9.0. The enzyme retained high activity after incubation in buffers of different pH values for 12 h. In particular, the residual activity was more than 80% in buffers in the pH range 7–10. The characteristics of the recombinant lipase were identical to those of the native lipase from *P. vulgaris*, which displayed favorable stabilities of both temperature and pH (Fig. 5).

TLC analysis of the hydrolysis products of triolein revealed that the recombinant lipase cleaved ester bonds not only at the 1,3-position but also at the 2-position (Fig. 6). Based on this, we concluded that the recombinant lipase can cleave ester bonds nonspecifically.

Conclusions

The objective of this study is to develop a system in which large quantities of active lipase can be produced in culture medium. We cloned the *PVL* gene from *P. vulgaris* T6 and expressed it in both *E. coli* and *B. subtilis*.

For lipase expression in *B. subtilis*, we constructed and utilized two vectors, pMMP43 and pHPQ. The *P43* promoter could enhance lipase expression, and a onefold increase was observed in comparison with the expression levels observed with the P_{xyl} promoter in pMM1525. pHPQ was a comparatively stable sucrose-inducible vector with a *P43*-directing *degQ* gene inserted at the upstream of the *sacB* promoter to enhance its efficiency. Utilization of the *sacB* promoter with the *P43-degQ* cassette resulted in high levels of lipase production. The host WB800 cells were essential for high expression efficiency because they lack most extracellular proteases. The lipase in the culture supernatant was very stable and was not cleaved by proteases.

The lipase from recombinant WB800 cells was similar to the native enzyme and had optimum temperature of

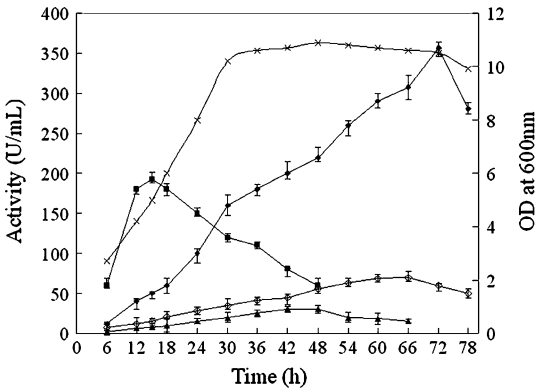

Fig. 3 Time-course profile of expression of *PVL* gene in *E. coli* (*filled squares*), *B. subtilis* (*filled diamonds* extracellular lipase, *open diamonds* intracellular lipase), and *P. vulgaris* (*filled triangles*), and the growth curve (*crosses*) of WB800 [pHPQ-PVL]. Maximum activity was 192.2 U/mL after induction by IPTG for 15 h at 15°C in *E. coli*. The extracellular PVL activity expressed in *B. subtilis* reached 356.8 U/mL at 72 h, while the maximum intracellular lipase activity of WB800 [pHPQ-PVL] was 40.0 U/mL. Maximum activity was 27.0 U/mL in *P. vulgaris*. The absorbance of WB800 [pHPQ-PVL] at OD_{600nm} was measured by inoculating overnight primary cultures into MSR medium

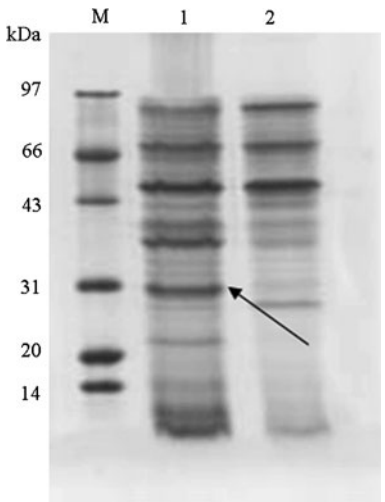

Fig. 4 SDS-PAGE of the supernatant of recombinant WB800. *Lane 1* induced expression of WB800 [pHPQ-PVL], *lane 2* induced expression of WB800 [pHPQ], *M* molecular mass markers. *Arrow* indicates the lipase protein expressed from pHPQ-PVL, which was about 31 kDa

50°C and optimum pH of 9.0. It could cleave all ester bonds of triacylglycerol.

In conclusion, the *P. vulgaris* lipase gene was successfully overexpressed in *B. subtilis*. We hope that the results of this study will provide a basis for future improvements and industrial applications of this lipase.

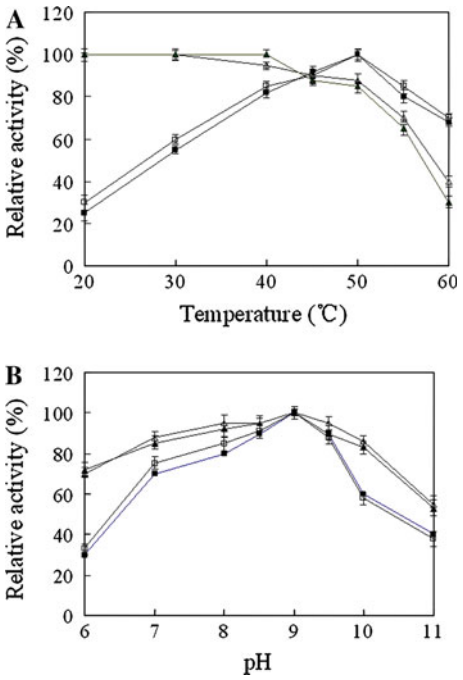

Fig. 5 Effects of temperature (**a**) and pH (**b**) on native and recombinant lipase activity and stability. **a** *Filled squares* effects of temperature on the activity of native lipase; *open squares* effects of temperature on the activity of recombinant lipase; *filled triangles* effects of temperature on the stability of native lipase; *open triangles* effects of temperature on the stability of recombinant lipase. **b** *Filled squares* effects of pH on the activity of native lipase; *open squares* effects of pH on the activity of recombinant lipase; *filled triangles* effects of pH on the stability of native lipase; *open triangles* effects of pH on the stability of recombinant lipase

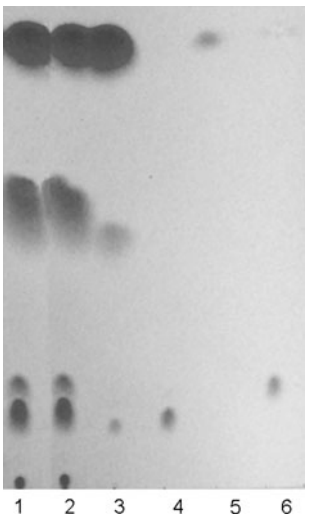

Fig. 6 Thin-layer chromatography of the hydrolysis products obtained after triolein catalysis by the lipase from WB800 [pHPQ-PVL] cells. *Lane 1* hydrolysis products of triolein catalyzed by lipase from *P. vulgaris* T6, *lane 2* hydrolysis products of triolein catalyzed by lipase from WB800 [pHPQ-PVL] cells, *lane 3* hydrolysis products of triolein catalyzed by Lipozyme, *lane 4* 1,2-diolein, *lane 5* triolein, *lane 6* 1,3-diolein

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