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Molecular Identification of Lipase LipA from *Pseudomonas protegens* Pf-5 and Characterization of Two Whole-Cell Biocatalysts Pf-5 and Top10lipA

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To identify lipase LipA (PFL_0617) from *Pseudomonas protegens* Pf-5, a *lipA* deletion mutant (Pf0617) and a complementary strain (Pf0617lipA) were constructed, and their effects on the lipase production were examined. Pf0617 remarkably decreased its whole-cell lipase activity, whereas Pf0617lipA made its whole-cell lipase activity not only restore to wild-type level but also get a further increment. However, the deletion and overexpression of *lipA* did not affect the extracellular lipase activity. In addition, the unbroken whole cells of these strains were able to catalyze the hydrolysis of membrane-permeable *p*-nitrophenyl esters, but could not hydrolyze the membrane-impermeable olive oil. These results confirmed that LipA was an intracellular lipase and Pf-5 could also be used as a natural whole-cell biocatalyst. To evaluate the potential of Pf-5 as a whole-cell biocatalyst and separately characterize the whole-cell LipA, the properties of the whole-cell lipases from Pf-5 and Top10lipA were characterized. The results demonstrated that both Pf-5 and Top10lipA exhibited high tolerance to alkaline condition, high temperature, heavy metal ions, surfactants, and organic solvents. Taken together, *lipA* can realize functional expression in *E. coli* Top10, and Pf-5 and Top10lipA as whole-cell biocatalysts may have enormous potential in applications.

Keywords: *Pseudomonas protegens*, intracellular lipase, whole-cell biocatalyst, markerless deletion mutant, high tolerance.

Introduction

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3), belonging to the α/β hydrolase fold superfamily, is an important class of esterases, which are responsible for the hydrolytic cleavage of carboxyl ester bonds in mono-, di-, and triglycerides to liberate fatty acids and alcohols in aqueous solutions [1, 6]. Moreover, in micro- or non-aqueous environments, lipase can also catalyze various reactions, such as esterification, alcoholysis, aminolysis, or transesterification [23]. Therefore, lipases have been becoming key biocatalysts in a wide variety of applications, such as foods, detergents, biodiesel, fine chemicals, and waste treatment, in recent years [3, 5, 8, 22, 29].

Lipases, produced by various plants, animals, and microorganisms, are ubiquitous in nature. Most of them used in biocatalysts are microbial enzymes, either of fungal or bacterial origin [2, 14, 23]. Although fungal lipases are very useful, they are intolerant in industrial applications. However, lipases from bacteria are more tolerant. In addition, they can be easily produced through genetic manipulation, and couple with some novel additional features, which render them of much interest for industrial applications, biomedical sciences, and academic research [1]. Amongst the bacterial lipases, the most widely used are those originating from the genus *Pseudomonas* [6, 23]. Owing to their versatility and high tolerance, they have attracted wide attention, as more and more research is being focused on them to attempt to find more lipases with good properties and potential practical applications [3, 10],

and to study the expression regulation to produce more lipase proteins [16, 20, 27].

Besides the excellent properties of lipases, the cost of lipases is another important factor to be considered in the practical application. Compared with free biocatalysts, whole-cell biocatalysts demonstrate more advantages, such as simpler purification, higher stability and resistance, and reusability [28]. Owing to their property of natural immobilization, whole-cell biocatalysts can overcome most shortcomings of chemically or physically immobilized biocatalysts, such as easy loss of enzyme activity and/or natural properties, difficulty to find suitable supports, complicated immobilization procedures, and high production costs. Therefore, whole-cell biocatalysts appear to be the best candidates for industrial applications [9].

P. protegens Pf-5 (previously called *P. fluorescens* Pf-5) is a rhizosphere inhabitant that was isolated from soil in College Station, Texas in 1979. As a well-known biocontrol bacterium, it produces secondary metabolites that suppress plant diseases caused by a wide variety of pathogenic bacteria, fungi, and oomycetes [7, 26]. However, its lipases have not yet been formally studied. In this study, for the first time, we identified LipA as an intracellular lipase, by deletion and overexpression of *lipA*. Subsequently, the properties of Pf-5 were characterized by determining its whole-cell lipase activity in various reaction conditions in order to evaluate its potential as a whole-cell biocatalyst. In consideration of Pf-5 having more than one lipase, the properties of *E. coli* Top10lipA were further characterized by heterologous overexpression to separately characterize the whole-cell LipA because of *E. coli* Top10 being a lipase-free host.

Materials and Methods

Strains, Culture Conditions, and General Methods

P. protegens (28°C) and *E. coli* (37°C) strains were propagated in liquid or solid (1.5% (w/v) agar) LB medium. Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), gentamicin (50 μg/ml), and kanamycin (40 μg/ml) for *P. protegens*, and spectinomycin (100 μg/ml), gentamicin (50 μg/ml), and kanamycin (40 μg/ml) for *E. coli*. PrimeSTAR HS DNA Polymerase, restriction enzymes, and DNA ligation kit ver. 2.0 were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). Genomic DNA extraction, plasmid preparation, and DNA gel extraction were carried out with commercial kits (Omega Bio-Tek, Doraville, GA, USA) according to the manufacturer's protocols. Oligonucleotide primers were synthesized from Wuhan Anygene Biological Technology Co., Ltd. (Wuhan, China). DNA sequencing was performed by Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China).

