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ORIGINAL ARTICLE

In vivo functional expression of an extracellular Ca^{2+}-independent *Bacillus pumilus* lipase in *Bacillus subtilis* WB800N

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Abstract A strain showing distinct lipase activity was isolated from food factory sewage and identified as *Bacillus pumilus* (named *Bacillus pumilus* Nws-bp1) by 16S rRNA sequence analysis. The wild-strain Nws-bp1 showed maximum lipase activity of 2.91 U/ml. Meanwhile, the lipase gene (named lip_{BP}) was obtained from strain Nws-bp1 with the assistance of homology analysis. The gene has an open reading frame of 648 bp encoding 215-amino-acid lipase (Lip_{BP}) with 34-amino-acid putative signal peptide, and shows highest identity with the lipase from *Bacillus pumilus* MTCC B6033 (CP007436.1). Also, the lip_{BP} gene without signal peptide sequence was expressed in *Bacillus subtilis* WB800N using *amyQ* (encoding an amylase) signal peptide. The lipase total enzyme activity was 44.15 U/ml which was about 15 times higher than that of the parent strain, and in supernatant was 32.29 U/ml (about 73 % of the total activity). The pH and temperature optima were pH 10.0 and 40 °C, respectively. Moreover, the recombinant Lip_{BP} showed apparent stability under alkaline conditions especially at pH 9.0–11.0. Also, Lip_{BP} showed stability under normal temperature and retained 85 % of the residual activity after incubation at 40 °C for 8 h without substrate. The specific activity of purified Lip_{BP} was 2650±117 U/mg (pNPP substrate). The K_m and V_{max} values of purified Lip_{BP} were 1.36 mM and 208.25 μmol/(ml·min), respectively. This is the first report of *Bacillus pumilus* lipase expressed in *Bacillus subtilis* using *amyQ* signal peptide, and the pH stability and organic solvent tolerance recombinant lipase provide its potential value in industrial applications.

Keywords Strain screening · Lipase · Gene cloning · Enzymatic properties · *Bacillus subtilis* expression

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Introduction

Lipase (triacylglycerol acylhydrolases, EC 3.1.1.3) is one of the most commonly used enzymes catalyzing both the hydrolysis and synthesis of a variety of acylglycerols at the interface of lipid and water (Pahoja and Sethar 2002). Owing to the properties like wide substrate specificity, and high enantio- and regioselectivity, lipases have a wide range of potential applications in the food, pharmaceutical, detergent and fine chemical industries (Schmid and Verger 1998). Diverse lipases from bacteria (*Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp., *Propionibacterium* sp., *Acinetobacter* sp., *Burkholderia* sp., *Serratia* sp., *Proteus* sp.), fungi (*Candida* sp., *Aspergillus* sp., *Rhizopus* sp., *Rhizomucor* sp. and *Penicillium* sp.), plants, animals and in the colonic region of humans have been reported in previous research. Although the sequences of lipase substrate-binding sites have high homology, the lipases from different microbes have great differences in substrate specificity and optimal reaction conditions. So, cloning novel enzymes with distinct features, such as thermostable, alkaline, and marked activities and stabilities in organic solvents, is of interest for industrial applications (Pennisi 1997; Niehaus et al. 1999).

Recently, lipases from *Bacillus* species have attracted more attention since they have the potential to be used in the food and chemistry industries (Kim et al. 2002). Lipases from

Bacillus sp. are considered to be lidless and small (actually the smallest lipases known). These lipases seem to be particuilarly suited for the synthesis of chiral drugs (Westers et al. 2005). *Bacillus* lipases have been purified and biochemically characterized from *B. subtilis*, *B. pumilus* (Cho et al. 2000), *B. stearothermophilus* (Kim et al. 2000; Massadeh and Sabra 2013), *B. thermocatenulatus* (Schmidt-Dannert et al. 1996) and *B. thermoleovorans* (Cho et al. 2000). However, the expression efficiency of lipases in wild-type *Bacillus* species is low, and this has limited their wide utilization. During recent years, the production of lipase in recombinant *Escherichia coli* has been well studied (Cho et al. 2000; Rahman et al. 2003; Liu et al. 2012). However, there have been few reports of the lipase expression in *B. subtilis*. Ma et al. (2006) used plasmid pBSR2 expressed lipase (from *B. subtilis* strain IFFI10210) in *B. subtilis* A.S.1.1655 in 2006 for the first time.

Previously, using enrichment procedures, the strains which can utilize olive oil were isolated from food factory sewage-contaminated soil samples. Among the isolated strains, the strain Nws-bp1 with the distinct lipase activity was identified as *B. pumilus* by morphological, physiological, biochemical tests and 16S rRNA sequence analysis. In this study, we obtained the lipase gene (named lip_{BP}) from the strain Nws-bp1. Then, the gene was successfully expressed in *B. subtilis* and the enzyme characterization was also described through protein purification. To the best of our knowledge, this is the first report of *B. pumilus* lipase expressed in *B. subtilis* and secreted to culture medium by *amyQ* signal peptide. Meanwhile, the alkaline-adapted Lip_{BP} showed potential value in industrial applications according to its pH stability and organic solvent tolerance.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Bacillus pumilus* strain Nws-bp1 was isolated from the food factory sewage-contaminated soil samples in Shanghai China. The replicative vector pHT43 was used to express recombinant proteins in *B. subtilis* WB800N. *Escherichia coli* DH5a was used to amplify all the vectors in this study.

