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ORIGINAL PAPER

Functional expression of a novel alkaline-adapted lipase of *Bacillus amyloliquefaciens* from stinky tofu brine and development of immobilized enzyme for biodiesel production

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Abstract Using enrichment procedures, a lipolytic strain was isolated from a stinky tofu brine and was identified as *Bacillus amyloliquefaciens* (named *B. amyloliquefaciens* Nsic-8) by morphological, physiological, biochemical tests and 16S rDNA sequence analysis. Meanwhile, the key enzyme gene (named lip_{BA}) involved in ester metabolism was obtained from Nsic-8 with the assistance of homology analysis. The novel gene has an open reading frame of 645 bp, and encodes a 214-amino-acid lipase (Lip_{BA}). The deduced amino acid sequence shows the highest identity with the lipase from *B. amyloliquefaciens* IT-45 (NCBI database) and belongs to the family of triacylglycerol lipase (EC 3.1.1.3). The lipase gene was expressed in *Escherichia coli* BL21(DE3) using plasmid pET-28a. The enzyme activity and specific activity were 250 ± 16 U/ml and 1750 ± 153 U/mg, respectively. The optimum pH and temperature of the recombinant enzyme were 9.0 and 40 °C respectively. Lip_{BA} showed much higher stability under alkaline conditions and was stable at pH 7.0–11.0. The *Km* and *V*max values of purified Lip_{BA} using 4-nitrophenyl palmitate as the substrate were 1.04 ± 0.06 mM and 119.05 ± 7.16 μmol/(ml min), respectively. After purification, recombinant lipase was immobilized with the optimal conditions (immobilization time 3 h at 30 °C, with 92 % enzyme recovery) and the immobilized enzyme was applied in biodiesel production. This is the first report of the lipase activity and lipase gene obtained from *B. amyloliquefaciens* (including wild strain and recombinant strain) and the recombinant Lip_{BA} with the detailed enzymatic properties. Also the preliminary study of the transesterification shows the potential value in biodiesel production applications.

Keywords *Bacillus amyloliquefaciens* · Lipase · Gene cloning · Functional expression · Immobilization

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Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a class of enzymes that can catalyze the hydrolysis of long-chain triacylglycerols into fatty acids and glycerol. They are widely distributed in bacteria, yeasts, fungi, plants and animals (Pahoja and Sethar 2002). Among these, various microbial lipases produced by *Bacillus* sp. showing conspicuous commercially significant due to its numerous unique characteristics: region specificity, chiral selectivity, and substrate

specificity (Schmid and Verger 1998). The lipases are widely used in various industries like food, dairy, chemical, textile, pharmaceutical, cosmetic, detergent production, and especially in biodiesel production and synthesis of new polymeric materials. As containing so many applications, cloning the novel enzyme with distinct features such as thermostable, alkaline, high activities and stabilities in organic solvents is of interest for industrial applications (Niehaus et al. 1999; Pennisi 1997). Jaeger et al. (1999) classified the bacterial lipases into six families (families I–VI) and family I was further classified into six subfamilies (subfamilies I.1–I.6). Lipases from *Bacillus* sp. were considered to be the lidless and small (actually the smallest lipases known). These lipases seem to be well-suited for biotechnological applications, the synthesis of chiral drugs in particular.

Lipases from *Bacillus* species have attracted more attention since they have the potentials to be used in food and chemistry industries. Although many *Bacillus* lipases (Kim et al. 2002) genes from *Bacillus subtilis*, *Bacillus pumilus* (Cho et al. 2000), *Bacillus stearothermophilus* (Kim et al. 2000), *Bacillus thermocatenulatus* (Schmidt-Dannert et al. 1996) and *Bacillus thermoleovorans* (Cho et al. 2000) have been cloned and sequenced, there is no report of *Bacillus amyloliquefaciens* lipase including wild strain lipase activity, lipase gene cloning from *B. amyloliquefaciens* and recombinant strain. Recently, the increased global demand for biofuels has prompted the search for alternatives to edible oils for biodiesel production (Hama and Kondo 2013). Biodiesel is produced by transesterification of oils or fats with chemical catalysts or lipase (Tan et al. 2010). Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time (Datta et al. 2013). Although many immobilized microbial lipases have been used in the production of biodiesel from vegetable oil (Hama and Kondo 2013). There is no report of transesterification catalyzed by *Bacillus amyloliquefaciens* lipase.

Previously, we have obtained a lipolytic strain which was identified as *B. amyloliquefaciens* (named *B. amyloliquefaciens* Nsic-8). In this study, a novel lipase gene (*lip*$_{BA}$) was obtained from *B. amyloliquefaciens* and in vivo functional expression of the active lipase was successfully achieved. The enzyme characterizations including the enzyme activity/stability, optimum temperature, optimum pH and substrate specificity were also described through protein purification. Meanwhile, recombinant lipase Lip$_{BA}$ was immobilized and the biodiesel production using immobilized Lip$_{BA}$ lipase was studied with preliminary results in this research. Also the optimal conditions of immobilization and transesterification were analyzed in our research. To our best knowledge, this is the first report of lipase gene cloning from *B. amyloliquefaciens* and the recombinant enzyme with detailed enzymatic properties. This alkaline-adapted and thermal-adapted lipase showed potential value in industrial applications according to the enzyme characterizations especially in the detergent industry.

Materials and methods

Bacterial strain and plasmids

An alkaline thermostable lipase-producing bacterial strain used in this study was isolated from a stinky tofu brine by three steps (Kumar et al. 2011) and identified as *B. amyloliquefaciens*. *Escherichia coli* DH5α (Invitrogen) and plasmid pMD19-T (TaKaRa) were used for gene cloning and sequencing. Plasmid pET-28a (Novagen) was the vector used to construct the protein expression plasmid in *E. coli* BL21 (DE3).

Chemicals, strain screening and culture conditions

Methyl heptadecanoate and the substrate 4-nitrophenyl palmitate were purchased from Fluka and Sigma, respectively. Methanol (AR grade), n-hexane (AR grade) and olive oil (saponification value 175–195) used for transesterification and all other chemicals (analytical grade) were purchased at local market. Yeast extract and tryptone were obtained from Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China).