Construction of Plasmids

To construct the markerless deletion mutation cassette of the coding region of *lipA*, homology arms of 754 bp upstream (bp -2 to -755 relative to the translational start site) and 824 bp downstream (bp 884 to 1,707 relative to the translational start site) were respectively amplified using primers lipAU-U-*Bam*HI/lipAU-L-*Kpn*I (5′-TTG GAT CCT GGG TCG ATC ACA TC-3′/5′-TAG GTA CCG GCG ACA TCC TTG TG-3′) and lipAD-U-*Kpn*I/lipAD-L-*Xba*I (5′-TTG GTA CCT CTG ATC TCA GCG TTG-3′/5′-TGT CTA GAT CGG CTT CTA TCG CT-3′). *Bam*HI-*Kpn*I-digested lipAU, *Kpn*I-*Xba*I-digested lipAD, and *Bam*HI-*Xba*I-digested pJQ200SK were ligated, and then followed by *E. coli* Top10 transformation. Transformants were screened on LB plates containing 50 μg/ml gentamicin and further verified by colony PCR. Then, the plasmid DNA was isolated and sequenced. The recombinant plasmid was named as pJQΔlipA.

In order to construct the *lipA* expression plasmid, PCR amplicon encoding *lipA* (904 bp) was amplified using primers lipAF-BamHI/lipAR-HindIII (5′-AAG GAT CCA TGT CCC AAG AGC TTG C-3′/5′-GGA AAG CTT GCA ACG CTG AGA TCA GAG-3′). Then, the *Bam*HI-*Hin*dIII-digested amplicon was cloned into the same digested pBBRKm containing $lacI^q$-P_{lac}, creating the recombinant plasmid pBBRKlipA.

Construction of Markerless Deletion Mutant and Complementary Strain

A gene replacement system with the plasmid pJQ200SK was used for the construction of the markerless deletion mutant of the coding region of *lipA* through a double-crossover recombination event (Fig. 1) [15, 25]. Briefly, pJQΔlipA was introduced into *P. protegens* Pf-5 by triparental mating with pRK2073 as a helper plasmid [17]. For mating assays, overnight cultures of helper, donor, and recipient strains (*i.e.*, *E. coli* Top10 (pRK2073), *E. coli* Top10 (pJQΔlipA), and *P. protegens* Pf-5) were mixed at the ratio of 6:6:1 (v/v), and collected by centrifugation at 8,000 rpm for 1 min. The cell pellets were successively resuspended and washed three times with sterile distilled water, and grown on LB agar at 28°C overnight. Then, the cells were washed off the plate with 0.5 ml of liquid LB medium, and spread at 10^{-2} dilutions onto LB agar supplemented with 100 μg/ml ampicillin and 50 μg/ml gentamicin. Single-crossover colonies were verified by colony PCR using primers lipA-672U/lipA946L (5′-CAG GGC AAG AAG GTG TT-3′/5′-CTC TCG GAA CGC TGG AC-3′). Subsequently, the selected colonies were grown in LB broth at 28°C overnight and then diluted $1{:}10^2$ into 5 ml of LB broth supplemented with 10% sucrose. After 6 h of incubation, cultures were plated at 10^{-4} dilutions onto LB agar supplemented with 100 μg/ml ampicillin and 10% sucrose and incubated at 28°C. Double-crossover colonies were confirmed by colony PCR with the above primers and sequenced. Furthermore, the double-crossover colonies were grown overnight in LB medium containing 50 μg/ml gentamicin to confirm gentamicin sensitivity and loss of the pJQ200SK plasmid. Finally, the verified double-crossover colony (*i.e.*, markerless

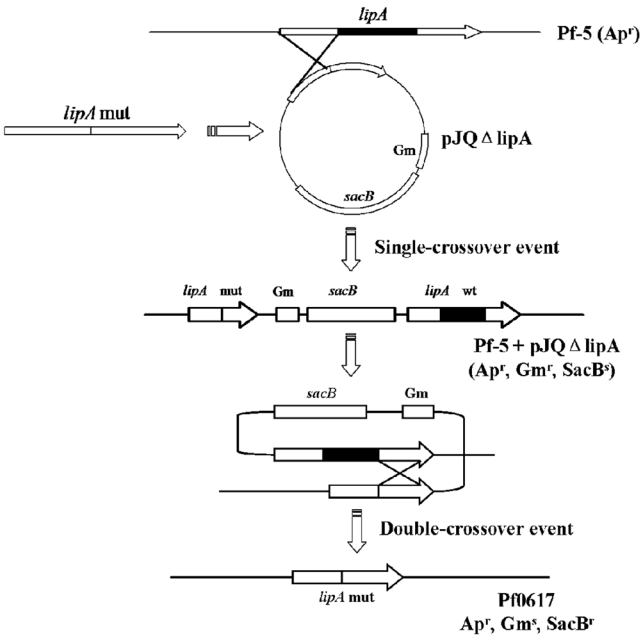

Fig. 1. Schematic diagram depicting the construction of a markerless deletion mutant of the coding region of *lipA* through a double-crossover event.