Chemicals and culture conditions

LA Taq polymerase, T4 DNA ligase, DNA marker and restriction enzymes were purchased from TaKaRa Biotechnology (Otsu, Japan). Protein marker was purchased from MBI Fermentas (Vilnius, Lithuania). Isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin and chloromycetin were purchased from Amresco (Shanghai Genebase, China). DNA Mini kit and Plasmid Mini Prepare kit were purchased from Axygen Biosciences (Union City, CA, USA). The substrate 4-nitrophenyl palmitate (pNPP) was purchased from Sigma. Other chemicals were obtained commercially and were of reagent grade.

Luria Bertani medium was used for *E. coli* and *Bacillus* spp. propagation with appropriate resistance. Rhodamine B agar plates (yeast extract 0.5 %, tryptone 1 %, NaCl 1 %, olive oil 1 %, rhodamine B 0.001 % and agar 1.5 %) were used for strain screening. Lipase basal medium (olive oil 1 %, tryptone 0.5 %, yeast extract 0.25 %, KH_2PO_4 0.07 %, K_2HPO_4 0.03 %) was used for wild-strain lipase activity analysis. Kanamycin was used at 50 μg/ml, chloromycetin was used at 10 μg/ml, and ampicillin was used at 100 μg/ml.

Strain screening, identification and wild-strain activity analysis

In this work, an enrichment culture technique was applied. LB medium with added 1 % olive oil emulsion was used to isolate potential bacterial strains. A 1-ml sample of food factory sewage was added to a flask with 100 ml sterile distilled water. After activation on a rotary shaker for 40 min, 0.1 ml activation culture broth was withdrawn and inoculated into 100 ml enriched medium in a flask, which then was shaken at 120 rpm and 30 °C for 48 h. After several rounds of enrichment, inocula were serially diluted and plated onto Rhodamine B agar plates. The microbes showing obviously hydrolysis circles were isolated, purified and transferred to maintenance slants. The strains were selected and identified by 16S rRNA sequence analysis using primers 16S-U CCTACGGG AGGCAGCAG and 16S-D ACGGGCGGTGTGTAC.

The lipase activity of wild-strain Nws-bp1 was measured in lipase basal medium. The cell-free broth supernatant collected by centrifugation at 8000 *g* and 4 °C for 10 min was used as the crude enzyme sample.

Gene cloning of lipase and DNA manipulation/propagation

Based on the information of *Bacillus pumilus* lipase in GeneBank, degenerate primers (BP-L-U: ATGAAAGTGA TKYKWTTTAAGA, BP-L-D: TTARTTYGTATTYTGTCC TCCG) for the lipase gene sequence were designed to clone the complete sequence of *B. pumilus* Nws-bp1 lipase gene (Fig. 1b). According to NCBI search, the following gene sequences of lipase were analyzed to design primers: *B. pumilus* L21 (GI:408690816), *B. pumilus* ArcL5 (GI:586962947), *B. pumilus* sp. (GI:40362981), *B. pumilus* B106 (GI:169639780) and *Bacillus* sp. B26 (GI:17224341). The genomic DNA isolated from *B. pumilus* Nws-bp1 was used as the template. The PCR amplification was enforced with polymerase LA Taq in the GC buffer. The scheme of

Table 1 The bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Source or reference
Strains		
E. coli DH5α	$F^-\psi80lacZ\Delta M15\Delta$ (lacZYA-argF) U169 endA1 recA1 hsdR17 (r_k^-,m_k^-) supE44λ^-thi-1 gyrA96 relA1 phoA	BRL
B. pumilus Nws-bp1	wild strain screened from food factory sewage (CCTCC AB 2013050)	CCTCC AB 2013050
B. subtilis WB800N	apr his npr eglS (DELTA) 102 bglT/bglS (DELTA) EVCm^r	Mo Bi Tec (Phan et al. 2006)
WB800N-pHT43-*lip*$_{BP}$	*B. subtilis* WB800N, carrying plasmid pHT43-*lip*$_{BP}$, Cm^r	This study
Plasmids		
pMD19-T	Cloning vector; Amp^r	Takara
pMD-*lip*$_{BP}$	pMD19-T vector, carrying mature lipase gene, Amp^r	This study
pHT43	*Bacillus*-*E. coli* shuttle vector; vector for extracellular expression; Amp^r and Cm^r	Mo Bi Tec (Phan et al. 2006)
pHT43-*lip*$_{BP}$	pHT43 based vector, carrying mature lipase gene; Amp^r and Cm^r	This study

amplification included 30 cycles with the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 56 °C) and extension (50 s at 72 °C), and final elongation step (10 min at 72 °C). The target fragments with the approximate length with our hope were cloned into the pMD19-T vector (Fig. s-1 in supplementary materials). The constructed plasmids pMD-*lip*$_{BP}$ was sequenced in two directions by Invitrogen, Shanghai, China.

