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PII: S1381-1177(14)00008-3
DOI: http://dx.doi.org/doi:10.1016/j.molcatb.2014.01.006
Reference: MOLCAB 2863

To appear in: *Journal of Molecular Catalysis B: Enzymatic*

Received date: 22-8-2013
Revised date: 30-11-2013
Accepted date: 1-1-2014

Please cite this article as: X.-L. Li, Y. Shi, W.-H. Zhang, Y.-J. Dai, H.-T. Zhang, Y. Wang, H.-K. Wang, F.-P. Lu, A High-Detergent-Performance, Cold-Adapted Lipase from *Pseudomonas stutzeri* PS59 Suitable for Detergent Formulation, *Journal of Molecular Catalysis B: Enzymatic* (2014), http://dx.doi.org/10.1016/j.molcatb.2014.01.006

A High-Detergent-Performance, Cold-Adapted Lipase from *Pseudomonas stutzeri* PS59 Suitable for Detergent Formulation

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Abstract

A high-detergent-performance and cold-adapted lipase was purified and characterised from *Pseudomonas stutzeri* PS59, which was isolated from Daqing oil fields (Heilongjiang, PR China). The lipase was purified to homogeneity using ammonium sulphate precipitation, dialysis, freeze-drying, ion exchange chromatography and gel filtration chromatography. The molecular weight of the lipase was approximately 55 kDa, as measured by SDS-PAGE. The lipase showed optima activity at pH 8.5 and 20 °C. The lipase activity was activated by metal ions, such as Ca^{2+} and Mn^{2+}, and surfactants, such as Tween 80, Tween 20, sodium dodecyl benzene sulfonate and urea. Oxidising agents, such as H_2O_2 and NaClO, were found to have little effect on the activity of the lipase, and most organic solvents can enhance the activity of the lipase. The broad substrate specificity and the compatibility of the lipase in the presence of surfactants, oxidising agents, and other detergent additives clearly indicate its potential application in the laundry industry. The hydrolysis resolution of (R, S)-ethyl 2-Methylbutyrate by *P. stutzeri* PS59 lipase was carried out with the yield of 31.2% for R-ethyl 2-Methylbutyrate, the enantiomeric excess of residual substrate (ee_s) was 85.7%. Thus, the lipase also showed an attractive potency for application in biocatalysis.

Keywords: *Pseudomonas stutzeri*; Lipase; Purification; Washing performance; Detergent.

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1. Introduction

Microbial lipases are one of the largest groups of industrial enzymes and have applications in a wide range of industrial and household products, including detergents, leather, silk, food and pharmaceuticals [23,10]. However, the single largest market for their use is in the detergent industry. For this reason, increasing numbers of lipases have been exploited by researchers in the detergent industry. Lipases from *P. alcaligenes* (Lipomax®) and *P. mendocina* (Lumafast®) are produced as detergent additives by Genencor [15]. These enzymes have been widely used in the detergent industry since their introduction in 1913 by Rohm. Various commercial detergent lipases, such as Lipolase and Polarzyme (Novozymes, Denmark), Lumafast (Genencor, USA), and Lipofast (Advanced Biochemicals, India) are available in the market [11]. The lipases produced by *Candida cylindracea* and *Aspergillus niger* have been tested to determine their efficiency in an aqueous solution of lipase with or without surfactants under various conditions for the removal of olive oil from cotton fabric [7,18]. A novel alkaline lipase from *Burkholderia cepacia* RGP-10, which is used in detergent formulations, exhibits better stability toward commercial detergents and oxidants [26]. All of these lipases are used in the detergent industry for the removal of fatty residues in laundry.

The performance of lipases in detergents depends on various factors, such as the pH of the detergent, ionic strength, washing temperature, detergent composition, washing procedure, and wash-water hardness. However, one of the most important factors for the application of detergent enzymes is the detergency of the enzymes. There is always a need for novel enzymes with novel properties that can further enhance the washing performance of the currently used enzyme-based detergents; however, few novel enzymes have been recently exploited based on their detergency.

To generate a novel lipase with a high washing performance, Lipolase was modified by novozymes using protein engineering, and Lipolase Ultra was achieved by replacing serine 96 in the active centre of Lipolase with leucine. In washing experiments, the detergency of Lipolase Ultra was found to be four-fold higher than that of Lipolase under the same washing conditions [30]. Lipases with cold activity, thermostability, alkaline stability and resistance to metal ions, organic solvent, and various detergent additives are attractive to researchers and have been isolated for use in detergents [21,2,14]. However, the washing performance was usually ignored. The *Ralstonia pickettii* lipase retains high activity in the presence of most detergents but achieves only a 39.5% removal rate of

olive oil from cotton fabric in the same washing test [12]. The removal rates of olive oil from cotton fabric in the presence of lipase from *Cryptococcus* sp. S-2 under its optimum conditions was found to be only 19.6 and 43.1 % at 20 and 37 °C, respectively [31]. Kamini et al. demonstrated that the oil removal rate from cotton fabric by the lipase produced by *Aspergillus niger* is approximately 55% [18]. In this artile, the lipase from *Pseudomonas stutzeri* PS59 shows about 75% of removal rate of olive oil. The lipase shows the maximum activtiy at 20°C and keeps high stablity at a wide range of pH 4-11. Based on these reports, all novel detergent lipases should be evaluated based on their washing performances. In addition, even though these novel lipases exhibit good properties, it is obvious that the lipase with superior washing performance is the most desirable lipase to be included in the formulation of a detergent.

In present study, a number of bacterial strains were isolated, and the washing performances of their lipases were evaluated. A strain producing a high-washing-performance lipase was isolated and identified as *Pseudomonas stutzeri*. This lipase from the novel isolated bacterium ***P. stutzeri*** PS59 was also purified and characterised.

2. Materials and Methods

2.1. Materials

p-Nitrophenyl palmitate (p-NPP) was obtained from Sigma (St. Louis, OH, USA). The surfactants Tween® 80, Tween® 20, Triton® X-100, sodium dodecyl sulphate (SDS), sodium dodecyl sodium sulphate (SDBS) and sodium lauryl sulphate (IPC-SDS) were obtained from Sinopharm Chemical Reagent Co. Ltd. All other chemicals were of analytical grade.

2.2. Screening and isolation of high-detergent-performance and cold-adapted lipase producing strains

Soil samples were collected from the areas contaminated by crude oil in the Daqing oil fields in PR China. In the first screen, those microorganisms with high detergent performance were screened using an enriched medium (1% yeast extract, 0.1% K_2HPO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 2% olive oil emulsion, pH= 9.0). The flasks were incubated at 30 °C and 180 rpm for 72 h. The cultures were then acclimated by their successive transfer to a fresh enrichment medium three times. The enriched culture was spread after its serial dilution on Victoria blue agar plates (0.1% K_2HPO_4, 0.3% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 2% olive oil emulsion with 0.2% Victoria blue B, and 1.5% agar, pH= 9.0). The screening

plates were incubated at 30 °C for 48 h. The colonies with dark blue zones were isolated and transferred to slant medium (1% peptone, 0.5% yeast extract, and 1% NaCl, pH= 8.0).