In this work, enrichment culture technique was applied. LB medium added with 1 % olive oil emulsion was used to isolate potential bacterial strains. One ml sample (stinky tofu brine) was added to a flask with 100 ml sterile distilled water. With activating on a rotary for 40 min, 0.1 ml activation culture broth was inoculated into 100 ml enriched medium. Then the medium was shaken at 120 rpm, 30 °C for 48 h. After several rounds of enrichment, inocula was serially diluted and plated onto Rhodamine B agar

plates. The microbes showing obviously hydrolysis circle were isolated, purified and transferred to maintenance slants. The strains showing high ratios of hydrolysis circles were selected and identified by 16S rDNA sequence analysis.

Gene cloning of lipase and DNA manipulation/propagation

Based on the information of *Bacillus* sp. lipase in GeneBank, degenerate primes (BA-L-U: ATGAAA CAWATAAAARCAAAATYC. BA-L-D: TTAAT TYGTATTTTGTCCGCCGCCG) for the lipase gene sequence were designed. According to NCBI search, the following gene sequences of lipase were analyzed to design primers: *Bacillus amyloliquefaciens* strain E1PA (GI:511774067), *Bacillus amyloliquefaciens* SQR9 (GI:631799361), *Bacillus amyloliquefaciens* TA208 (GI:328551700) and *Bacillus amyloliquefaciens* XH7 (GI:341825903).

The DNA manipulations were carried out following the standard procedures. The *Bacillus amyloliquefaciens* Nsic-8 was harvested after overnight growth in LB medium and DNA extraction was performed using the DNA Mini kit (Axygen). The primes (forward primer P-NOSIG-F(5′-GCGGGATCCTCCTCAGGGCATAA CCCT-3′, reverse primer P-NOSIG-R(5′-CTACTCGA GTTAATTTGTATTTTGTCC-3′, underlined nucleotides indicate restriction enzyme sites) were used for lipase gene (without signal peptide) amplification, and it was carried out by the following steps: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension of 8 min at 72 °C.The PCR products were cloned into pMD-l9T simple vector after recovering by DNA gel extraction kit (Axygen, China), and then transferred into *E. coli* DH5α. The nucleotide sequence and predicted amino acid sequence were analyzed by the programs of Blast (NCBI). The open reading frame (ORF) was predicted using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide was predicted by the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Enzyme Mw and pI were predicted using the ExPASy proteomic server program compute pI/Mw (http://web.expasy.org/compute_pi/). Multiple sequence alignments were performed using DNAMAN and CLUSTAL W. The unrooted phylogenetic tree was constructed using the MEGA program and the three-dimensional structure of this enzyme was predicted by the SWISS-MODEL server (http://swissmodel.expasy.org/SWISS-MODEL.html).

Gene expression of lipase in *E. coli*

Plasmid pET-28a was used for gene expression in *E. coli*. After digestion by *Bam*HI/*Xho*I, the lip_{BA} gene was reclaimed and connected with pET-28a vector, which were digested by the same restriction endonuclease. The positive clones were selected by restriction enzyme digestion and finally confirmed by sequencing (Fig. s-1 in supplementary materials). The recombinant plasmid pET-28-lip_{BA} was transformed into *E. coli* BL21 (DE3). Recombinant cell (BL21-pET-28-lip_{BA}) was grown to saturation in LB medium supplemented with appropriate antibiotic (Kanamycin 50 μg/ml).

The recombinant lipase strain was incubated in 5 ml Luria–Bertani (LB) medium containing kanamycin (50 μg/ml) at 37 °C for 12 h and then transferred into 50 ml LB medium for propagation. IPTG (0.1 mM) was added to the medium until the absorbance at 600 nm was 0.6. With the cultivation condition at 20 °C for 18 h, the crude enzyme was collected by ultrasonic broken after the strains were harvested by centrifugation (12,000×*g*, 10 min).

Purification of lipase and SDS–PAGE analysis

After induction by IPTG, cells were separated by centrifugation, dispersed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and heated to 100 °C. Finally, proteins were separated by SDS–PAGE (12.5 % acrylamide/bis-acrylamide) and stained with coomassie blue.

The previous articles described the methods for heterologous protein expression and Ni–NTA purification procedures in our group (Tan et al. 2010). After lysed by sonication, the crude enzyme was passed through a 0.22 μm filter and then applied to a Ni–NTA sperflow column (1 ml, Qiagen). After equilibrated with the lysis buffer (NPI 10: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), the column was subsequently washed with 10 ml of wash buffer (NPI 20: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) to remove the impurity protein. The fusion protein (His-tagged Lip_{BA}) was eluted with a linear gradient of washing buffer (from NPI 50 to NPI 250). The eluted protein was desalted

and concentrated by ultrafiltration using a 50 ml Amicon Ultra Centrifugal Filter Device with a molecular weight cut-off of 10 kDa (Millipore, USA). The purified enzyme was resuspended in sodium phosphate buffer (pH 7.0) containing 20 % glycerol and stored at −40 °C. The crude extract and the pure enzyme were analyzed by SDS–PAGE. All purification steps were carried out at 4 °C. Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as the standard.

Enzymatic properties analysis

Enzyme activities of purified Lip_{BA} solution were assayed by measuring the absorbance at 405 nm of liberated p-nitrophenol. 1 U is defined as the amount of enzyme releasing 1 μmol p-nitrophenol per min under the assay conditions. The reaction mixture (0.5 ml) contained 50 μl of 4-Nitrophenyl palmitate solution (final concentration of 25 mM in the solution of isopropanol and dimethyl sulfoxide with volume ratio 3:1) as the substrate, 440 μl of lipase assay buffer (50 mM Glycine-NaOH buffer, pH 9.0) and 10 μl of appropriately diluted enzyme sample. The reaction mixture was incubated at 40 °C for 5 min, cooled in ice-bath and the absorbance was measured at 405 nm.