The nucleotide sequence of gene was aligned with the sequences of lipase genes of various *Bacillus* species available in GenBank using the program CLUSTAL W (Wei et al. 2014). The nucleotide sequence and predicted amino acid sequence were analyzed by the programs of Blast (NCBI). 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/). 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 *B. subtilis*

The *B. subtilis* expression vector pHT43 was used to express foreign proteins in this study. The lipase gene without signal peptide sequence was amplified by PCR using primers pHT43-lip-U/pHT43-lip-D (pHT43-lip-U: CGGGATCCCACCATCACCATCACCATGCTGAGCATAATCCGGT, pHT43-lip-D: CGTGGACGTCTTAATTCGTATTCTGTCCTCCGC). After digestion by *Bam*HI/*Aat*II, the lipase gene was reclaimed and connected with the pHT43 vector, which was digested by the same restriction endonuclease (Fig. 2).

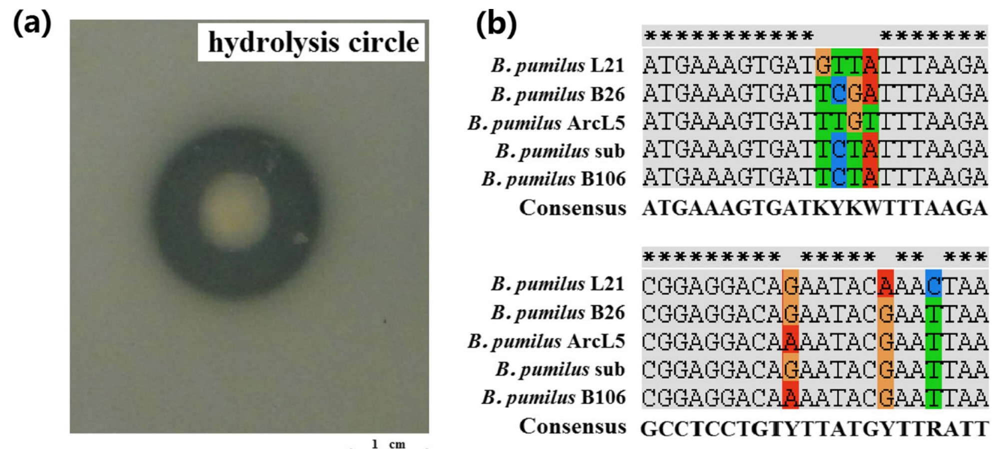

Fig. 1 Lipase in wild-strain and degenerate primers design. **a** Hydrolysis circle on lipase screening culture. **b** Comparison of five lipase genes from different resource. Sequences listed include lipase genes from *Bacillus pumilus* L21 (GI:408690816), *B. pumilus* ArcL5 (GI:586962947), *B. pumilus* sp. (GI:40362981), *B. pumilus* B106 (GI:169639780) and *Bacillus* sp. B26 (GI:17224341)

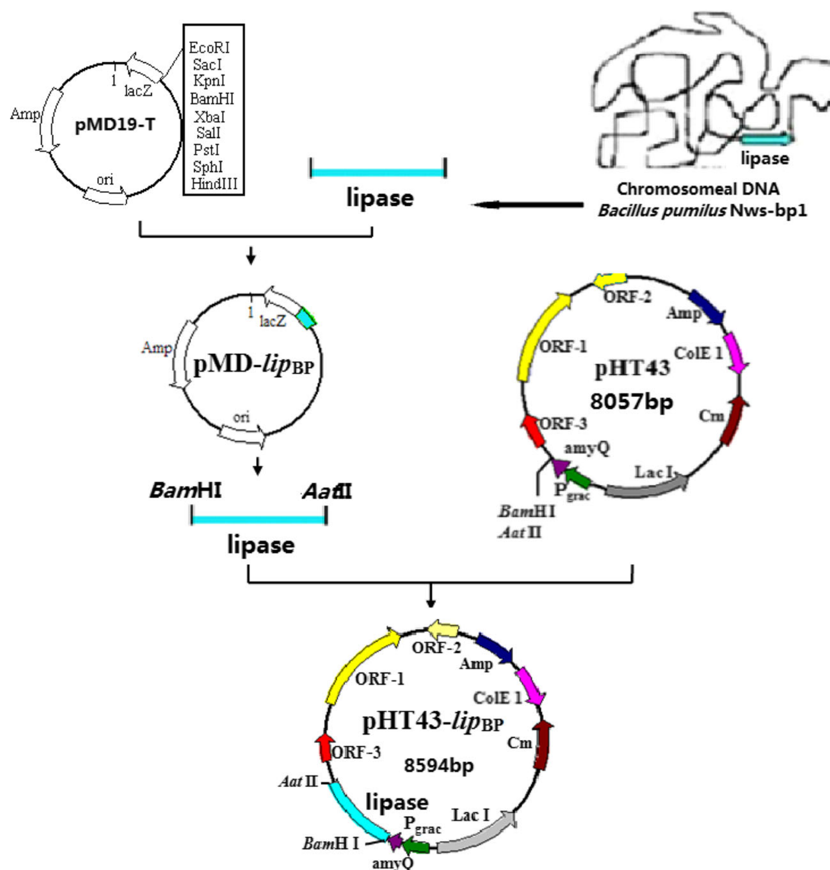

Fig. 2 Cloning strategy for the construction of lipase expression vectors pHT43-*lip*$_{BP}$

The recombinant plasmid named pHT43-*lip*$_{BP}$ was confirmed by restriction enzyme digestion and finally confirmed by sequencing (Fig. s-1 in supplementary materials). Transformation of *B. subtilis* WB800N was carried out using an electroporation method. The positive strains (WB800N-pHT43-*lip*$_{BP}$) were selected based on 10 μg/ml chloromycetin and shaken at 37 °C overnight in Luria-Bertani (LB) broth.