In the second screen, those strains identified in the first screen were inoculated into the second screen medium (4% peptone, 2% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 1% olive oil, pH= 8.0). The flasks were incubated at 30 °C and 180 rpm for 36 h in a rotary shaker. The crude lipases were obtained by centrifugation at 8000 rpm and 4 °C for 15 min. Cloth washing experiments were then performed using the crude lipases as the detergent. In particular, the high-detergent-performance lipase producing strains were screened through repeated washing experiments. The strain producing the lipase with higher detergent performance was selected for further studies.

2.3. Assay of lipase activity

The lipase activity was determined by alkali titration using olive oil as the substrate, as described by Wang et al. with some modifications [34]. In brief, an assay mixture consisting of 1 ml of olive oil, 3 ml of 2% polyvinyl alcohol, 5 ml of phosphate buffer (20 mM, pH= 8.5), and 1 ml of suitably diluted culture broth was incubated at 30 °C and 180 rpm for 15 min. The reaction was terminated by the addition of 15 ml of 95% ethanol, and the amount of liberated fatty acids was titrated with 50 mM NaOH in the presence of two or three drops of phenolphthalein (0.5%, w/v in ethanol) as the indicator. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 µmol of free fatty acid per minute under the experimental conditions.

A different lipase assay was performed using spectrophotography, as described previously by Wang et al. with some modifications [33]. The substrate solution was prepared by dissolving 30 mg of pNPP in 10 ml of isopropanol. The assay mixture consisted of 100 µl of suitably diluted enzyme, 810 µl of buffer, and 90 µl of the substrate solution. The mixture was incubated at 30 °C for 15 min, and the amount of liberated p-nitrophenol was determined at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per minute under the assay conditions.

2.4. Evaluation of enzymes for detergent performance

To determine the efficiency of the employed lipases, which were produced from the strains identified in the first screen, for use as a bio-detergent additive, 10 X 5 cm white cotton fabric cloths were stained with olive oil and washed in phosphate buffer (pH = 8.0) in the presence of the studied lipases.

The cotton cloths were previously degreased in boiling chloroform to ensure that all lipids were removed from the cotton cloths prior to soiling. Both sides of the cotton cloth were impregnated with 0.5 ml of olive oil in acetone solution (100 mg/ml) and dried for 15 min at room temperature. The stained cloth pieces were incubated for 1 h at 30 °C on a rotary shaker (180 rpm) in 250 ml Erlenmeyer flasks containing 100 ml of the washing solution, which consists of phosphate buffer (pH= 8.0) and the crude lipases (total of 100 U).

The weight of the cotton cloths was measured before and after the addition of olive oil. The weight of the stained cotton cloth was also measured after washing. The efficiency of olive oil removal, ω, was calculated taking into account the weight of the cotton cloths before and after the addition of olive oil and the weight of the stained cotton cloths after washing and is expressed by the following equation:

$$\%\omega = \frac{Wb - Wa}{Wb - Wc} \times 100 ,$$

Where *Wa* and *Wb* represent the weights of the cotton cloth before and after the addition of olive oil, respectively, and *Wc* is the weight of the stained cotton cloth after washing. All of the washing tests were performed three times, and the standard error was also calculated [31,18,9].

2.5. Bacterial strain identification

Strain PS59 was identified through 16S ribosomal DNA (rDNA) analysis. The 16S rDNA sequence of the isolated strain was determined by Shanghai Sunny Biotechnology Co, Ltd. A homology search using the reference strains registered in DDBJ/EMBL/GenBank was performed using NCBI BLAST.

2.6. Enzyme production medium and culture condition

P. stutzeri strain PS59 was cultured in the seed medium (Luria-Bertani broth medium) containing (w/v) 1% peptone, 0.5% yeast extract, and 0.5% NaCl (pH= 8.0). A 2% inoculum of the seed medium was then transferred into the lipase-producing medium (1% peptone, 0.8% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2$, and 1% olive oil, pH= 8.5). The cultivations were performed in 500 ml Erlenmeyer flasks containing 60 ml of medium on a rotary shaker (180 rpm/min) for 36 h at 30 °C. The crude lipase was obtained by centrifugation at 8000 rpm and 4 °C for 15 min.

2.7. Purification of the lipase

The cell-free supernatant (800 ml) was precipitated overnight at 60% saturation with $(NH_4)_2SO_4$ at 4 °C, and the pellet was collected after centrifugation. The precipitate was dissolved in 120 ml of phosphate buffer (pH= 8.0) and dialysed in deionised water for two days. The removal of ammonium sulphate was monitored with $BaCl_2$. A partially purified lipase powder was obtained by freeze-drying the lipase solution on a vacuum freeze drier. The partially purified lipase was then subjected to ion exchange chromatography. Approximately 5 ml of the partially purified enzyme was loaded onto a Hitrap Q HP column (5 X 5 cm) pre-equilibrated with 20 mM phosphate buffer (pH= 7.0). The fractions were eluted from the column using a linear NaCl gradient (0-1 M). Fractions of 1 ml each were collected at a flow rate of 1 ml min^{-1}. The active fractions were pooled and concentrated by ultrafiltration. The concentrated fraction was then chromatographed on a Superdex 200 column pre-equilibrated with 20 mM phosphate buffer (pH= 8.0) at a flow rate of 0.5 ml min^{-1}. The fractions were monitored continuously at 280 nm for protein and also assayed individually for lipase activity. The active fractions were stored at -20 °C. The purity of the fractions showing maximum activity were further analysed by electrophoresis.

2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was conducted to determine the purity and molecular weight of the enzymes using 5% (w/v) stacking and 12% (w/v) separating gels. The samples were prepared by mixing the purified enzymes (v/v) with distilled water containing 10 mM Tris-HCl (pH 8.0), 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. The samples were heated at 100 °C for 5 min before electrophoresis. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol/ 10% acetic acid and destained with 10% ethanol/ 10% acetic acid. The molecular weights of the purified lipases were estimated using a low-molecular-weight calibration kit as the marker.

2.9 Effects of pH on the activity and stability of the lipase

The optimum pH of the lipase was measured by varying the pH of the assay reaction mixture using the following buffers (0.1 M): sodium phosphate (pH 6-9) and glycine NaOH (pH 9.0-10.5). The stability of the lipase was analysed after the lipase was pre-incubated for 20 min, 40 min, and 1 h in the various buffer solutions (pH 4-11). The residual enzyme activity was then measured under the standard assay conditions.

2.10 Effects of temperature on the lipase activity and stability

The optimal temperature of the lipase was evaluated by measuring the lipase activity at different temperatures (0-50 °C) in 0.1 M phosphate buffer (pH= 8.0). The effect of temperature on the lipase stability was determined by measuring the residual activity of the lipase after its incubation in 0.1 M phosphate buffer (pH 8.0) at temperatures ranging from 30 to 80 °C for 15 min, 30 min, and 60 min.