The pH optimum for the enzyme activity was studied over a range from pH 2–10 for 5 min (40 °C). The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 12 h and incubated at 40 °C. The following buffer systems were used: pH 2.0–3.0 with 50 mM glycine–HCl,pH 4.0–5.0 with 100 mM citric acid-sodium citrate, pH 6.0–7.0 with 200 mM sodium phosphate, pH 8.0 with 50 mM Tris–HCl, 9.0–10.0 with 50 mM glycine-NaOH and pH 11.0 with 50 mM sodium hydrogen phosphate-NaOH. The temperature optimum for the enzyme activity was assayed at 10–50 °C (pH 9.0). The thermal stability of Lip_{BA} was evaluated by assaying its residual activity after incubation of the enzyme at various temperatures for 12 h in sodium phosphate buffer (pH9.0). The effects of metal ions on the lipase activity were determined with various metal ions (1 and 8 mM) such as Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} (pH 9.0). Meanwhile, effects of surfactants (Tween 20, Tween 80, TritonX-100, sodium dodecyl sulfate (SDS), EDTA and DTT at the concentration of 1.0 mM) and various organic solvents (Methanol, Ethanol, Acetonitrile, Benzene, n-hexane, Chloroform, DMSO, Acetone, Are propanol and Isopropanol) (15 % (v/v) and 25 % (v/v)) were measured using the spectrophotometric assay as above. The reaction mixtures containing the enzyme sample were incubated at 40 °C for 60 min in 50 mM glycine-NaOH buffer, pH 9.0. The enzyme sample without any additives was considered as control (100 %).

Immobilized lipase by embedded in sodium alginate

The conditions of the immobilized lipase using sodium alginate as carrier were studied. First, purified lipase solution (10, 20, 30, 40 and 50 ml) was mixed with 50 ml sodium alginate (0.5 g) solution (dissolved with hot distilled water). Then, the mixed solution was dropped into aseptic $CaCl_2$ (1 %) solution using an injector. After immobilized for different time (0–6 h, every 30 min) with shaking (20–60 °C), filtration, washing and drying, the immobilized lipase was finally obtained (Datta et al. 2013). Then the residual lipase activity and thermal stability of the immobilized enzyme was measured.

Preliminary study of enzymatic transesterification reaction

The immobilized lipase was used for transesterification reaction. The reaction mixture containing 5.0 ml n-hexane, 0.5 mM olive oil, 1.5 mM methanol (divided into three times to add), 5 mg immobilized lipase (about 500 U) and 20 μl H_2O. After shaking with 200 rpm at a certain temperature (20–60 °C) for 24 h, biodiesel products were detected in the samples. As control, the industrial enzyme Novozym 435 (lipase B from *Candida Antarctica*) was tested under the same conditions. To confirm a suitable organic solvent for the lipased-catalyzed transesterification, various organic solvents (Isooctane, n-heptane, n-hexane, Cyclohexane, Acetone and Benzene) were examined. A series of temperature (20, 25, 30, 35, 40, 45 and 50 °C) were tested simultaneously.

The products were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC) respectively. Silica gel TLC plates were used for TLC analysis. After activated at 110 °C for 1 h, prepared samples were spotted onto the plate. The plate was

placed in a thin-layer chromatography (TLC) chamber containing a solvent system of N-hexane and Diethyl (19:1). After the plate was dried, the products bands were dyed by solid iodine. For GC analysis, HP-5 capillary column (Agilent, 30.0 m × 320 um × 0.25 um) was used. The temperature of vaporizer and detection room was 260 and 280 °C, respectively. First, the column temperature kept 180 °C for 1 min, then heated up to 280 °C with a speed of 10 °C/min for detection.

Results

Strain identification and lipase gene cloning

Strain Nsic-8 was identified as *Bacillus amyloliquefaciens* by 16S rDNA phylogenetic analysis and the morphology (Table 1). The GeneBank accession numbers for the *Bacillus amyloliquefaciens* Nsic-8 lipase gene is KF040967. The lipase gene lip_{BA} was amplified by primers BA-L-U/BA-L-D.

Sequence analysis revealed that the sequence of the lip_{BA} ORF uses ATG as the start codon and the G+C content (%) of lip_{BA} is 48.2 mol%. The lip_{BA} gene consists of 645 nucleotides and encodes a deduced protein of 214 amino acids. Analysis of signal peptide showed that a possible signal peptide of 32 amino acids in the N-terminal region was found and the peptide bond between 32th and 33th amino acids (SKA-SS) would be cleaved by signal peptidase (SignalP Server). The molecular weight of Lip_{BA} was estimated to be 22.76 kDa (19.22 kDa without the signal peptide), and the pI value was calculated to be 9.74 (9.66 without the signal peptide) by the ExPASy compute pI/Mw program algorithm.

Using the neighbor-joining method (CLUSTAL W), the lip_{BA} gene in *Bacillus amyloliquefaciens* Nsic-8 was aligned with the other confirmed lipase from other *Bacillus* sp.. An unrooted phylogenetic tree was constructed using the MEGA program and the phylogeny relationships of closely related microorganisms were shown in Fig. 1a. Homology analysis revealed that Lip_{BA} in *Bacillus amyloliquefaciens* shared the most identity with predicted triacylglycerol lipase in *Bacillus amyloliquefaciens* CAU B946 (GI: 375360932) and 78 % identical to the lipase in *Bacillus* sp. JS GI: 489334049 (74 % to *Bacillus pumilus* GI:169639781, GI:489310171, 73 % to *B. licheniformis* GI:51507670, 69 % to *B. megaterium* GI:515136309, 68 % to *B. megaterium* QM B1551 GI:294500508 and *B. megaterium* GI:18857878). Also, Lip_{BA} contains a single catalytic domain of the alpha/beta hydrolase family and belongs to the family of triacylglycerol lipase (EC 3.1.1.3) (Fig. 1b). The catalytic triad Ser^{110}, Asp^{166}, and His^{189} residues were seen in the regions. The conserved region, Ala-Xxx-Ser-Xxx-Gly (the feature of the lipase sequences from *Bacillus* sp.) is boxed. The sequence of signal peptide is underlined (Fig. 1c). Meanwhile, as no report of this lipase has been made, the secondary and three-dimensional structure of Lip_{BA} was predicted by the SWISS-MODEL server and the protein structure was viewed by PdbViewer (Fig. s-2 in supplementary materials). The catalytic triad Ser^{110}, Asp^{166}, and His^{189} residues and the distributions of α-helix/β-sheet were seen in the Fig. s-2.