Protein purification of lipase and SDS-PAGE analysis

The transformants were grown in LB medium (with 10 μg/ml chloromycetin) at 37 °C at 200 rpm. When the optical density (OD) at 600 nm reached about 1.0, IPTG was added to a final concentration of 1.0 mM. After incubation at 30 °C for 20 h, the samples were harvested by centrifugation (8000 *g*, 5 min at 4 °C). Then, samples were separated by being 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.

In a previous article, Wei et al. (2014) described the methods for heterologous protein expression and Ni-NTA purification procedures in our group. After 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) induction, the cells were harvested by centrifugation and resuspended in buffer A [20 mM phosphate buffer (pH 8.0), 500 mM NaCl, and 50 mM imidazole]. After being lysed by sonication, the liquid was applied to a Ni^{2+}-chelating Sepharose Fast Flow column (Amersham Biosciences). After washing to baseline, the protein was eluted with buffer B [20 mM phosphate buffer (pH 8.0), 500 mM NaCl, and 200 mM imidazole]. Finally, a desalting column (Amersham Biosciences) was used to remove the salts. The purity of the Lip$_{BP}$ preparation exceeded 80 %, and was judged by SDS-PAGE.

Enzymatic properties analysis

Enzyme activities of Lip$_{BP}$ were measured in triplicate spectrophotometrically using para-nitrophenyl palmitate (pNPP) as substrate. The pNPP was dissolved in 2-propanol to produce a concentration of 3 mg/ml. The substrate solution consisted of 0.1 ml of pNPP solution and 2.8 ml Tris–HCl buffer (20 mM, pH 8.0). The reaction was carried out at 30 °C by adding 0.1 ml of appropriately diluted enzyme solution to the substrate solution preincubated at 30 °C for 5 min, and

incubation was continued for a further 5 min. The test tubes were immersed in ice until the optical density was taken at 405 nm. Also, spontaneous substrate hydrolysis without enzyme was measured and taken as blank. One unit (U) was defined as the amount of enzyme that liberated 1 μmol pNP per min under the standard assay conditions. This method was used to assay the characterization of the lipase. The average values of triplicate measurements were used as each activity value. All the recombinant lipase activities (supernatant of fermentation broth expressed from *B. subtilis*) were converted to the whole fermentation volume (volumetric activities U/ml or mg/ml medium).

The temperature optimum for the enzyme activity was studied over a range from 10 to 70 °C in 50 mM Tris–HCl buffer (pH 8.0) with pNPP as the substrate. The thermal stability of Lip_{BP} was evaluated by assaying its residual activity after incubation of the enzyme at various temperatures for different times (2, 4 and 8 h) in Tris–HCl buffer (pH 8.0). The pH optimum for the enzyme activity was studied over a range from pH 4 to 11 (40 °C). The pH stability of the enzyme was determined by incubating the enzyme in various buffers for different times (2, 4 and 8 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, pH 9.0–10.0 with 50 mM glycine-NaOH, and pH 11.0 with sodium hydrogen phosphate-NaOH. The temperature stability for the enzyme activity was assayed at 20–70 °C (pH 8.0). The effects of metal ions and other reagents on enzyme activity were determined in a standard assay medium containing each reagent. The metal ions were at two different concentrations (1 and 10 mM). Meanwhile, the 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, n-propanol and isopropanol 10 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 Tris–HCl buffer (pH 8.0). The enzyme sample without any additives was considered as control (100 %). Each experiment was repeated three times and each experiment included three replicates. The average values of triplicate measurements were used as each activity value. All values are means± SD from three independent experiments (repeats with SD of ≤5 %).

The kinetic parameters K_m/V_{max} were determined for the substrate pNPP at 40 °C in 50 mM Tris–HCl buffer (pH 8.0) for 5 min with the substrate over a range of 0.2–2.0 mM, and then calculated from Lineweaver-Burk reciprocal plots.

Results

Cloning of the lipase and sequence analysis

After 48 h growing on the lipase screening culture medium plate at 30 °C, significant hydrolysis circles appeared around the bacterial colony (Fig. 1a). The strain Nws-bp1 showing distinct lipase activity was identified as *B. pumilus* by 16S rDNA phylogenetic analysis and the morphology. The GeneBank accession number for the *B. pumilus* Nws-bp1 16S rDNA gene is KC568200, and KF229753 for lipBP gene. The wild-strain Nws-bp1 showed maximum lipase activity of 2.91 U/ml by preliminary culture medium optimization.