2.11 Effect of various metal ions, surfactants, and oxidising agents on the stability of the lipase.

The effects of different metal ions (Zn^{2+}, Cu^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+}, K^{+}, Ca^{2+}, and Mg^{2+}) and EDTA were investigated by pre-incubating the lipase with 1 and 10 mM solutions of these ions and EDTA for 4 h at 30 °C, and aliquots were withdrawn at half-hour time intervals and analysed for residual lipase activity. The enzyme activity was determined as a percentage of the relative activity compared to that of the control (without metal ions and EDTA), which was considered 100%.

To evaluate the stability of the lipase in the presence of additives, the lipase preparation was incubated in the presence of various surfactants and oxidising agents at a concentration of 1% (v/v) in assay buffer (0.1 M phosphate buffer, pH= 8.0). Bile salt, sodium deoxycholate, SDS, CTAB, and SDBS were used at a concentration of 0.1% (w/v), and sodium perborate was used at concentration of 0.1% and 0.5%. The experiment was performed on a rotary shaker (150 rpm) for 4 h to simulate laundry-washing conditions. Lipase samples were collected at 30 min intervals to analyse the lipase activity by the pNPP method. The control test was performed under the same conditions without the additives. The residual activity in each sample was calculated with respect to that of the control, which was considered 100%.

2.12 Effect of organic solvents on the lipase activity

The effects of organic solvents at a concentration of 15% (v/v) on the activity of the lipase were investigated. The lipase was mixed with the organic solvents (DMSO, acetone, glycerol, ethanol, isopropanol, dichloromethane, butyl alcohol, caprylic alcohol, and caproic acid) and incubated at 30 °C and 150 rpm on a shaker. The residual lipase activity with respect to that of the control, which was considered as 100%, was measured at appropriate time intervals over a period of 4 h under the optimised assay conditions.

2.13 Analysis of the substrate specificity of the *P. stutzeri* PS59 lipase

An assay mixture consisting of 1 ml of liquid ester or 1 g of solid ester, 3 ml of isopropanol, 5 ml of phosphate buffer (pH= 8.0), and 1 ml of the lipase solution was incubated for 15 min at 30 °C with stirring at 180 rpm. The reaction was terminated by the additon of 95% ethanol, and the amount of liberated fatty acids after incubation was determined by titrating with 50 mM NaOH in the presence of two drops of phenolphthalein solution as the indicator. The control experiment was performed under the same conditions with the addition of 95% ethanol prior to the reaction. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 μmol of free fatty acid per minute under the experimental conditions.

2.14 Bioresolution of (R, S)-ethyl 2-Methylbutyrate by *P. stutzeri* PS59 lipase

The kinetic resolution of (R, S)-ethyl 2-Methylbutyrate was carried out in 10 ml phosphate buffer (50mM) containing 2 mM (R, S)-ethyl 2-Methylbutyrate catalyzed by 10 mg *P. stutzeri* PS59 lipase at 30 °C with shaking at 180 rpm.

The amounts of optical isomers were determined by GC using Agilent-β-DEX-120-Chiral cyclodextrin capillary GC column (30 m × 0.25 mm × 0.25 μm). The chromatographic conditions employed were: carrier gas, hydrogen at 60 kPa; the flow rate, 2.0 ml/min split ratio, 1:100; injection volume, 1ul; injector temperature, 250 °C; detector temperature, 300°C; oven temperature program, initially at 40 °C and increased at 10 °C/min to 120 °C; total time of analysis, 10 min.

2.14 Statistical analysis

The experimental results are expressed as the means ± standard deviation (S.D.). In this study, all of the experiments were performed in triplicate, and the samples were analysed in duplicate or triplicate. The Origin8.0 software (OriginLab, USA) was used for the statistical evaluations.

3. Results and Discussions

3.1 Isolation of high-washing-performance-lipase-producing bacteria

The first screen analysed a total of 278 lipase-producing bacterial strains that were isolated from the soil samples collect for this study. Forty-five of these strains, which produced larger fading rings, were isolated and subjected to the washing performance test. In the first washing experiment, the lipases of the tested eight strains showed superior washing performance (Fig. 1a). Of these eight strains, five lipase-

producing strains exhibiting high washing performance were used for the repeated washing test (Fig. 1b), which revealed that strain PS59 secreted an extracellular lipase with high washing performance at low temperature and high alkaline conditions. Thus, this strain was therefore selected for the subsequent experiments.

Fig. 1.

Strain PS59, which produces a high-washing-performance, cold-adapted lipase, was identified as *Pseudomonas stutzeri* based on an analysis of its 16S rDNA sequences, which revealed 100% homology.

3.2 Enzyme purification

Table 1

The lipase from *P. stutzeri* PS59 was purified to homogeneity, and the results are shown in Table 1. The lipase was precipitated using ammonium sulphate (60% saturated) to yield an active fraction with a 93% yield and a 3.75-fold increase in its specific activity. The precipitate was dissolved in double distilled H_2O, and the enzyme solution was dialysed using a MD77 bag to remove the salt. During dialysis, a yield of 88.7% was achieved with 3.64-fold purification. The lipase solution was freeze-dried to yield the crude lipase with a loss of approximately 5.0% of the enzyme activity. The crude lipase powder was used as a starting material for further purification. After ion exchange chromatography (Q-Sepharose), a specific activity of 330.6 U/mg was obtained with 11.7-fold purification. The purification using ion exchange chromatography resulted in a peak of the active fraction at 60% NaCl. Further purification using gel filtration chromatography resulted in two peaks, and the first peak contained the active fraction with a specific activity of 631 U/mg and 22.45-fold purification. SDS-PAGE analysis of the first peak obtained through gel filtration chromatography clearly revealed a single protein band corresponding to a molecular mass of approximately 55 kDa (Fig. 2). The SDS-PAGE analysis indicated that the first peak exhibited a high purity of the active lipase fraction.

Fig. 2

The lipases of *Pseudomonas* were mainly classified into three groups [15]. Group □ lipases have a higher molecular mass (approximately 55 kDa) than the other lipases. Thus, based on the molecular mass of the lipase investigated in our study, the lipase produced by *P. stutzeri* PS59 is a group □ lipase.

3.3 Effects of pH on the lipase activity and stability

Fig. 3.

The lipase characterised herein was found to be functionally active in the pH range of 7.0 to 11.0. The lipase showed optimal activity at pH 8.5 (100%) and showed approximately 80% residual activity at pH 9.0 (Fig. 3a). The *P. stutzeri* PS59 lipase was found to be active and stable over a wide pH range of 4 to 11, which indicates its potential applicability in the laundry industry (Fig. 3b). These findings are in accordance with several earlier reports. For example, the lipase from *Ralstonia pickettii* was stable at high pH [12], and a novel lipase from the fungus *Talaromyces thermophilus* was stable in a wide pH range of 9 to 11 [2].