Expression and purification of the recombinant enzyme in *E. coli*

Recombinant strain (BL21-pET-28-lip_{BA}) was grown to saturation in LB medium supplemented with appropriate antibiotic to express the recombinant protein. The SDS-PAGE results showed that the recombinant protein appeared mostly as inclusion bodies (induction temperature: 37 °C, induction time 18 h) in recombinant strains BL21-pET-28a-lip_{BA} and no lipase activity was detected. When the induction temperature dropped to 30 °C, the protein was expressed both in precipitation and supernatant. Using low temperature induction (20 °C), the recombinant protein appeared mostly as the soluble protein (Fig. 2a). As shown in Fig. 2b, the expressed protein Lip_{BA} increased with the extension of the culture time within a certain range at 20 °C. However, when cells were fermented for more than 18 h, there was no more significant accumulating of the recombinant protein. So we chose 18 h as the culture time to collect recombinant strains (BL21-pET-28a-lip_{BA}). The optimal expression conditions were found to be an IPTG concentration of 0.1 mM, an induction temperature of 20 °C and an induction time of 18 h. The optimal enzyme activity of Lip_{BA} was 250 ± 16 U/ml. Through Ni–NTA purification procedures, the specific activity of purified Lip_{BA} was 1750 ± 153 U/mg (about 7-purification fold). The purified Lip_{BA} migrate a single band on SDS–PAGE with an apparent

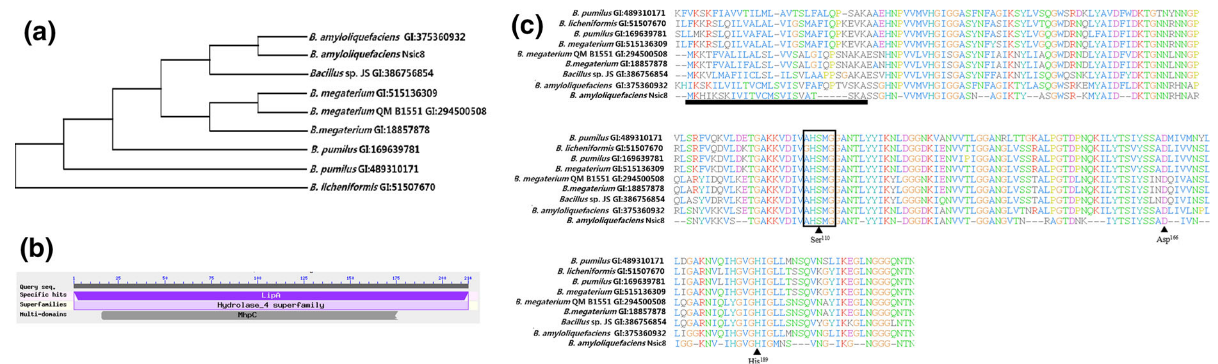

Fig. 1 The amino acid residues analysis. **a** Rooted phylogenetic tree of Lip_{BA}. The consensus amino acid residues of Lip_{BA} and some homologous lipase were aligned with FASTA. The sequences used in this alignment were obtained from GenBank as following. 1: Lipase from *Bacillus amyloliquefaciens* subsp. plantarum CAU B946 (Accession No: GI375360932), 2: Lipase from *Bacillus amyloliquefaciens* Nsic-8 (this study), 3: Lipase from *Bacillus* sp. JS (Accession No:GI386756854), 4: Lipase from *Bacillus megaterium* (Accession No: GI515136309), 5: Lipase from *Bacillus megaterium* QM B1551 (Accession No: GI294500508), 6: Lipase from *Bacillus megaterium* (Accession No: GI18857878), 7: Lipase from *Bacillus pumilus* (Accession No: GI169639781), 8: Lipase from *Bacillus pumilus* (Accession No: GI489310171), 9: Lipase from *Bacillus licheniformis* (Accession No: GI51507670). **b** Putative conserved domains in Lip_{BA}. **c** Conserved sequence alignment of Lip_{BA}. The structures are denoted as follows: *filled triangle*, the catalytic site (Ser110, Asp166 and His189). The reserved amino acid motif AHSXG is boxed. The consensus amino acid residues for the signal peptide are shown in underscore character

molecular mass of about 20.0 kDa, which was identical to the calculated value.

Biochemical characterization of the purified recombinant lipase

The optimum temperature of the purified Lip_{BA} was examined by assaying enzyme activity at different temperatures (20, 25, 30, 35, 40, 45, 50, 55 and 60 °C) in 50 mM Glycine-NaOH buffer (pH 9.0). The purified Lip_{BA} exhibited higher lipase activities over a temperature range of 30–45 °C (over 80 % of the highest activity), among which the highest specific enzyme activity was at 40 °C. Residual activities were determined with standard assay conditions. The Lip_{BA} was entirely stable at 20–60 °C and more than 50 % of its activity was retained after 12 h in 60 °C (Fig. 3a).

The effect of pH on lipase activity was determined using 4-nitrophenyl palmitate as substrate at various pH at 40 °C. The purified Lip_{BA} exhibited higher lipase activities over a pH range of 7.0–11.0, among which the highest specific enzyme activity was at pH 9.0. The activity of Lip_{BA} decreased significantly below pH 6.0 and only about 30 % of the maximal activity in this condition (pH 6.0). Stability of the purified enzyme was investigated in buffer solutions over the pH range of 2.0–11.0. The enzyme solution was incubated at 4 °C for 12 h and the residual activity was determined at pH 9.0 (Fig. 3b). Results showed that the lipase is an alkaline pH stability enzyme which was most stable at pH 7.0–11.0, among which the highest stability was at pH 9.0 (retaining 70 % activity).