Sequence analysis revealed that the sequence of the lip_{BP} ORF (648 bp) uses ATG as the start codon and the G+C content (%) of the lip_{BP} is 44.9 moles %. The lip_{BP} gene consists of 648 nucleotides and encodes a deduced protein of 215 amino acids. Analysis of signal peptide showed that a possible signal peptide of 34 amino acids in the N-terminal region was found and the peptide bond between 34 th and 35 th amino acids (AKA-AE) would be cleaved by signal peptidase (SignalP Server). The molecular weight of Lip_{BP} was estimated to be 22.9 kDa (19.22 kDa without the signal peptide), and the pI value was calculated to be 9.88 (9.46 without the signal peptide) by the ExPASy compute pI/Mw program algorithm (http://www.expasy.org/cgi-bin/protparam). Homology analysis revealed that lip_{BP} in *B. pumilus* Nws-bp1 is 97 % identical to the gene of CP007436.1 in *B. pumilus* strain MTCC B6033, 86 % identical to the AJ297356 in *B. licheniformis*, and 74 % identical to the CP002927 in *B. amyloliquefaciens* XH7. Using the neighbor-joining method (CLUSTAL W), the lip_{BP} gene in *B. pumilus* Nws-bp1 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 are shown in Fig. 3b. From the phylogenetic tree, the Lip_{BP} protein in *B. pumilus* Nws-bp1 shared the most identity with predicted triacylglycerol lipase from *B. pumilus* strain MTCC B6033. The result that lip_{BP} belongs to the alpha/beta hydrolase family was further confirmed by the analysis of sequences and the phylogenetic tree (Fig. 3). The mature protein contains a single catalytic domain of the alpha/beta hydrolase family and belongs to the family of triacylglycerol lipase (EC 3.1.1.3). The catalytic triad of Ser^{111}, Asp^{167}, and His^{190} residues was 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. 3c). Meanwhile, the three-dimensional structure of Lip_{BP} was predicted by the SWISS-MODEL server and the protein structure was viewed by PdbViewer (Fig. s-2 in supplementary materials).

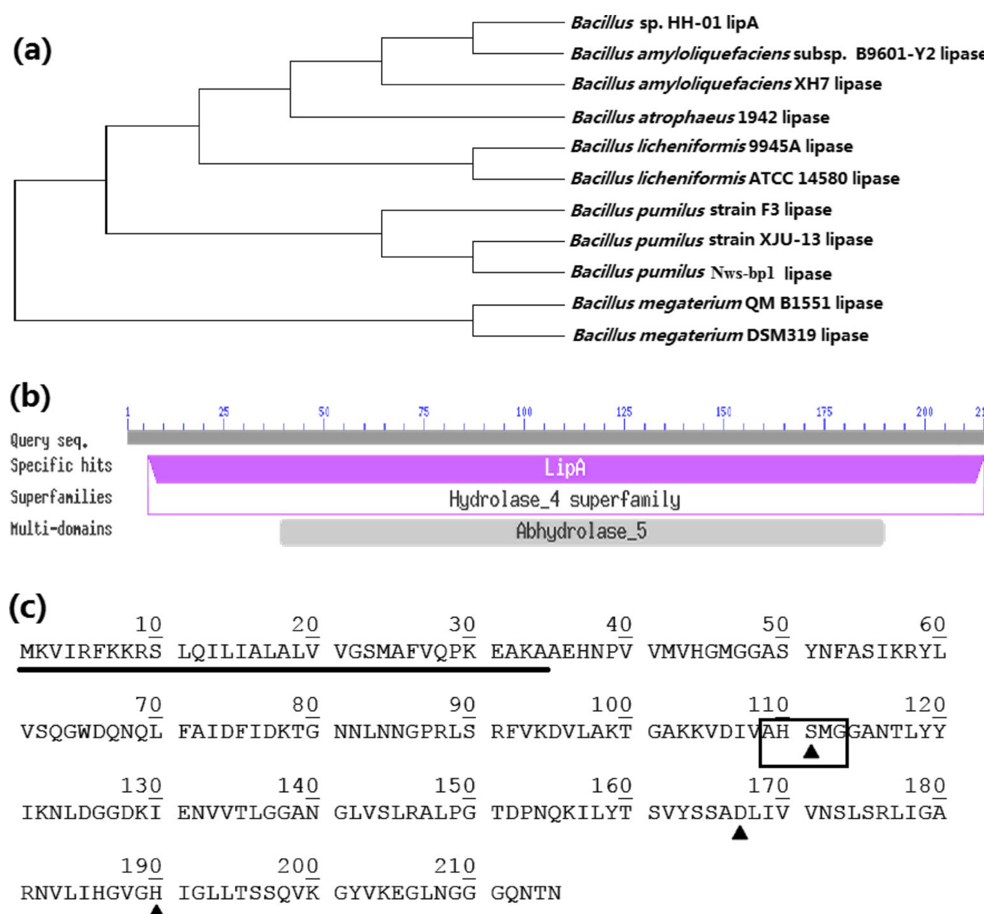

Fig. 3 The amino acid residues analysis. **a** Rooted phylogenetic tree of Lip_{BP}. The consensus amino acid residues of Lip_{BP} and some homologous lipase were aligned with FASTA and GENEDOC. The sequences used in this alignment were obtained from GenBank as follows. 1: Lipase from *Bacillus* sp. HH-01 (GI:300116986), 2: Lipase from *B. amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2 (GI:380496984), 3: Lipase from *B. amyloliquefaciens* XH7 (GI:341825903), 4: Lipase from *B. atrophaeus* 1942 (GI:310867486), 5: Lipase from *B. licheniformis* 9945A (GI:170878073), 6: Lipase from *B. licheniformis* ATCC14580 (GI:51507668), 7: Lipase from *B. pumilus* F3 (GI:118340982), 8: Lipase from *B. pumilus* XJU-13 (GI:130750924), 9: Lipase from *B. pumilus* Nws-bp1 used in this study (GI:544191646), 10: Lipase from *B. megaterium* QM B1551 (GI:294346812), 11: Lipase from *B. megaterium* DSM319 (GI:294799901). **b**, **c** Conserved sequence alignment of Lip_{BP}. The structures are denoted as follows: *black triangle*, the catalytic site (Ser^{111}, Asp^{167}, His^{190}). The reserved amino acid motif AHSXG is *boxed*. The consensus amino acid residues for the signal peptide are *underlined*