3.4 Effects of temperature on the lipase activity and stability

The activity of the lipase was determined at a wide range of temperatures (0-50 °C). The optimum temperature for lipase activity was determined to be 20 °C. The lipase activity was almost constant between 20-30 °C and gradually declined at temperatures below 15 °C and beyond 35 °C (Fig. 3c). Similar cold-active lipases with optimal temperatures ranging from 15 to 30 °C have been identified from other microbial strains, such as *Yarrowia lipolytica* NCIM 3639 (25 °C), *Acinetobacter* sp. strain no 6 (20 °C), and *Geotrichum* sp. SYBC WU-3 (two cold active lipases, 20 and 15 °C) [35,29,3]. Moreover, a cold-active lipase of marine Antarctic origin exhibited maximum activity at 20 °C [24]. A lipase from *Aeromonas* sp. LPB 4 was described to be highly active at 10 °C [20]. In general, fat stains are not easy to remove at low temperatures using conventional detergents; therefore, it is necessary to identify cold active lipases that can be used in detergent formulations. The cold active lipases used in detergent formulations can reduce the energy consumption as well as the wear and tear on textile fibres to maintain the texture and quality of fabrics [6]. The lipase investigated in this study was also found to be stable at a broad temperature range (30-60 °C) (Fig. 3d). The activity and stability profile of the lipase was better or comparable with that of other cold active lipases that have been reported to be compatible with detergent formulations [17,35].

In most developing countries like China and India, cold water was usually adopted for cloth washing. Therefore, a detergent enzyme, which exhibits high activity at low temperature, will be more practical than those thermophilic ones. In this article, a high-detergent-performance and cold-adapted lipase was

isolated and characterized. The lipase shows high specific activity of 631 U/mg, which is comparable to the commercialized Lipase TL (http://www5.mediagalaxy.co.jp/meito/kaseihin/lipase/data/lip_tl.html). But, compared with lipase TL, the newly isolated lipase exhibits the optimum activity at lower temperature of 20 °C and it shows high stability at a wide pH range from 4 to11. According to our research, the *P. stutzeri* PS59 lipase is more suitable for washing at low temperature than lipase TL. These superior properties make the lipase an ideal candidate for application in detergent industry.

3.5 Stability of the lipase in the presence of various metal ions, surfactants, and oxidising agents

Fig. 4.

The compatibility of the *P. stutzeri* PS59 lipase with various concentrations of metal ions, surfactants, and oxidising agents was investigated. Of the metal ions tested at concentration of 1 mM and 10 mM, the *P. stutzeri* PS59 lipase showed high activity and stability in the presence of Ca^{2+} and was inhibited by EDTA (Fig. 4a and 4b). In the initial phase, Ca^{2+} addition activated the lipase (up to 24.1% and 19% compared with the control at 1 mM and 10 mM, respectively). It suggests that the enzyme needs calcium as a catalytic activator. The activity of most lipases from *Pseudomonas* has been found to be enhanced by Ca^{2+} [37,1,19,4]. This increase in the activity of the lipase is observed because calcium plays a vital role in the construction of a stable catalytic enzyme structure, which is a result of the calcium ions binding to the internal structure of the enzyme. At metal ion concentrations of 1 mM and 10 mM, the lipase was also slightly activated by Zn^{2+}, Mn^{2+} and K^{+} during the first half-hour and repressed by Cu^{2+}, Fe^{2+}, Ba^{2+}, and Mg^{2+} (Fig. 4a and b). After 30 min of incubation with 1 mM and 10 mM Cu^{2+}, Fe^{2+}, Ba^{2+}, and Mg^{2+}, the lipase retained approximately 80 and 70% residual activity, respectively. Two hours later, the lipase exhibited more than 50% residual activity in the presence of most metal ions (except 10 mM Cu^{2+} and Fe^{2+}). The results shown in Fig. 4a and 4b reveal that Ca^{2+}, Zn^{2+}, and Mn^{2+} can enhance the enzyme activity, whereas Cu^{2+}, Fe^{2+}, Ba^{2+}, and Mg^{2+} inhibited the enzyme activity. K^{+} had little effect on the enzyme activity. Most *Pseudomonas* lipases are inhibited by heavy metal ions such as Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{+}, Cd^{+}, Fe^{2+}, and Co^{2+}, and activated by Ca^{2+} and Mg^{2+} [28]. An alkaline lipase from the newly isolated *Pseudomonas mendocina* PK-12CS was found to be inhibited by Hg^{2+}, Fe^{2+}, Zn^{2+} and activated by Mg^{2+} [16]. The activity of the lipase produced by *Pseudomonas gessardii* was found to be significantly enhanced by Ca^{2+}, slightly less enhanced by Mg^{2+}, and inhibited by other metal ions, such as Zn^{2+}, Fe^{2+} and Cu^{2+} [25].

Fig. 5.

The industrial applications of surfactants and oxidising agents are mainly related to detergents. These chemicals can destroy the activity of a lipase by changing the lipase's tertiary structure. However, these additives may preserve lipase activity and stability by inhibiting lipase aggregation. Hence their addition may enhance lipase activity. As shown in Fig. 5a, the lipase investigated in the present study was stable in the presence of most ionic and non-ionic surfactants. The lipase activity can be promoted by the presence of Tween 20, Tween 80, SDBS and urea by 35%, 47%, 27% and 33%, respectively for the first 15 min of incubation, and the lipase retained approximately 80% or more residual activity after longer incubation period. In addition, the lipolytic activity was inhibited by the presence of SDS, sodium deoxycholate, bile salt, IPC-SDS, and CTAB, whereas Triton X-100 had little effect on the lipase activity. It was obvious that the ionic interaction between most ionic surfactants (SDS, IPC-SDS, and CTAB) and the enzyme plays an important role in the inactivation of globular proteins [27,4]. In contrast, the absence of nonionic surfactants can reduce the harmful electrostatic interactions to preserve the activity of the enzyme. The activation and promotion of the enzyme by some nonionic surfactants such as Tween 80 (up to 126%), was due to the increase in the conformation flexibility of the active site, which is a result of the formation of hydrogen bond between the surfactant molecules and the enzyme [36]. As previously reported, Lipolase® maintains 68.5%, 52.3%, 31.6% and 26.7% residual activity after one hour of incubation in the presence of Triton X-100, Tween 80, Tween 20 and SDS [21], respectively, whereas the *P. stutzeri* PS59 lipase maintained 78.2%, 126%, 80.1%, and 40.8% residual activity, respectively. Compared with commercial Lipolase®, some properties of the *P. stutzeri* PS59 lipase suggest that it can potentially be applied in the detergent industry. In addition, the choice of surfactants is notably important in detergent formulations containing lipases. The activity of the lipase produced from indigenous *Pseudomonas aeruginosa* was found to be strongly inhibited by Tween 80 [9], whereas Tween 80 was found to significantly promote the activity of the lipase of *P. stutzeri* PS59 in our study.