The effects of different metal ions and chemical reagents on the lipase were examined (Table 2). At low concentration (1 mM), Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} partly inhibited or had no effects on the enzyme activity. However, at high concentration (8 mM), all of the metal ions showed significantly inhibited effects especially for Cu^{2+} (only 9 % of the highest activity). From the results of surfactants tests, the lipase activities had no significantly affected by EDTA and DTT, but significantly inhibited by Tween 20 and Tween 80 (Fig. 4). From the organic solvents test, the enzyme is fairly stable in alkanes, but is highly denatured in hydrophilic solvents such as acetone or short chain (C1–C3) alcohol (Methanol, Ethanol, Are propanol and Isopropanol). Also we found that the lipase was activated by 15 % (v/v) n-hexane (121 %), but inhibited by the concentration of 25 % (v/v) (73 %) (Fig. 4).

Immobilized lipase by embedded in sodium alginate

As shown in Fig. 5, the optimal temperature of immobilization was found to be 30 °C. Meanwhile,

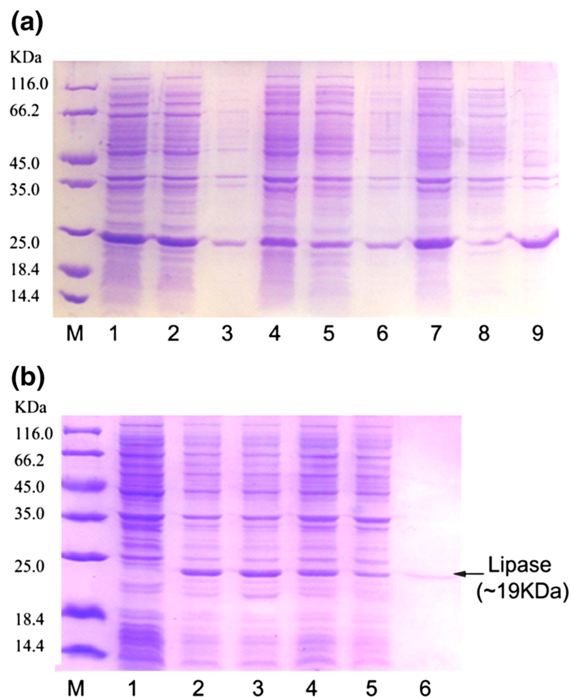

Fig. 2 SDS–PAGE analysis of the recombinant lipase and protein purification. **a** The SDS–PAGE analysis of the recombinant protein with different temperature (BL21-pET-28-lip_{BA}, 20, 30 and 37 °C for 16 h culture). *lane M* standard marker proteins; *lane 1* cell lysate (20 °C); *lane 2* precipitation of cell lysate (20 °C); *lane 3* supernatant of cell lysate (20 °C); *lane 4* cell lysate (30 °C); *lane 5* precipitation of cell lysate (30 °C); *lane 6* supernatant of cell lysate (30 °C); *lane 7* cell lysate (37 °C); *lane 8* precipitation of cell lysate (37 °C); *lane 9* supernatant of cell lysate (37 °C). **b** The SDS–PAGE analysis of the recombinant protein with different induction time (BL21-pET-28-lip_{BA}, culture temperature 20 °C). *lane M* standard marker proteins; *lanes 1–5* samples of the recombinant lipase (Lip_{BA}) cultured for 0, 6, 12, 18 and 24 h, respectively; *lane 6* the purified Lip_{BA} protein

the optimal immobilization time of Lip_{BA} was 3 h at 30 °C, with 92 % enzyme recovery was detected. The high enzyme recovery indicted that Lip_{BA} immobilized by embedded in sodium alginate was practical. The immobilized ability of 50 ml sodium alginate (0.5 g) solution was 30 ml lipase solution (45 mg protein). The thermostability of the immobilized lipase was studied by incubating the enzyme at 60 °C for 12 h, the residue activities of the immobilized lipase and free lipase were 75 and 52 %, respectively. Moreover, the immobilized lipase had good operational stability, which could be repeatedly used for three times with the relative activity as high as 90 %. Furthermore, the immobilized lipase was used for enzymatic transesterification reaction.

Enzymatic transesterification reaction

In biodiesel production, we applied the immobilized Lip_{BA} as biocatalyst. The TLC analysis revealed that band of methl ester (biodiesel) was detected in silica gel (Fig. 6a), also the results were further verified by gas chromatography analysis (Fig. 6b). In biodiesel production, three influence factors of the immobilized recombinant Lip_{BA} on the transesterification was investigated including temperature, reaction time and organic solvents. The results indicated that the optimal temperature of Lip_{BA} transesterification reaction was 35 °C (Fig. 6c). The conversion rate reached maximum after 18 h with n-hexane as organic solvents reaction system. Transesterification activities results showed that Lip_{BA} exhibited about equal conversion in non-polar solvents with only minor differences between them (Fig. 6d). However, immobilized enzyme Lip_{BA} led to very low transesterification in polar organic solvents such as acetone. The conversion increased with the response time extended, but the transesterification reaction was no longer continued after 18 h (Fig. 6e).

Determination of the kinetic parameters

The kinetic parameters were determined from Lineweaver–Burk plots (Fig. s-3 in supplementary materials). The values of *Km* and *Vmax* for 4-nitrophenyl palmitate were calculated to be 1.04 ± 0.06 mM and 119.05 ± 7.16 μmol/(ml min) by measuring the absorption at 405 nm for several concentrations of the substrate over a range of 0.2–2.0 mM, respectively. The data shows that the recombinant enzyme has high catalytic capacity.

Discussion

Lipase is an important enzyme which is used especially in biodiesel production and synthesis of new polymeric materials. Cloning of novel lipase genes with distinct features, especially from easily grown bacteria, is of interest for industrial applications. *Bacillus* species are well known for their ability to produce and secrete a large number of useful extracellular enzymes. Stinky tofu is a well-known and popular traditional fermented Chinese snack. Previous studies indicated that stinky brine fermentation is a type of alkaline fermentation because of the