Expression of the recombinant enzyme in *B. subtilis*

In this study, the *E. coli-B. subtilis* shuttle vector pHT43 were used to express lip_{BP} in *B. sublitis.* The strong synthetic promoter P_{grac} was fused the lac operator immediately downstream of the groESL promoter to make vector pHT43 controllable by IPTG (Phan et al. 2006). The gene lip_{BP} were fused downstream of P_{grac} of the vector pHT43, and the recombinant plasmid (named pHT43-lip_{BP}) was transformed into competent cells of *B. subtilis* WB800N. The recombinant strains which contain the positive plasmid were grown to saturation in LB medium supplemented with appropriate resistance.

When the optical density at 600 nm reached 0.8, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added to induce the *lac* promoter. The SDS-PAGE profile revealed the presence of an additional protein band with an estimated molecular mass of 20 kDa, which was close to the molecular mass of Lip_{BP}. After induction for 20 h with IPTG, enzyme activity in the supernatant of culture medium was detected while no enzyme activity was detected in uninduced transformant nor from transformant harboring the empty pHT43 plasmid (negative control). At this time, the lipase total enzyme activity was 44.15 U/ml which is about 15 times higher than that of parent strain (2.91 U/ml), and in supernatant was 32.29 U/ml (about 73 % of the total activity).

Purification and quantitative assay of recombinant Lip_{BP} by SDS-PAGE analysis

The recombinant lipase (Lip_{BP} from strain WB800N-pHT43-lip_{BP}) was overexpressed in *B. subtilis* WB800N cells under the optimal conditions and was purified by Ni-NTA

Table 2 Purification of Lip_{BP} by Ni-NTA affinity chromatography

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
Crude extract	1614.5	7.6	213.3	100.0	1.0
Ni-NTA	858.9	0.3	2650	53.2	12.4

The crude enzyme was obtained from 50 ml culture and 10 ml purified enzyme was obtained

purification procedures (Table 2). About 0.3 mg Lip_{BP} which was purified up to 12.4 times with a recovery of 53.2 % was obtained using Ni-NTA affinity chromatography. The crude extract enzyme specific activity was 213.3 U/mg, and specific activity was up to 2650 U/mg after purification. The purified enzyme migrated on SDS/PAGE as a single band with an apparent molecular mass of about 20.0 kDa (Fig. 4). The results indicated that Ni-NTA affinity chromatography was an appropriate method for the protein purification.

Biochemical characterization of the purified recombinant Lip_{BP}

To examine the properties of the lipase produced by *B. subtilis* WB800N/pHT43-lip_{BP}, the recombinant lipase was purified through Ni-NTA purification. After purification, the specific activity of Lip_{BP} was 2650±117 U/mg. The purified Lip_{BP} exhibited higher enzyme activities over a pH range of 7.0–11.0, among which the highest specific enzyme activity was at pH 10.0 (Fig. 5b). The enzyme retained more than 75 % of the maximal activity in the pH range of 9.0–11.0 and was highly stable at pH 9.0–11.0 (more than 70 % residul activity under pH 9.0–11.0 for 8 h) (Fig. 5d).

The effect of temperature on the activity and stability of recombinant Lip_{BP} is shown in Fig. 5. The optimal temperature of purified Lip_{BP} was 40 °C (pH 8.0). The recombinant Lip_{BP} retained 52 % of the maximal activity in 10 °C and 43 % in 70 °C (Fig. 5a). It retained more than 85 % of the residul activity after incubation at 40 °C for 8 h without substrate (Fig. 5c).

The effects of different metal ions and chemical reagents on the enzyme are shown in Fig. 6. At low concentration (1 mM), metal ions partly inhibited or had no effect on the enzyme activity. Meanwhile, at high concentration (10 mM), most metal ions showed significantly inhibited effects, especially for Fe^{2+} and Zn^{2+} (Fig. 6b). The Lip_{BP} activities were not significantly affected by DTT, SDS, TritonX-100 or EDTA and strongly inhibited by Tween-80 and Tween-20 (Fig. 6a). The recombinant proteins show higher organic solvent tolerance and retained more than 75 % of the maximal activity in 10 % (v/v) methanol, alcohol, acetonitrile, DMSO, hexane, and chloroform. However, 25 % methanol, alcohol and acetonitrile showed significantly inhibited effects. Of most interest is that the lipase Lip_{BP} was activated by 10 % (v/v) n-hexane (up to 115 % relative activity) (Fig. 6c).

The kinetics (V_{max} and K_m) of the purified lipase was determined by Lineweaver-Burk double reciprocal plot at varying substrate (pNPP) concentration. The K_m and V_{max} values of purified Lip_{BP} were 1.36 mM and 208.25 μmol/(ml·min), respectively.