Furthermore, the lipase produced by *P. stutzeri* PS59 was evaluated in the presence of oxidising agents (Fig. 5b). Stability of lipases in the presence of oxidising agents is an important property of lipases in detergent formulations and has been achieved through protein engineering or site-directed mutagenesis [8]. Compared with Lipolase® (Novozyme, Denmark), which maintains 82.5% and 46.3% residual activity after one hour of incubation in the presence of H_2O_2 and NaClO, respectively, the lipase produced

by *P. stutzeri* PS59 retained 77 and 56.6% residual activity. In addition, a low concentration (1%) of sodium perborate can promote the activity of the lipase during the first 1.5 h of incubation, the activity of the lipase declined to approximately 80% residual activity after 1.5 h. Moreover, the activity of the lipase was inhibited by a high concentration (5%) of sodium perborate.

3.6 Effect of organic solvents on the lipase

Fig. 6.

The hydrolysation of water-insoluble substrates by lipases must take place at an interface. Mixtures of water and organic solvents contribute to the bioconversion of water-insoluble substrates by lipases. Although organic solvents damage the activity of enzymes, the activity of enzymes in mixtures of water and organic solvents has been reported [5]. In general, enzymes would lose their activity in the presence of organic solvents at concentrations of 10-20%. The activity and stability of the enzymes in organic solvents depend not only on the properties and concentration of the organic solvents but also on the nature of the enzyme [32]. For this reason, the effects of various organic solvents at a concentration of 15% (v/v) on the lipase from *P. stutzeri* PS59 were determined, and the results are presented in Fig. 6.

The *P. stutzeri* PS59 lipase retained more than 100% residual activity (containing 100%) in the presence of isopropanol, ethanol, butyl alcohol, glycerol, DMSO, and caprylic alcohol during the first half hour of incubation and retained 139.1%, 126%, and 107.5% relative activity after 1 h of incubation in the presence of caprylic alcohol, glycerol, and DMSO, respectively (Fig. 6). However, the lipase activity declined to 69 and 58.2% after one hour of incubation in the presence of ethanol and butyl alcohol. Isopropanol had no effect on the lipase activity. In addition, the lipase activity suffered some inhibition in the presence of acetone, dichloromethane and caproic acid. The *P. stutzeri* PS59 lipase maintained high activity and stability in the presence of caprylic alcohol, glycerol, DMSO, butyl alcohol, ethanol, and isopropanol. The stability of the lipase in these organic solvents is due to the fact that these solvents play a key role in stabilising the enzymatic activity in the solution. However, the inhibition of the lipase by acetone, dichloromethane, and caproic acid may be due to the adsorption of the solvents on the substrate and the resulting repression of the enzyme's interaction with the substrate [22]. In addition, the residual activity of the lipase after one hour of incubation in several other organic solvents was listed in table 2.

Table 2

3.7 Substrate specificity of the lipase

Fig. 7.

In general, an oil stain consists of many different long-chain fatty acid esters. The washing performance of a lipase depends on the hydrolytic ability of a lipase. The substrate specificity of the lipase to various esters was detected through alkaline titration. As shown in Fig. 7, the lipase from *P. stutzeri* PS59 can hydrolyse most long-chain fatty acid esters. To study the substrate specificity of the lipase, the activity for triolein was considered to be 100%. The lipase showed 89.3%, 83.3%, and 78.6% relative activity for stearin, palmitin, and gallicin, respectively. In addition, the activity of the lipase for ethyl palmitate, ethyl linoleate, and ethyl oleate was 74.7%, 58.4%, and 53%, respectively. In addition, the lipase showed 72.6%, 51.6%, 33.1%, 31.0%, and 38.1% relative activity for methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl laurate, respectively. Based on these data, we determined that the lipase can effectively hydrolyse triglycerides and has a broad specificity for other esters. Furthermore, the lipase can be potentially used for the removal of oil stains, as reflected by the directly measured washing performance of the lipase.

3.8 Bioresolution of (R, S)-ethyl 2-Methylbutyrate by *P. stutzeri* PS59 lipase in an aqueous system

(R, S)-ethyl 2-Methylbutyrate is one of the typical flavor compounds used in food. The (R, S)-ethyl 2-Methylbutyrate was hydrolyzed by *P. stutzeri* PS59 lipase in an aqueous system, and the *P. stutzeri* PS59 lipase was found to be selective towards (S)-ethyl 2-methylbutyrate (Fig.8). The time course of resolution of (R, S)-ethyl 2-Methylbutyrate showed that the 66.4% of substrate was conversed, and the residual substrate, (R)-ethyl 2-methylbutyrate, remained with the yield of 31.2% after 12 h of reaction, and the enantiomeric excess of (R)-ethyl 2-methylbutyrate reached 85.7%, giving an E-value over 5 (Fig.9), which is higer than other lipases such as lipase (E=3.6) produced from *Candida cylindracea*[13]. Thus the *P. stutzeri* PS59 lipase exhibited a relatively high enantioselectivity for ethy 2-Methylbutyrate.

Fig.8.

Fig.9.

4 Conclusions

In conclusions, a high-detergent-performance, cold-adapted extracellular lipase from the P. stutzeri PS59 was purified and identified. The lipase has an optimum temperature and pH of 20 °C and 8.5, respectively. Moreover, the analysis of the stability of the enzyme in the presence of various metal ions, surfactants, oxidising agents, and solvents, as well as its broad substrate specificity, demonstrates that the enzyme exhibits potential as a commercial additive in detergents. And the lipase showed good enantioselectivity towards (R, S)-ethyl 2-Methylbutyrate. These indicated that the lipase can be a very attractive enzyme for potential application in biocatalysis. Further research is needed to study the mechanism of the high-washing performance of the *P. stutzeri* PS59 lipase.

Acknowledgments

Financial support was provided by the Hi-Tech Research and Development Program of China (863 Planning: Grant number: 2013AA102803; Task number: 2013AA102803C) and the program for Changjiang Scholars and Innovative Research Team in University (IRT1166).

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Figure captions

Fig. 1. (a) The washing performance of eight lipases analysed in the second screen of the isolated strains. (b) Repeated assay of five lipases that exhibited the highest washing performance in the second screen.
Fig. 2. SDS-PAGE of the purified lipase. Band 1: standard protein markers (Da); Band 2: crude lipase solution (20 mg/ml); Band 3: the lipase purified by Q-sepharose; Band 4: the lipase purified by Superdex 200.

Fig. 3. (a) Effect of pH on lipase activity. The lipase activities were evaluated at 20 °C in the pH range of 6.0 to 10.5 using buffers with different pH values. The maximum activity was obtained at pH 8.5 (considered 100%). (b) pH stability of the lipase. The lipase activity is determined after 20 min, 40 min, and 60 min of incubation at 30 °C in the presence of sodium phosphate buffer (pH 3.0-8.0) and glycine NaOH buffer (pH 9.0-11). (c) Effect of temperature on the activity of the lipase. The lipase activity was measured at various temperatures. The activities are shown relative to that measured at 20 °C, which was considered 100%. (d) Temperature stability of the purified lipase. The lipase activity was measured after 15 min, 30 min, and 60 min of incubation at pH 8.0 at the different temperatures. The activity of the lipase incubated at 30 °C was considered 100%.