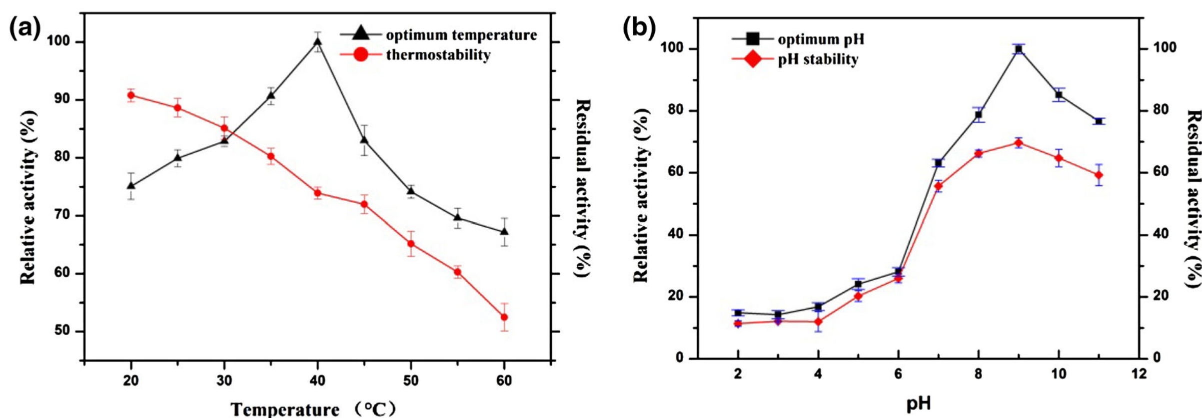

Fig. 3 Characterization of recombinant Lip_{BA}. **a** The temperature optimum and thermal stability of the recombinant lipase activity. (*filled triangle*) Optimum temperature of the recombinant lipase. (*filled circle*) Thermostability of the recombinant lipase. **b** The pH optima and pH stability of the recombinant lipase activity. (*filled square*) Optimum pH of the recombinant lipase. (*filled diamond*) pH stability of the recombinat lipase

Table 1 Phenotype info of *B. amyloliquefaciens* Nsic-8

Phenotype info	description
Gram stain	+
Cell shape	Rod
Motility	Yes
Sporulation	Yes
Temperature range	Mesophilic
Optimal temperature	37 °C
Oxygen requirement	Aerobic
Habitat	Terrestrial

Table 2 Effect of metal ions on the recombinant lipase activity

Metal ions	Relative activity (%)	
	1 mM	8 mM
Control	100	100
Fe^{2+}	76	12
Mg^{2+}	92	38
Ca^{2+}	62	25
Cu^{2+}	78	9
Zn^{2+}	96	10
Co^{2+}	80	13
Ni^{2+}	93	29
Mn^{2+}	87	18

The data represent the mean of three experimental repeats with SD of ≤5 %

production of NH_3 and referred to stinky tofu as an alkaline-fermented food. The pH of the food rises to 8.0–9.0 due to ammonia production during the fermentation process and *Bacillus* sp. commonly participates in the fermentation process. Selecting new bacterial strains or improving bacterial strains is a prerequisite and effective solution in industrial applications and will be important for maximal lipase production. In this work, a strain with the grease degradation ability was isolated from stinky tofu brine and identified as *Bacillus amyloliquefaciens*. Although many lipases from *Bacillus* sp. have been intensively investigated (Kim et al. 2002; Kamijo et al. 2011; Gupta et al. 2004), there was no report of lipase gene expression from *Bacillus amyloliquefaciens*. We obtained the lipase gene from the strain *Bacillus amyloliquefaciens* isolated from a stinky tofu brine and the mature lipase gene without the N-terminal signal peptide was successfully expressed in *E. coli* BL21. The novel recombinant enzyme Lip_{BA} with detailed enzymatic properties has preferable research significance.

Protein sequence alignment showed that Lip_{BA} belongs to lipase family I.4. Although, as representative strains in this subfamily, lipases from *B. subtilis* and *B. pumilus* have been studied for a long time, the novel lipase from *Bacillus amyloliquefaciens* was reported for the first time in this paper. Many of the lipases act on their substrates at the lipid-water

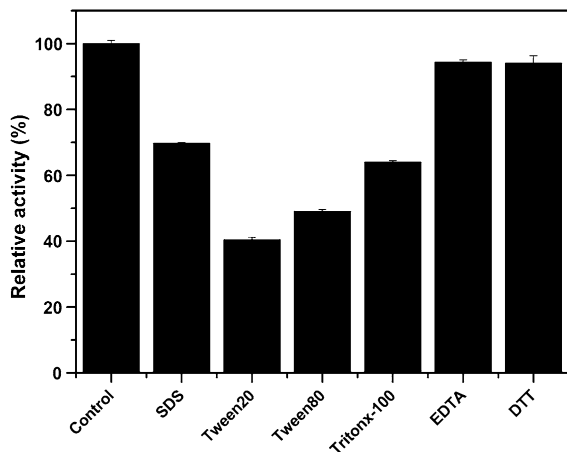

Fig. 4 Effects of surfactants on the recombinant lipase activity

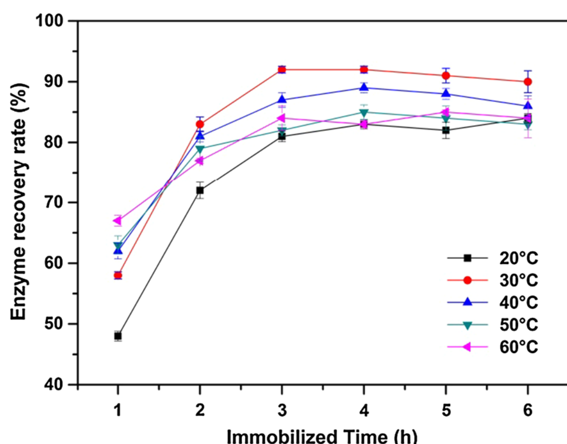

Fig. 5 Enzyme recovery of the enzyme immobilization at different temperature (*filled square*: 20 °C, *filled circle*: 30 °C, *filled triangle*: 40 °C, *filled inverted triangle*: 50 °C, *right slanted triangle*: 60 °C) for different time (1, 2, 3, 4, 5, 6 h)