Pf-5, wild type; Pf0617, markerless deletion mutant of coding region of *lipA*; *lipA* mut, a 1.578 kb DNA fragment with a deletion in the coding region of *lipA*; pJQΔlipA, pJQ200SK carrying *Bam*HI-*Xba*I *lipA* mut.

deletion mutant of coding region of *lipA*) was named as Pf0617.

For complementation of Pf0617, pBBRKlipA was introduced into Pf0617 by triparental mating to obtain the complementary strain Pf0617lipA, and the strain Pf-5K was designated as the control.

Lipases Preparation

P. protegens strains and *E. coli* Top10lipA were respectively grown overnight in LB medium; after that, 50 ml of the bacterial cultures was colleted by centrifugation (12,000 rpm, 5 min) at 4°C. The cell-free supernatant was the extracellular lipase production, whereas the cell pellets were washed three times with 0.9% (w/v) NaCl solution, subsequently resuspended in 5 ml of 0.9% NaCl solution, and the resuspended cells were the whole-cell lipase production. Sterile distilled water substituted for the NaCl solution when the effects of metal ions on the activity of whole-cell lipase were determined.

Lipase Activity Assay

The lipase activity on the membrane-permeable *p*-nitrophenyl caprylate (*p*NPC) (Sigma-Aldrich China Inc., Shanghai, China) was determined by a spectrophotometric method as described previously [18], with some modifications. The reaction system consisted of 1.45 ml of Tris-HCl buffer (50 mM, pH 9.0) and 15 µl of *p*NPC (10 mM *p*NPC in acetonitrile). The mixtures were pre-incubated at 55°C for 5 min, and 35 µl of the lipase productions was subsequently added. NaCl solution (35 µl, 0.9%) substituted for equivalent amounts of lipase productions when the blank reaction was performed. The reaction mixtures were centrifuged (12,000 rpm, 2 min) at 4°C after 5 min of incubation at 55°C, and the OD_{410} was measured. One unit of lipase activity was defined as the amount of enzyme needed to release 1 µmol of *p*-nitrophenol per minute. Activity for the whole-cell lipase was expressed as U/ml·OD_{600}. The lipase activity on the membrane-impermeable olive oil emulsified with 2% (w/v) polyvinyl alcohol solution was determined at 55°C and pH 9.0 as described previously [22], with some modifications. The reaction system contained 4 ml of 25% (v/v) olive oil emulsion and 5 ml of Tris-HCl buffer (50 mM, pH 7.5). The mixture was pre-incubated at 55°C for 10 min, and 1 ml of lipase productions was subsequently added. The blank reaction was performed by 1 ml of 0.9% NaCl solution substituting equivalent amounts of the lipase productions. After 30 min of incubation at 55°C with shaking at 150 rpm, the reaction was immediately terminated by the addition of 15 ml of ice-cold 0.9% (w/v) NaCl solution. The liberated free fatty acid amount was measured by titration with 50 mM NaOH using phenolphthalein as an indicator. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of fatty acid per minute.

Characterization of the Whole-Cell Biocatalysts Pf-5 and Top10lipA

The substrate specificity was determined using a series of *p*-nitrophenyl esters purchased from Sigma-Aldrich China Inc. (Shanghai, China), included *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl decanoate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), and *p*-nitrophenyl palmitate (C16), and the maximum activity was considered as 100%. The lipase activity assay was done as described above.

The optimum pH was examined in buffers with pH values between 5.0 and 10.5, and the maximum activity was considered as 100%. The pH stability was also investigated by measuring the residual activity after incubating the cell suspension in the buffers with pH values from 5.0 to 10.5 at 28°C for 24 h, and the residual activity in the buffer with pH 7.0 was considered as 100%.

The optimum temperature was detected at different temperatures from 20°C to 80°C, and the maximum activity was considered as 100%. To determine the thermostability, whole-cell samples were pre-incubated at various temperatures ranging from 40°C to 70°C for 1 h. Then, the residual activity was determined at 55°C and pH 9.0, and the activity without pre-incubation was considered as 100%.

The effects of metal ions, EDTA, and surfactants on the activity of the two whole-cell lipases were analyzed by detecting the residual activity after incubating whole-cell samples with various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, Fe^{3+}, and

Al^{3+}) (5 mM), EDTA (5 mM), and surfactants (SDS, CTAB, NP-40, Triton X-100, Tween 20, Tween 40 and Tween 80) (0.1% (w/v)) at 28°C for 1 h, respectively; whole-cell samples with sterile distilled water were designated as the controls.

To observe the effects of organic solvents on the activity of the two whole-cell lipases, various organic solvents (methanol, ethanol, tert-butanol, glycerol, acetone, chloroform and *n*-hexane) were respectively added to whole-cell samples to a final concentration of 30% (v/v). The residual activity was measured after incubation at 28°C for 1 h; whole-cell samples with sterile distilled water were set as the controls.