Fig. 4. Percentage of residual lipolytic activity as a function of time in the presence of (a) 1 mM metal ions and (b) 10 mM metal ions.

Fig. 5. Percentage of residual lipolytic activity as a function of time in the presence of (a) various surfactants and (b) various oxidising agents.

Fig.6. Percentage of residual lipolytic activity as a function of time in the presence of 15% organic solvents.

Fig.7. Substrate specificity of the lipase. The activity of the lipase toward various esters was determined, and the activityof the lipase for triolein was considered 100%.

Fig.8. Kinetic resolution of (R, S)-ethyl 2-Methylbutyrate catalyzed by *P. stutzeri* PS59 lipase in an aqueous system

Fig.9. Time course of bioresolution of (R, S)-ethyl 2-Methylbutyrate catalyzed by *P. stutzeri* PS59 lipase.

Table 1

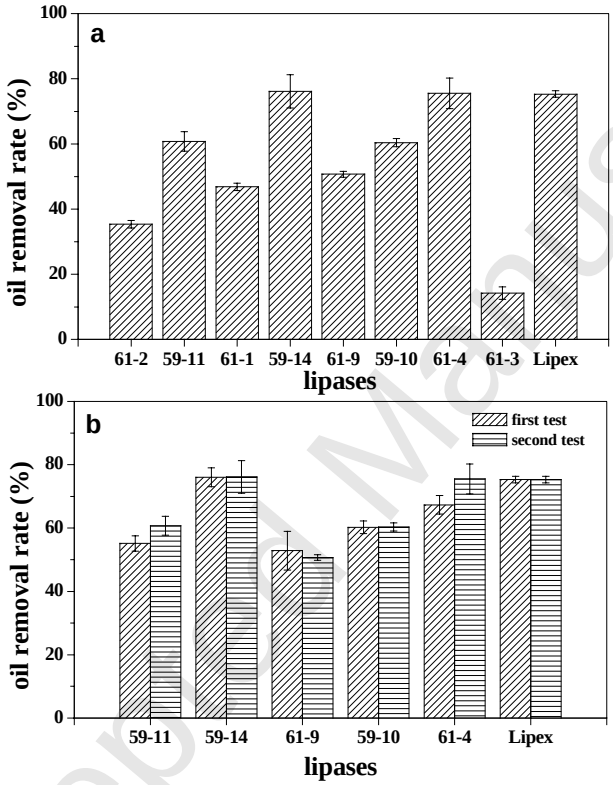
Summary of the steps used to purify the extracellular lipase produced by *Pseudomonas stutzeri*

Purification step	Enzyme activity (U ml^{-1})	Protein (mg ml^{-1})	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)	Fold purification
Cell-free culture supernatant	43	1.53	28.1	27950	100	1
Ammonium sulphate 60% saturation	216.7	2.05	105.4	26004	93	3.75
Dialysis	173.4	0.59	102.3	24800	88.7	3.64
Freeze drying	-	-	-	23400	83.7	
Purified by Q-sepharose	334	1.01	330.6	16200	57.9	11.77
Purified by Superdex 200	120	0.19	631	12040	43.1	22.45

Table 2

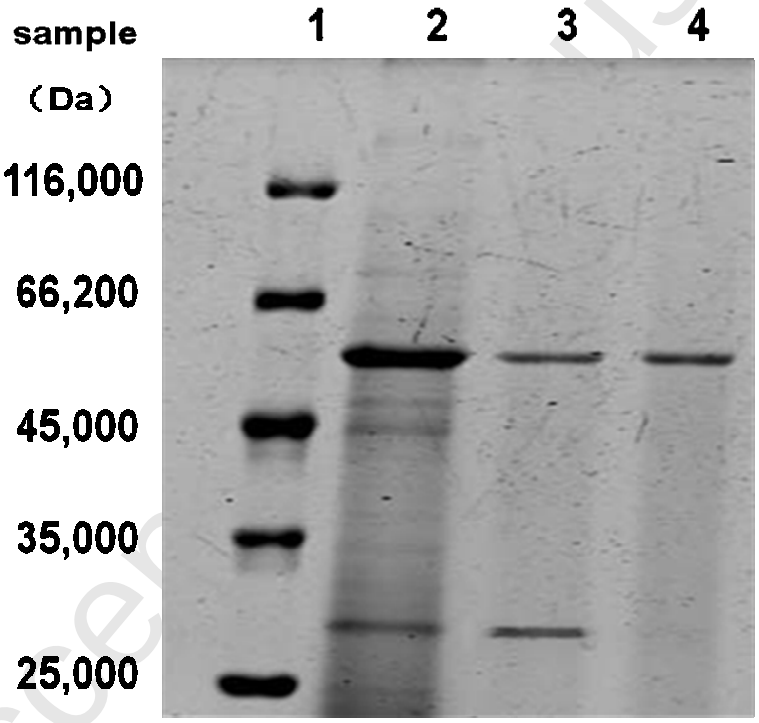
Effect of 15% organic solvents on the activity of the lipase from *P. stutzeri* PS59

Organic solvents	Residual activity (%)	Organic solvents	Residual activity (%)
Methyl benzene	98±3.5	Acetic acid	79±3
Cyclohexane	102±2.5	Isoamylol	109±3.8
Tetrahydrofuran	108±5	Glycol	114±5
n-Hexane	93±8.5	Diacetone	65±3
Ethyl acetate	84±1	Diethyl ether	77±3.6



Lipex® 100L is produced by Novozymes*

Fig. 1.



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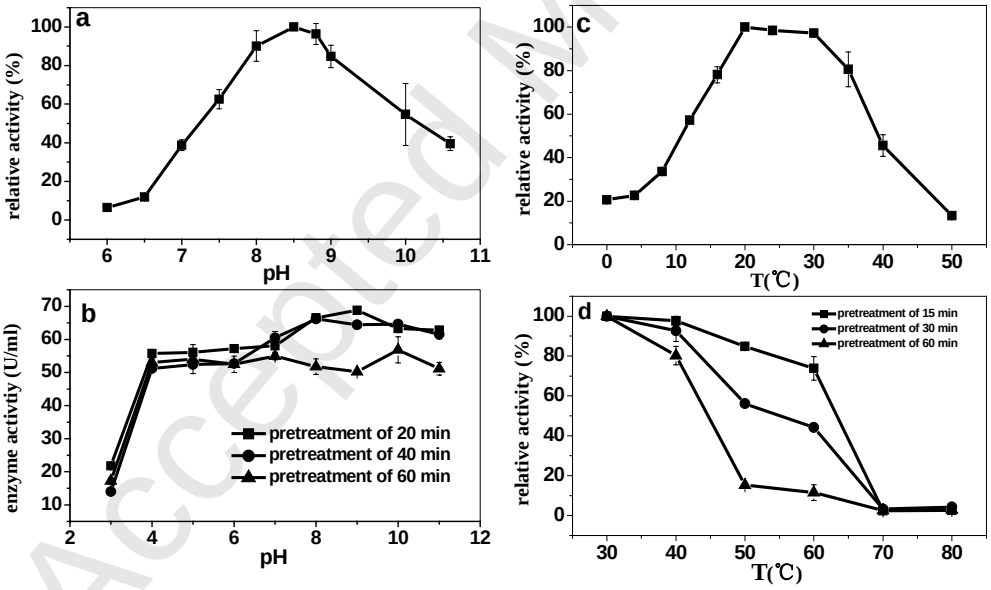