interface (called interfacial activation). This activation was enhanced by a lid-like polypeptide, which covers the active site of the lipase. Meanwhile, not all lipases show this interfacial activation. There are lipase family members that lack the lid and do not show activation at oil–water interfaces. From the three-dimensional structure of Lip_{BA}, there was no lid above the catalytic triad residues (Ser^{110}, Asp^{166}, and His^{189}) (Fig. s-2 in supplementary materials). These small, lidless lipases seem to be well-suited for biotechnological applications (Jaeger et al. 1999). Homology analysis revealed that Lip_{BA} in *Bacillus amyloliquefaciens* shared the 60–80 % identity with predicted triacylglycerol lipase in *Bacillus* sp. The homology results reveal the remarkable research significance of the novel recombinant enzyme including enzyme characterizations and kinetic parameter. The Lip_{BA} was stable at pH 7.0–11.0 compared with that of *B. alcalophilus* (pH 10.0–10.5) (Gupta et al. 2004), *B. licheniformis* (pH 9–11) (Gupta et al. 2004), *B. alcalophilus* (pH 10.6) (Ghanem et al. 2000) and *B. subtilis* (pH 7.0–9.5) (Ma et al. 2006). The optimum temperature (40 °C) of the recombinant lipase is similar to that of lipase from *B. pumilus* B106 (50 °C) (Zhang et al. 2009) and *B. subtilis* (35 °C) (Lesuisse et al. 1993). As for the thermal stability, *B. pumilus* lipase B26 retained 100 % activity after 15 min at 70 °C (Kim et al. 2002), *B. subtilis* lipase 168 retained 100 % activity after 30 min at 40 °C (Lesuisse et al. 1993). In this study, lipase Lip_{BA} kept 53 % residue activity at 60 °C for 12 h. Even at 70 °C for 12 h, the enzyme attained 46 % residue activity. The results indicated that the recombinant Lip_{BA} is thermotolerant and was favorable for industrial or diagnostic use (Sugihara et al. 1991).

In this study, various metal ions (Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+}) were used to test the influences on the enzyme activity. All the tested metal ions (1 mM) were found to inhibit the enzyme activity. Analysis of the effects of Ca^{2+} on the lipase activity indicated that Lip_{BA} is a Ca^{2+}-independent lipase. The presence of Ca^{2+} (1 mM) strongly inhibited the enzyme activity (62 %). Meanwhile, many lipases were stimulated by Ca^{2+}, such as *Bacillus cereus* C7 lipase (1 mM, 200 %) (Dutta and Ray 2009), *B. subtilis* lipase (10 mM, 116 %) (Ma et al. 2006) and *Pseudomonas xuorescens* lipase. Although most lipases have the phenomenon of interfacial activation, some lipases lack it, such as *B. subtilis* lipase and *Candida antarctica* lipase B, which do not have a lid domain. On the other hand, the activity of *Bacillus pumilus* lipase B26 (Ca^{2+}-independent lipase) was almost constant at a wide range of Ca^{2+} concentration (Kim et al. 2002). The protein structure that Lip_{BA} do not contain calcium binding sites may provide the presence of this reason.

In this research, Lip_{BA} was inhibited by various surfactants especially for Tween 20 and Tween 80. However, among the reported lipases, the influences of the surfactants on enzyme activities are quite different. Neda Akbari found that only SDS and EDTA had

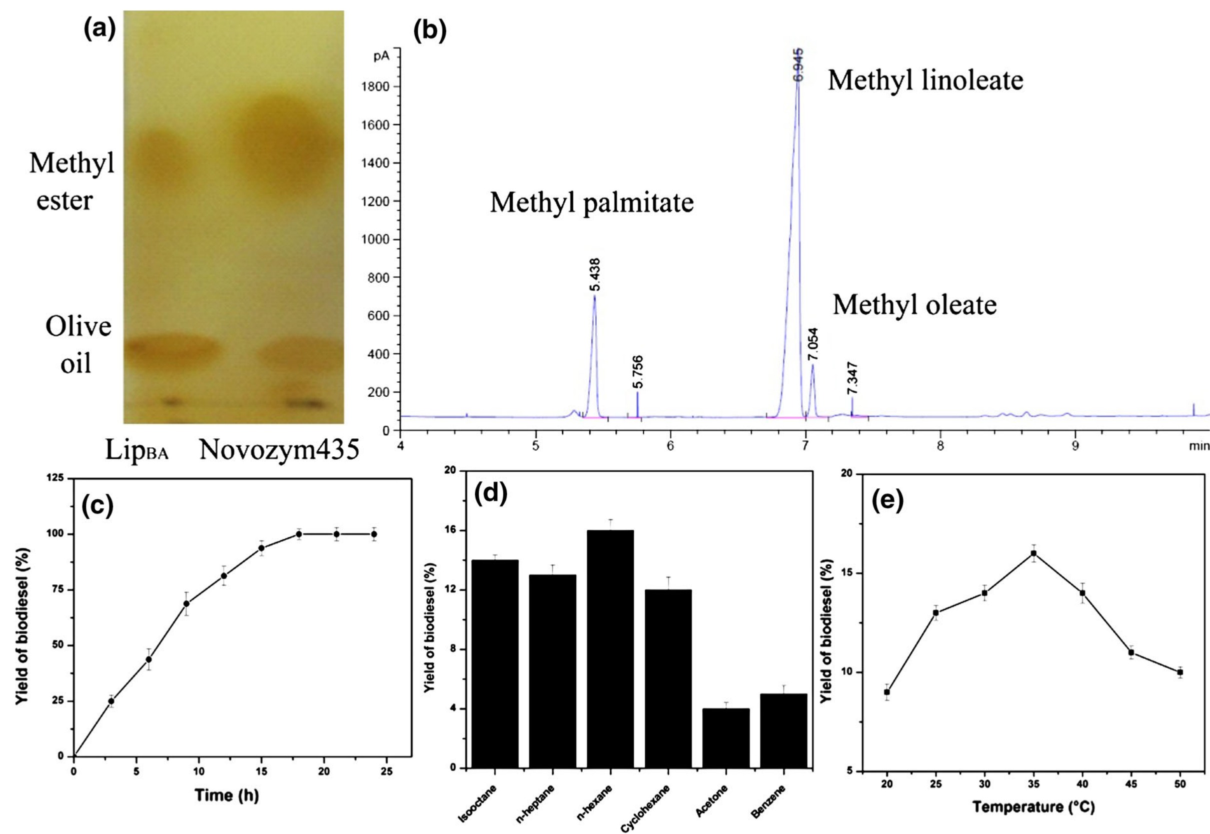

Fig. 6 Effects of some key parameters on the yield of biodiesel. **a** TLC analysis of product. **b** GC analysis of product. **c** Effect of temperature on the yield of biodiesel. **d** Effect of different grease on the yield of biodiesel. **e** Effect of different time on the yield of biodiesel