Results and Discussion

Construction of Markerless Deletion Mutant of Coding Region of *lipA*

To identify LipA, a markerless deletion mutant of the coding region of *lipA* was constructed by a gene replacement system with the plasmid pJQ200SK (Fig. 1), which utilized double-crossover recombination events to knock out the target sequence and clear the plasmid pJQ200SK by using the *Bacillus subtilis sacB* (levansucrase) gene for counterselection [31]. The primers lipA-672U/lipA946L produced specific fragments of 1,634 bp for wild-type Pf-5 and 749 bp for markerless deletion mutant Pf0617 by colony PCR. Then, the Pf0617 was further verified by sequencing. As expected, Pf0617 had no DNA sequence from the coding region of *lipA* and the plasmid pJQ200SK, which meant that the construction of the markerless deletion mutant of the coding region of *lipA* was successful. Construction of the desired gene knockout strain by insertion of a drug resistance marker into the target gene would be easier than the markerless deletion strategy. However, this strategy limits the number of different gene deletion mutations in a single strain because of the limitation of usable drug resistance markers, and may also interfere with expression of co-cistronic genes downstream from the disruption as a result of polar effects or frameshift mutations. Therefore, markerless deletion mutations would be preferable for the analysis of multiple genes operons or multigene knockout in the same strain [31].

Identification of LipA

After successfully constructing the markerless deletion mutant of the coding region of *lipA*, the *in vivo* biological function of *lipA* was verified through determining the lipase activity of wild-type Pf-5 and mutant Pf0617. Total lipase activity is composed of the extracellular lipase activity and the whole-cell lipase activity. Compared with Pf-5, Pf0617 remarkably reduced its whole-cell lipase activity to 47.29% (Fig. 2). Notably, Pf0617 still had almost half of the whole-cell lipase activity on membrane-permeable *p*NPC, which might be due to intracellular lipase-associated proteins hydrolyzing the intracellular *p*NPC. GenomeNet search (http://www.genome.jp/) showed that Pf-5 includes 16 lipase-associated proteins, in which LipA (PFL_0617) and PFL_2722 are lipases, PFL_5879 is a GDSL-like lipase/ccylhydrolase, and others are phospholipases. Generally, lipases have a conservative GXSXG motif containing the active-site serine residue, which is found in LipA and PFL_2722. Unlike the GXSXG motif-containing lipases, GDSL-like lipases include a GDS(L) motif containing the active-site serine residue [2]. Moreover, LipA respectively shares 10.1% and 12.9% identities with PFL_2722 and PFL_5879, which means that they may have quite different properties. Subsequently, the *lipA* expression in the Pf0617 complemented with pBBRKlipA (*i.e.*, Pf0617lipA) was investigated along with Pf-5K as the control. The whole-cell lipase activity of Pf0617lipA was 205.37% of that of Pf-5K (Fig. 2), suggesting that the *lac* promoter was effective in *P. protegens*, and the multiple copies of plasmid pBBRKlipA increased the enzyme activity. It was worth noting that both the deletion and overexpression of *lipA* had no effect on the extracellular lipase production (data not shown). Additionally,

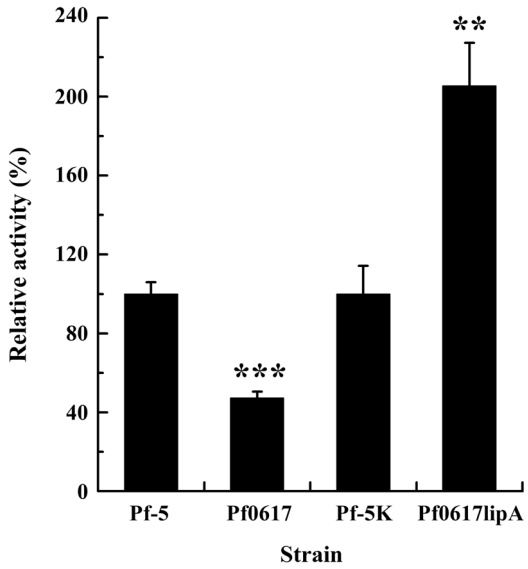

Fig. 2. Relative whole-cell lipase activity of different strains.
Pf-5, wild type; Pf0617, markerless deletion mutant of the coding region of *lipA*; Pf-5K, Pf-5 with pBBRKm; Pf0617lipA, Pf0617 with pBBRKlipA. The height of each bar and the error bars show the mean and standard deviation, respectively, from three independent experiments. $^{**}P < 0.01$, compared with Pf-5K, $^{***}P < 0.001$, compared with Pf-5.

the whole-cell lipase activity of these strains on the membrane-impermeable olive oil was not detected, but the recombinant LipA (*i.e.*, rPFL from *P. fluorescens* JCM5963) was able to hydrolyze olive oil [33]. Taken together, these results confirmed that LipA was an intracellular lipase and Pf-5 could also be utilized as a natural whole-cell biocatalyst. It is reported that LipA is a member of subfamily I.1 lipases [33], but subfamily I.1 lipases are generally secreted into the extracytoplasmic space through the type II secretion pathway [27]. That LipA is not an extracellular lipase may be due to the absence of its signal peptides for secretion and its cognate lipase-specific foldase for folding in the periplasm into an enzymatically active conformation.