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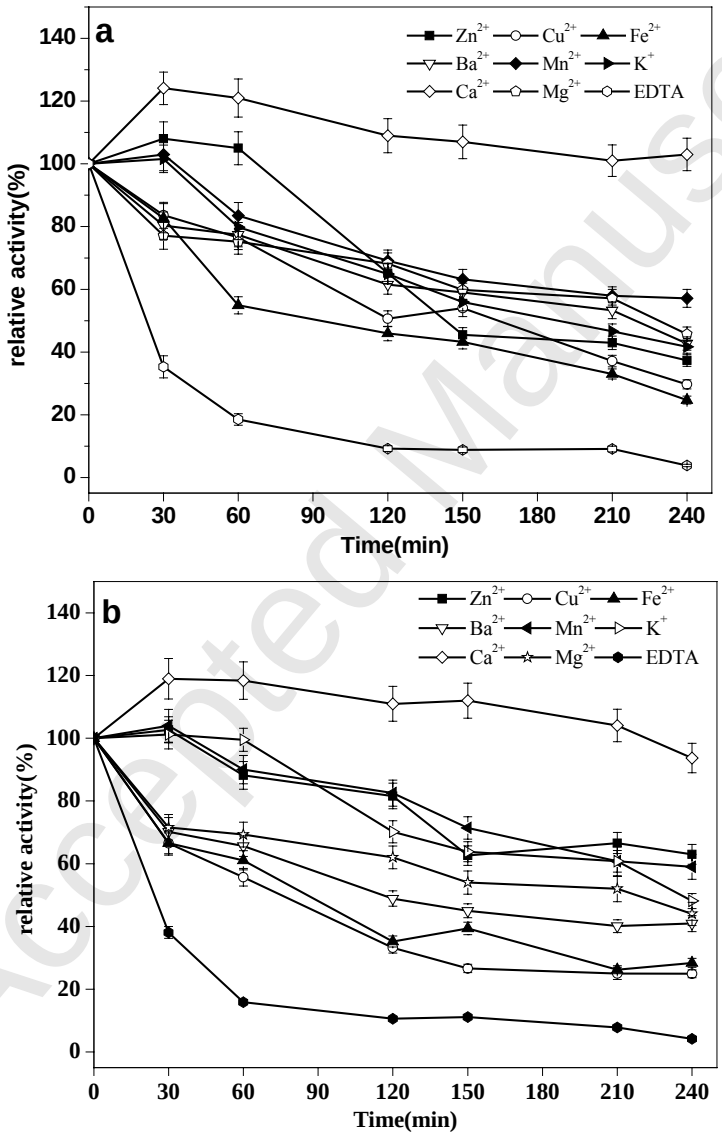

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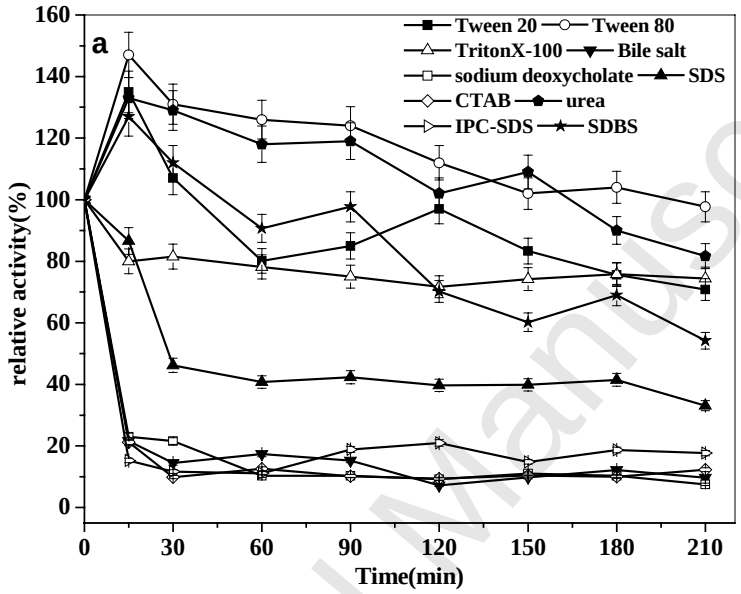


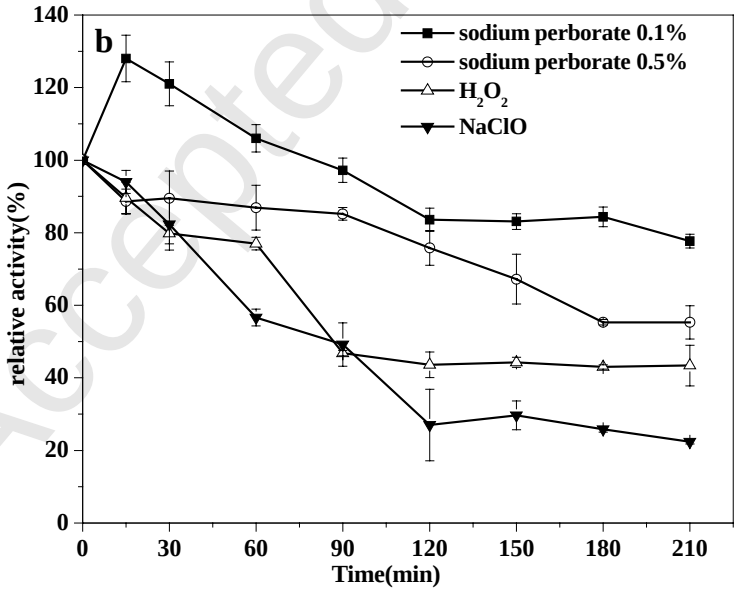
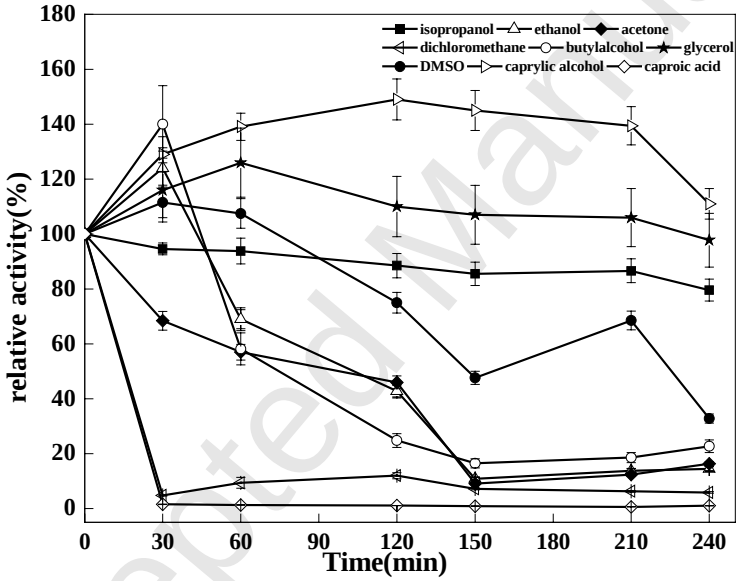