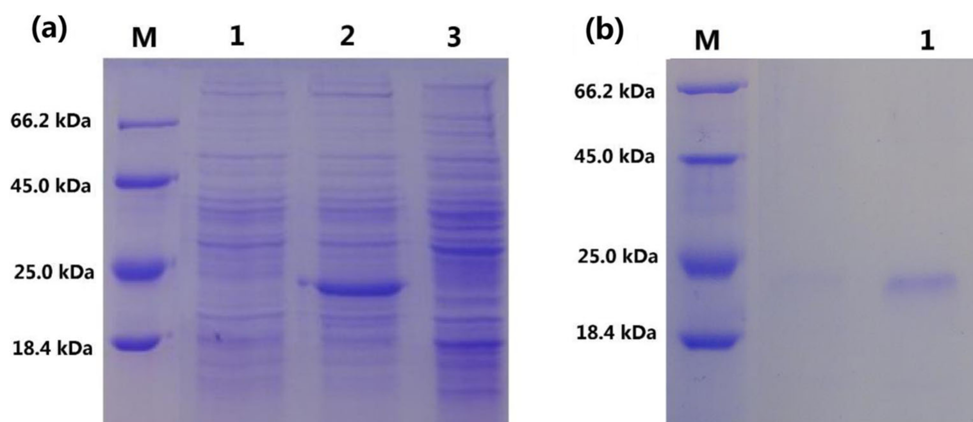

Fig. 4 SDS-PAGE of Lip_{BP} in *B. subtilis* WB800N and recombinant protein purification. **a** SDS-PAGE of Lip_{BP}. *Lane M* standard marker proteins; *lane 1* supernatant of fermentation broth (pHT43); *lane 2* supernatant of fermentation broth (WB800N-pHT43-lip_{BP}) after induction for 20 h; *lane 3* cell lysate supernatant of WB800N-pHT43-lip_{BP} after induction for 20 h. **b** Recombinant protein purification. *Lane M* standard marker proteins; *lane 1* purification of Lip_{BP} by Ni-NTA affinity chromatography

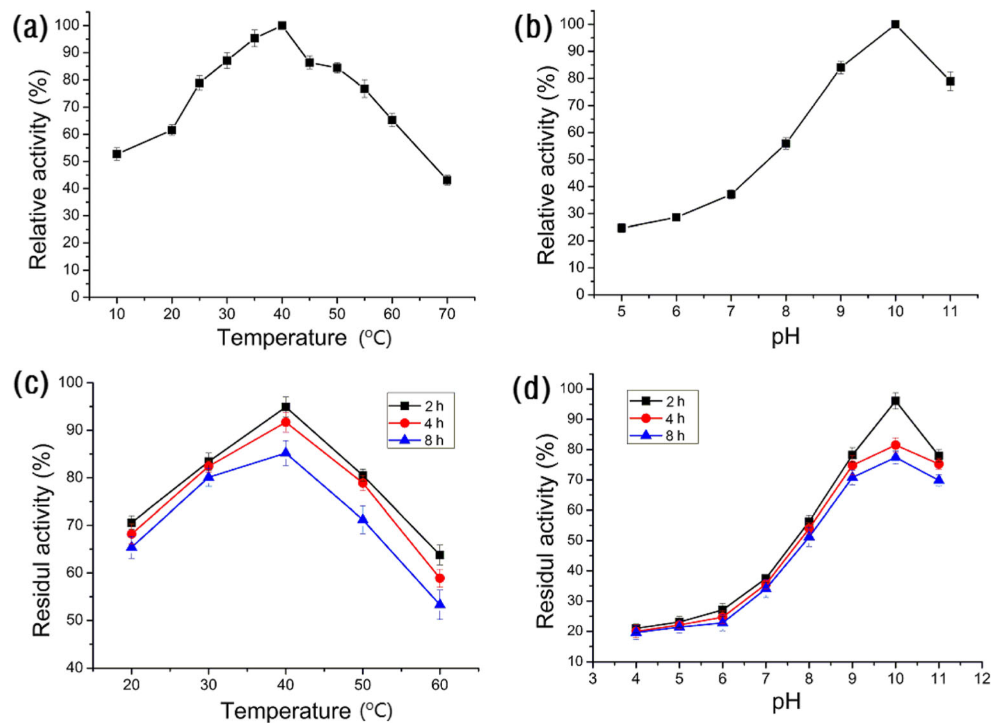

Fig. 5 Characterization of recombinant Lip_{BP}. **a** The temperature optimum of Lip_{BP} lipase activity (pH 8.0). **b** The pH optima of Lip_{BP} lipase activity (40 °C). **c** The thermal stability of Lip_{BP} lipase activity. The thermal stability of enzyme was evaluated by incubating the enzyme in various temperature for different times (2, 4 and 8 h) and incubated in Tris–HCl buffer (pH 8.0). **d** The pH stability of Lip_{BP} lipase activity. The pH stability of the enzyme was determined by incubating the enzyme in various buffers for different times (2, 4 and 8 h) and incubated at 40 °C. Each value represents the mean± SD of triplicates