Characterization of the Whole-Cell Biocatalysts Pf-5 and Top10lipA

As mentioned above, the deletion of *lipA* could not completely eliminate the whole-cell lipase activity of *P. protegens* Pf-5. In order to avoid the interference of other lipases from Pf-5, Top10lipA as a whole-cell lipase was also characterized as a result of *E. coli* Top10 being a lipase-free host. Their properties, including substrate specificity, optimal pH and pH stability, optimal temperature and thermostability, and the effects of metal ions, EDTA, surfactants, and organic solvents, were characterized by measuring the whole-cell lipase activities of Pf-5 and Top10lipA.

Pf-5 and Top10lipA, having similar substrate specificity, preferentially catalyzed the *p*-nitrophenyl esters of different fatty acids with short to medium chains. The best substrate of Pf-5 was observed for C8 (100%), followed by C10 (74.86%), C4 (65.71%), C12 (41.14%), C14 (28.71%), and C16 (24.14%) (Fig. 3). With regard to Top10lipA, the best substrate was also C8 (100%), followed by C10 (79.56%), C4 (66.74%), C12 (43.28%), C14 (21.33%), and C16 (17.47%) (Fig. 3). Likewise, a similar report was also found in the recombinant LipA [33].

The optimum hydrolytic activity (21.37 ± 0.50 U/ml·OD_{600}) of Pf-5 was examined at pH 9.5 and the hydrolytic activity within pH 9.0–10.0 had no significant difference. After incubating the whole-cell samples of Pf-5 in the buffers with pH values from 5.0 to 10.5 at 28°C for 24 h, it showed more than 80% of its maximum activity (30.32 ± 0.95 U/ml·OD_{600}) at pH 7.0 in a range of pH 6.0–10.0, and 71.63% and 79.03% of its maximum activity at pH 7.0 was detected at pH 5.0 and 10.5, respectively (Fig. 4A). The temperature-activity curve of Pf-5 showed that the optimal reaction temperature was 55°C (20.68 ± 0.23 U/ml·OD_{600}) and the difference between 50°C and 55°C was not significant.

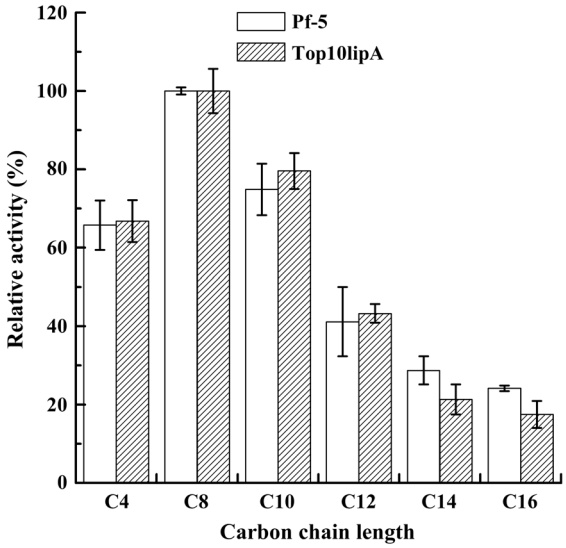

Fig. 3. Substrate specificity of whole-cell lipases from Pf-5 and Top10lipA.

Pf-5, wild type; Top10lipA, *E. coli* Top10 with pBBRKlipA. C4, *p*-nitrophenyl butyrate; C8, *p*-nitrophenyl caprylate; C10, *p*-nitrophenyl decanoate; C12, *p*-nitrophenyl laurate; C14, *p*-nitrophenyl myristate; C16; and *p*-nitrophenyl palmitate. The height of each bar and the error bars show the mean and standard deviation, respectively, from three independent experiments.

Compared with the initial activity before incubation, the residual activity after incubation for 1 h at temperatures ranging from 40°C to 60°C increased slightly. However, the residual activity declined sharply when the temperature was beyond 60°C, and Pf-5 only retained 52.42% of its original activity at 70°C for 1 h (Fig. 4C). As shown in Fig. 4B, Top10lipA had optimum hydrolytic activity (35.80 ± 2.07 U/ml·OD_{600}) at pH 9.0–9.5, and more than 80% of its maximum activity at pH 9.5 in a range of pH 8.0–10.0. The whole-cell samples of Top10lipA were incubated in buffers with pH values from 5.0 to 10.5; the lipase was stable over a broad pH range of 5.0–10.5 and showed more than 80% of its maximum activity (42.89 ± 3.47 U/ml·OD_{600}) at pH 7.0 in a range of pH 5.0–8.0 after 24 h of incubation at 28°C. Furthermore, the optimal temperature for Top10lipA towards hydrolysis of *p*NPC was 55°C, and it kept more than 90% of the maximum activity (33.56 ± 0.70 U/ml·OD_{600}) at 55°C in a temperature range of 50–60°C. After incubation for 1 h at temperatures ranging from 40°C to 70°C, Top10lipA was stable and retained over 80% residual activity compared with the initial activity before incubation (Fig. 4D). Generally speaking, the whole-cell lipase, being restricted by the cell, is subjected to protection against environmental interference.