Fig. 5



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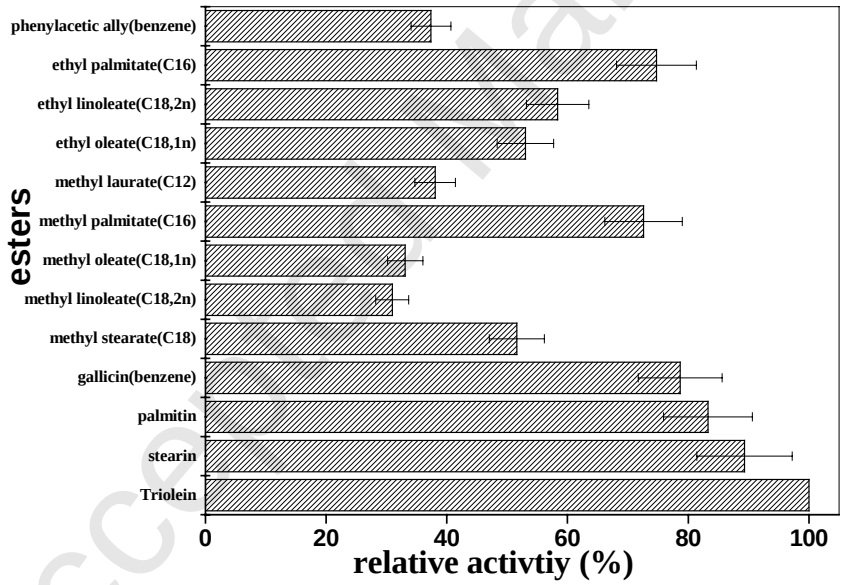

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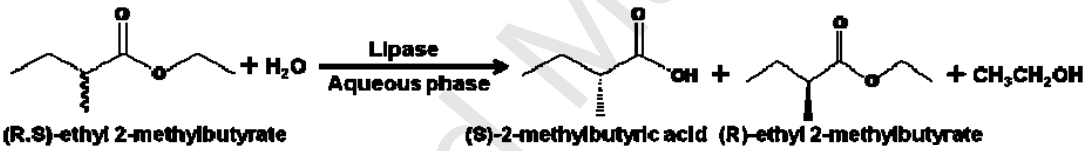

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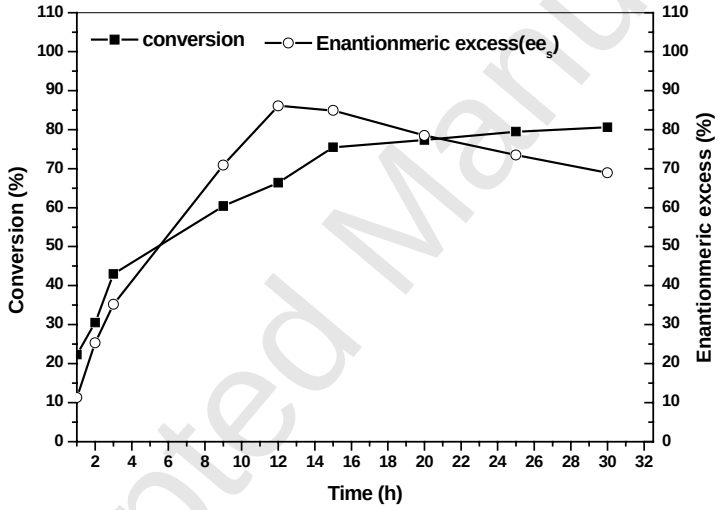

Fig.9.

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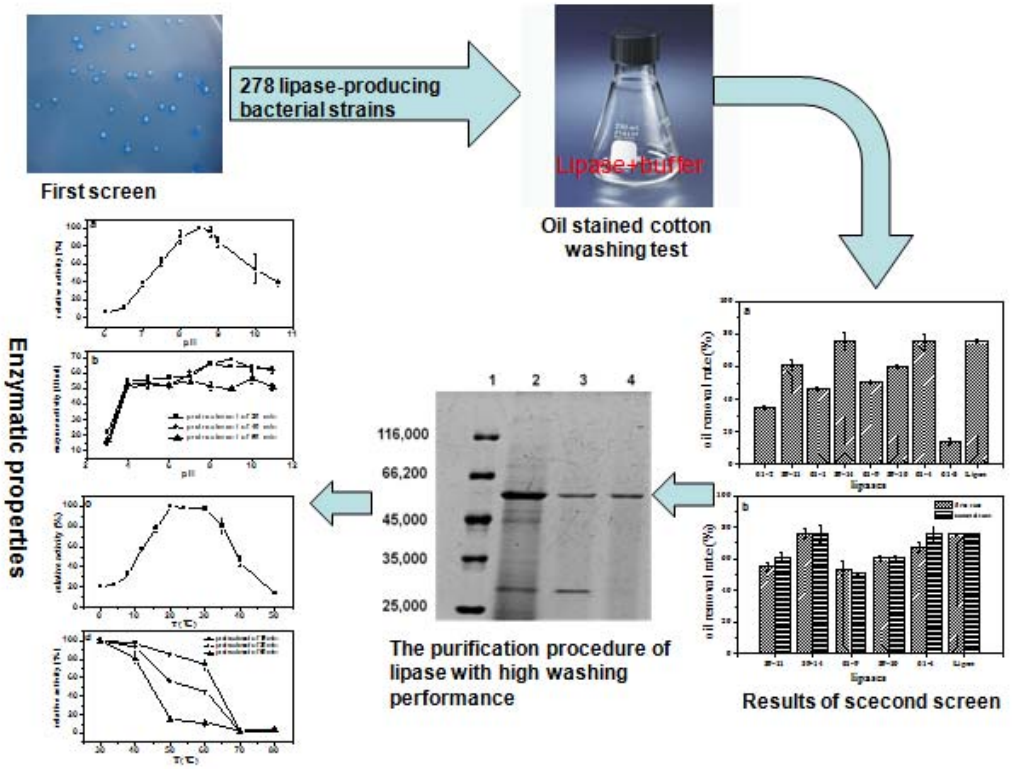

2

3

Highlights

- a novel method to isolate novel lipase-producing strains for formulation detergent.
- the lipase produced from *P. stutzeri* PS59 keeps high activity at lower temperature.
- the better stability of the lipase to metal ions and detergent additives.
- the lipase showed superior washing performance.
- the lipase also showed an attractive potency for application in biocatalysis.