significant effect on *Pseudomonas* sp. lipase activity. The CALB (lipase from *Candida antarctica* ZJB09193) activities were enhanced in the presence of Tween-20 and sorbitol, inhibited by SDS and Tween-80 (Liu et al. 2012). We chose many organic solvents with different log *P* to measure the value of the enzyme as a biocatalyst in organic solvents. For it is useful to compare activity in organic solvent to that in aqueous buffer (Snellman and Colwell 2008). Log *P* is a measure of the polarity of an organic solvent, which is defined as the logarithm of its partition coefficient in a standard n-octane/water two phase system. Laane et al. (1987) concluded that the solvent parameter that correlates best with enzyme activity was log *P*. In this study, various solvents with different log *P* were used to test the enzyme stability (Table 3). Methanol (log *P*: −0.69), ethanol (log *P*: 0.32), acetonitrile (log *P*: −0.34), chloroform (log *P*: 1.97) and acetone (log *P*: −0.24) inhibited the enzyme activity. The results can be explained that organic solvents with log *P* values <2.0 are considered to be unfavorable for biocatalysis. However, the lipase Lip_{BA} was activated by 15 % (v/v) n-hexane (log *P* 3.6), but inhibited by the concentration of 25 % (v/v). According to the reported enzymes, n-hexane slightly enhanced the *Bacillus sphaericus* 205y lipase activity (Rahman et al. 2003) and *Bacillus megaterium* lipase activity (Lima et al. 2004). On the other hand, the enzyme is fairly stable in alkanes and long chain alcohols but is highly denatured in hydrophilic solvents such as acetone or short chain (C1-C3) alcohol.

Immobilized lipase as the biocatalyst draws high attention because that process is "greener". Cross-linking of alginate with divalent ions (like Ca^{2+}) and glutaraldehyde improves the stability of enzymes. So the material sodium alginate was used for fabrication of immobilization in our experiments. In this research, the immobilized lipase Lip_{BA} with 92 % enzyme recovery

Table 3 Effect of various organic solvents on recombinant lipase activity

Solvent	Log *P*	Relative activity (%)	
		15 %	25 %
Control	–	100	100
Methanol	−0.69	54	48
Ethanol	0.32	57	50
Acetonitrile	−0.34	60	54
Benzene	2.0	91	70
n-Hexane	3.6	121	73
Chloroform	1.97	72	69
DMSO	−1.3	76	66
Acetone	−0.24	67	56
Are propanol	0.28	63	54
Isopropanol	0.13	60	44

15 and 25 % organic solvent was added to the reaction system, respectively

showed better temperature tolerance (about 44 % improvement in 60 °C for 12 h) and operational stability (90 % residual enzyme activity through 3 batches used). Several methods are used for immobilization and various factors influence the recovery rate of immobilized enzymes activities (Datta et al. 2013). *Candida rugosa* lipase adsorbed on biodegradable poly (3-hydroxybutyrate-co-hydroxyvalerate) showed 94 % residual activity after 4 h at 50 °C and reusability till 12 cycles (Cabrera-Padilla et al. 2012). *Rhizopus oryzae* Lipase immobilized on the HPMCPVA (a blend of hydroxypropyl methyl cellulose (HPMC) and polyvinyl alcohol (PVA)) showed 86–94 % immobilized efficiency, which was very high without any washing procedure (Dhake et al. 2011). Increasing environmental concerns have led to the use of immobilized biocatalysts for biodiesel production (Datta et al. 2013). Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. The maximum biodiesel yield of lipase from *Thermomyces lanuginosus* was 97 % at 50 °C in 24 h reaction (Dizge et al. 2009). The immobilized lipase of *P. cepacia* can catalyze biodiesel production with the final conversion was around 67 % (Noureddini et al. 2005). The immobilized of *Pseudomonas fluorescens* can catalyze vegetable oil and cooking oil with yield higher than 87 % (Salis et al. 2008). Although many microbial lipases in biodiesel production had been studied, few lipases from *Bacillus* sp. were investigated. The biodiesel yield of lipase from *Bacillus subtilis* reached about 90 % after 72 h reaction in a solvent-free system (Ying and Chen 2007). The transesterification activity of the isolated *Bacillus* sp. lipase showed 76 % of fatty acid methyl esters yield in 40 h (Sivaramakrishnan and Muthukumar 2012). In our study, the transesterification yield of olive oil was much lower than industrial enzyme Novozym 435 (lipase B from *Candida Antarctica*) (Fig. 6a). In this research, we found that the enzyme activity was inhibited by n-hexane (25 % concentration). Meanwhile, the enzyme is highly denatured in short chain (C1–C3) alcohol such as methanol. In the reaction system of transesterification, n-hexane and methanol were used as solvent and reactant respectively. This may be the reasons of the low conversion in transesterification. Furthermore, in non-aqueous systems, there is an optimum water content required for providing conformational flexibility. The immobilized lipase used in our study may be contains too many water, and lead to the low conversion in transesterification. As no report of lipase cloning from *Bacillus amyloliquefaciens*, we just provide the useful exploration in Lip_{BA} transesterification.

In this study, we obtained the novel lipase gene (lip_{BA}) from the strain *Bacillus amyloliquefaciens* Nsic-8 isolated from a stinky tofu brine and the gene was functional expressed in *E. coli*. The lipase gene (lip_{BA}) is the first reported lipase gene cloned from *Bacillus amyloliquefaciens.* Meanwhile, the detailed report of Lip_{BA} enzymatic properties and the preliminary study of recombinant enzyme immobilization supplied the novel data for *B. amyloliquefaciens* lipase research. Also the alkaline thermostable lipase reveals the potential value in industrial applications. Future investigations will focus on the application of this enzyme in the enantioseparation and studies on structure–function relationships. In addition, enzyme directed evolution including DNA-shuffling and site directed mutagenesis will be studied.

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