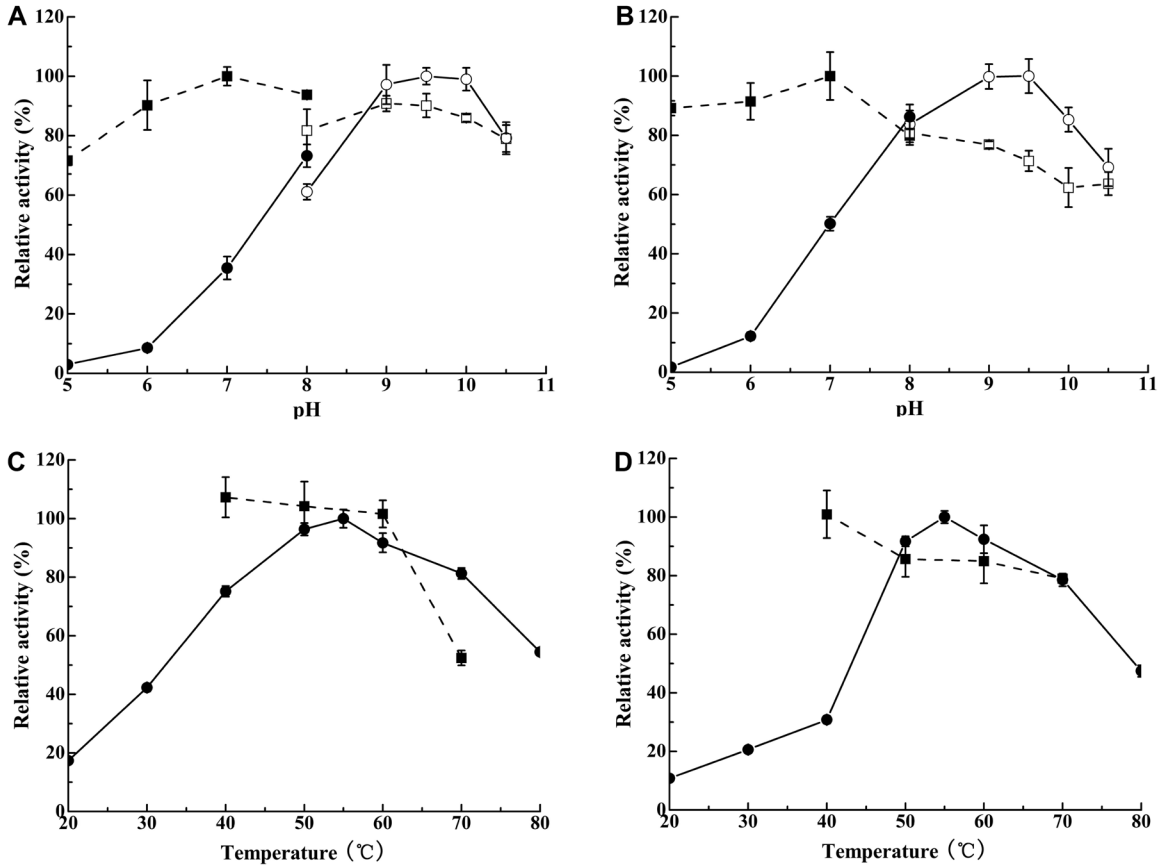

Fig. 4. Effects of pH and temperature on the activity (solid line, ● and ○) and stability (dashed line, ■ and □) of whole-cell lipases from Pf-5 (**A** and **C**) and Top10lipA (**B** and **D**).

Pf-5, wild type; Top10lipA, *E. coli* Top10 with pBBRKlipA. The following buffers were used: 50 mM disodium hydrogen phosphate-citric acid buffer (pH 5.0–8.0); 50 mM Tris-HCl buffer (pH 8.0–10.5). Data correspond to the mean ± SD of three independent experiments.

Therefore, it is expected to show higher thermostability and higher tolerance against ionic stress or pH change [21]. This was true of Pf-5 and Top10lipA, which had higher activity and stability in wide ranges of temperatures and pH values compared with the recombinant LipA (Table 1). As we all know, high activity and excellent stability under high temperature and alkaline conditions are useful characteristics for the lipases to be used in biotransformation fields [4, 28]. Moreover, Pf-5 and Top10lipA exhibited similar properties in pH and temperature, but they also had some differences, which might be caused by other lipase-associated proteins of Pf-5.

The effects of various metal ions and EDTA on the activities of the two whole-cell lipases are shown in Fig. 5. For Pf-5, Mg^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, K^{+}, and Na^{+} significantly increased its activity by 30.67%, 18.05%, 13.12%, 12.73%, 9.58%, and 7.68%, respectively, with respect to the control. On the contrary, Fe^{3+}, Zn^{2+}, Cu^{2+}, and EDTA significantly decreased its activity by 37.43%, 16.57%, 15.09%, and 11.02%, respectively, whereas Al^{3+} had no remarkable effect on its activity. For Top10lipA, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+} significantly increased its activity by 70.29%, 27.65%, 26.91%, 13.24%, and 10.93%, respectively, with respect to the control. Na^{+}, K^{+}, Ba^{2+}, Al^{3+}, and EDTA did not show remarkable effect on its activity; however, the presence of Fe^{3+} significantly decreased its activity by 37.42%. It was reported that most of the heavy metal ions inactivated lipases from *Pseudomonas* sp. [24] and recombinant LipA [33], but that of Pf-5 was enhanced by Mn^{2+} and Ba^{2+}, and of Top10lipA by Zn^{2+}, Cu^{2+}, and Mn^{2+}, and they also had high tolerance against other heavy metal ions used. This may be due to the fact that the cell more or less protects the whole-cell lipase from being inactivated by those heavy metal ions. Moreover, the enhanced effects of Mg^{2+}, Mn^{2+}, and

Table 1. Remarkable differences between whole-cell LipA and its free form.

	Whole-cell LipA[a]	Recombinant LipA[b]
Optimal pH	9.0–9.5	9.0
Thermostability	60–70°C > 80%	over 60°C < 80%
Mg^{2+}	+	N
Cu^{2+}	+	–
Mn^{2+}	+	–
Zn^{2+}	+	–
EDTA	N	–
SDS	+	–
Chloroform	+	–

[a]The data from *E. coli* Top10lipA.

[b]The data from rPFL of *P. fluorescens* JCM5963 [33].

+, Activation; –, inhibition; N, no effect.

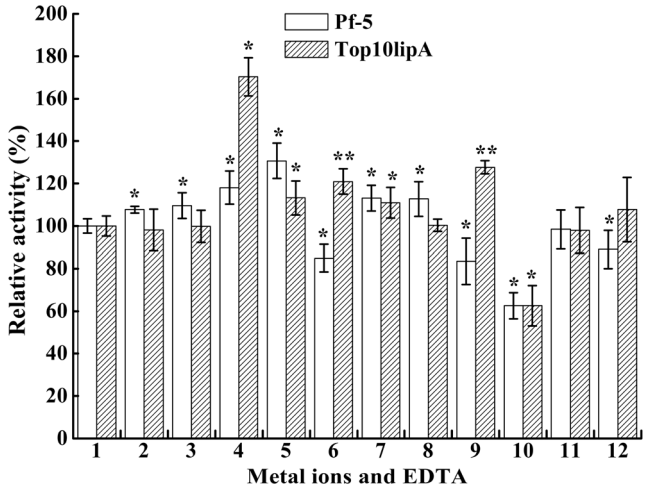

Fig. 5. Effects of metal ions and EDTA on the activity of whole-cell lipases from Pf-5 and Top10lipA.

Pf-5, wild type; Top10lipA, *E. coli* Top10 with pBBRKlipA. 1, control; 2, Na^{+}; 3, K^{+}; 4, Ca^{2+}; 5, Mg^{2+}; 6, Cu^{2+}; 7, Mn^{2+}; 8, Ba^{2+}; 9, Zn^{2+}; 10, Fe^{3+}; 11, Al^{3+}; 12, EDTA. The height of each bar and the error bars show the mean and standard deviation, respectively, from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

Ca^{2+} may be caused by the formation of insoluble salts of fatty acids released in hydrolysis, and thus avoiding product inhibition [11]. However, the activation effects of Ca^{2+} on Pf-5 and Top10lipA were not consistent with some lipases greatly activated by Ca^{2+} [30, 32], suggesting that LipA is not highly dependent on Ca^{2+}, which coincides with the effects of EDTA on the Pf-5 and Top10 lipA. Additionally, the differences between Pf-5 and Top10lipA may be attributed to Pf-5 containing other lipase-associated proteins.

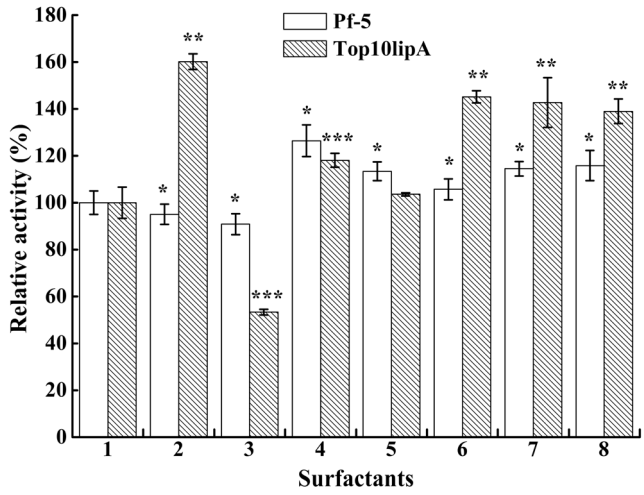

Fig. 6. Effects of surfactants on the activity of whole-cell lipases from Pf-5 and Top10lipA.

Pf-5, wild type; Top10lipA, *E. coli* Top10 with pBBRKlipA. 1, control; 2, SDS; 3, CTAB; 4, NP-40; 5, Triton X-100; 6, Tween 20; 7, Tween 40; 8, Tween 80. The height of each bar and the error bars show the mean and standard deviation, respectively, from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control.

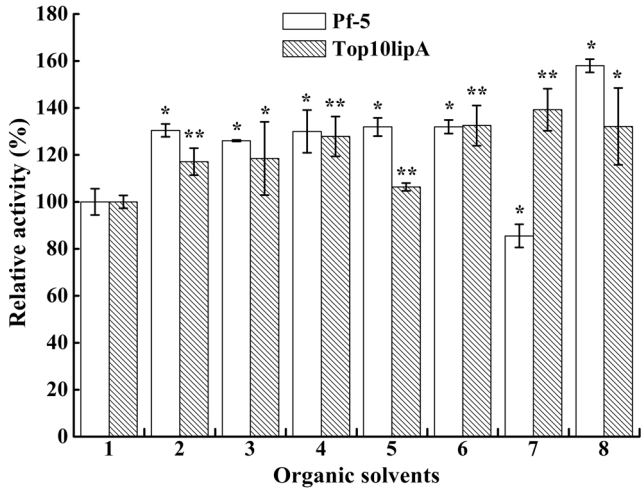

Fig. 7. Effects of organic solvents on the activity of whole-cell lipases from Pf-5 and Top10lipA.

Pf-5, wild type; Top10lipA, *E. coli* Top10 with pBBRKlipA. 1, control; 2, methanol; 3, ethanol; 4, *tert*-butanol; 5, glycerol; 6, acetone; 7, chloroform; 8, *n*-hexane. The height of each bar and the error bars show the mean and standard deviation, respectively, from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

The effects of various surfactants on the activities of the two whole-cell lipases were determined (Fig. 6). The results

of Pf-5 indicated that ionic surfactants SDS and CTAB remarkably inhibited its activity by 4.93% and 9.12%, respectively, whereas non-ionic surfactants NP-40, Triton X-100, Tween 80, Tween 40, and Tween 20 significantly increased its activity by 26.38%, 13.36%, 15.78%, 14.52%, and 5.70%, respectively. The results of Top10lipA demonstrated that anionic surfactant SDS significantly enhanced its activity by 60.17%, whereas cationic surfactant CTAB remarkably inhibited its activity by 46.69%. Furthermore, nonionic surfactants Tween 20, Tween 40, Tween 80, and NP-40 significantly increased its activity by 45.16%, 42.76%, 39.96%, and 18.09%, respectively; and Triton X-100 had no remarkable activation on its activity. However, SDS completely inhibited the activity of the recombinant LipA [33], which may be because of the whole-cell lipase being subjected to the protection of cell structure. The negative effects of ionic surfactants may be due to ionic interactions between the surfactant and the lipase, which induce unfolding and denaturing of the enzyme. However, the difference between CTAB and SDS may be due to the stronger ability of CTAB to damage the membrane of the bacterium than SDS. For nonionic surfactants, its positive effects, to some extent, may be attributed to hydrogen-bonding and hydrophobic interactions with the lipase [19]. Furthermore, Pf-5 having other lipase-associated proteins may be the reason for the differences existing between Pf-5 and Top10lipA.

The industrial applications of lipases can be broadened and enhanced greatly by using them in micro- or non-aqueous systems. Thus, high stability and activity of lipases in the presence of organic solvents are a requisite and desirable for biotransformation [24]. In view of that, the activities of the two whole-cell lipases in various organic solvents were also investigated (Fig. 7). Pf-5 was only inhibited in the presence of chloroform (85.53%), whereas other organic solvents stimulated its activity to some extent, and the maximum activity was in the presence of *n*-hexane (157.98%), followed by acetone (132.02%), glycerol (131.91%), methanol (130.47%), *tert*-butanol (130.04%), and ethanol (126.07%). All organic solvents used significantly stimulated the activity of Top10lipA, and the maximum activity was observed in the presence of chloroform (139.24%), followed by acetone (132.54%), *n*-hexane (132.15%), *tert*-butanol (127.89%), ethanol (118.47%), methanol (117.09%), and glycerol (106.33%). Both Pf-5 and Top10lipA had high stability and activity in organic solvents, which was similar to other organic solvent-tolerant lipases [11, 12, 33]. The activation effects of organic solvents on lipases can be explained from several aspects, such as inducing beneficial protein conformations and increasing the α-helix content, increasing access of the substrate to the active site of lipases, decreasing enzyme aggregation, modifying the substrate-water interface, *etc*. [11, 13, 24, 34]. In general, application of lipases in organic media instead of water has exhibited many advantages: (i) increased activity, stability, regio- and/or stereo-selectivity; (ii) higher solubility of substrate and relative ease of products recovery; (iii) possibility of reducing the degree of undesirable substrate and/or product inhibition; and (iv) ability to shift the reaction equilibrium to a synthetic direction [11, 24, 33]. Thus, in consideration of the high stability and activity in various organic solvents, Pf-5 and Top10lipA both show great potential in biotransformation fields.

In conclusion, in this study, LipA was identified to be an intracellular lipase, through constructing a markerless deletion mutant of the coding region of *lipA* and complementary strain Pf0617lipA with a lipase expression plasmid pBBRKlipA. Our results sufficiently confirmed that the properties of whole-cell LipA were more excellent than that of its free form (*i.e.*, recombinant LipA) (Table 1). The two whole-cell biocatalysts *P. protegens* Pf-5 and *E. coli* Top10lipA exhibited high stability and activity under moderate temperatures, alkaline conditions, heavy metal ions, surfactants, and organic solvents. In light of the excellent properties, the simple preparation, and reusability of the whole-cell lipase, biocontrol bacterium *P. protegens* Pf-5 and *E. coli* Top10lipA can be used as cost-effective whole-cell biocatalysts for various applications in biotransformation fields.

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