Discussion

Lipases are an important group of biotechnologically relevant enzymes and one of the most commonly used enzymes in bioconversion processes (Rahman et al. 2003). Many lipase genes have been obtained from cultured microorganisms or metagenomes of environmental samples (Rhee et al. 2005; Kamijo et al. 2011). Cloning the novel lipase gene with distinct features, especially from easily grown bacteria, is of interest for industrial applications. Among these, various microbial lipases produced by *Bacillus* sp. show conspicuous commercially significance due to their numerous unique characteristics: region specificity, chiral selectivity, and substrate specificity. *B. pumilus* has simple nutritional requirements and exhibits considerably enzyme yield. Also, some research on *B. pumilus* lipase have been reported in previous articles especially for the wild strain. Jose and Kurup (1999) reported the purification and characterization of an extracellular lipase from a newly isolated thermophilic *B. pumilus*. In 2002, the Ca^{2+}-independent lipase was cloned from *B. pumilus* B26 isolated in soil samples collected near Taejon, Korea (Kim et al. 2002). In 2009, the lipase was cloned from *B. pumilus* B106 associated with the South China Sea sponge *Halichondria rugosa*, and the optimal medium was optimized for higher lipase activity (Zhang et al. 2009). Sunsequently, identification of variables and value optimization for optimum lipase production in *B. pumilus* RK31 was applied using statistical methodology in 2011 (Kumar et al. 2011). Lipase from *B. pumilus* RK31 was purified and some properties were analyzed in the wild-strain (Rakesh et al. 2012). Ismael et al.

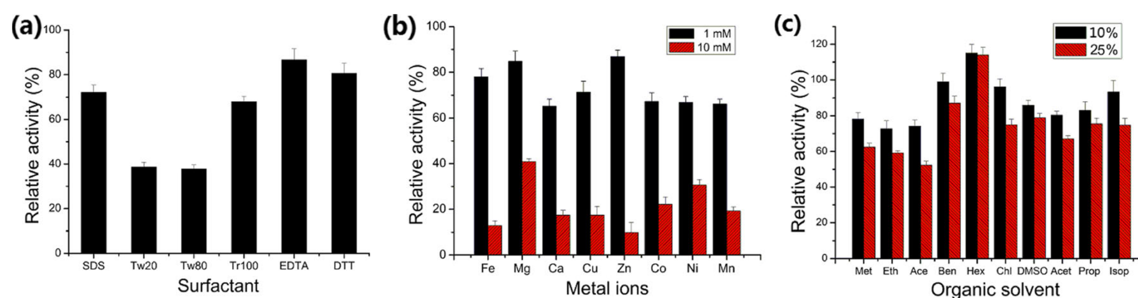

Fig. 6 Effect of metal ions and chemical compounds on the lipase activity. **a** The concentration of SDS, Tween 20, Tween 80, Tritonx-100, EDTA and DTT was at 1.0 mM. **b** The metal ions were at two different concentrations (1 and 10 mM). **c** The organic solvents were at two different concentrations (10 and 25 %) (*Met* methanol, *Eth* ethanol, *Ace* acetonitrile, *Ben* benzene, *Hex* n-hexane, *Chl* chloroform, *Acet* acetone, *Pro* n-propanol, *Isop* isopropanol). The reaction mixtures containing the enzyme sample were incubated at 40 °C for 60 min in Tris–HCl buffer (pH 8.0). The enzyme sample without any additives was considered as control (100 %)

(2013) reported the *B. pumilus* lipase for the hydrolysis of (R, S)-1-phenylethyl acetate production in ionic liquids. Although there has been some research involving the lipase from *B. pumilus*, there has been no report of *B. pumilus* lipase expressed in *B. subtilis* or secreted to culture medium by *amyQ* signal peptide. In this study, the strain *B. pumilus* Nws-bp1 was isolated from food factory sewage and the lipase gene was cloned and expressed in *B. subtilis* using *amyQ* signal peptide.

Many of the lipases act on their substrates at the lipid–water interface (called interfacial activation). This activation is 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. The lipases from *B. subtilis* and *B. pumilus* are actually the smallest lipases known (Westers et al. 2005). From the three-dimensional structure of Lip_{BP}, there was no lid above the catalytic triad residues (Ser^{111}, Asp^{167}, and His^{190}) (Fig. s-2 in supplementary materials). These small, lidless lipases seem to be well suited for biotechnological applications. Jaeger et al. (1999) classified bacterial lipases into six families (families I–VI) and further classified family I into six subfamilies (subfamilies I.1–I.6). Within family I, *Bacillus* lipases belonged to either subfamily I.4 or subfamily I.5 based on sequence homology and protein molecular size. Protein sequence alignment showed that Lip_{BP} belongs to lipase family I.4. Homology analysis revealed that Lip_{BP} in Nws-bp1 shared highest identity with lipase from *B. pumilus* (95 % identical to the gene of EF434173 in *B. pumilus* strain XJU-13). The recombinant Lip_{BP} had an optimum pH of 10, and retained more than 75 % of the maximal activity in the pH 11, which was similar to the lipase from *B. alcalophilus* (pH 10.0–10.5) (Ghanem et al. 2000), *B. licheniformis* (pH 9–11) (Gupta et al. 2004), *B. pumilus* (pH 9–11) (Gupta et al. 2004) 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 the lipase from *B. pumilus* B106 (50 °C) (Zhang et al. 2009) and *B. subtilis* (35 °C) (Lesuisse et al. 1993). As for thermal stability, *B. pumilus* lipase B26 retained 100 % activity after 15 min at 70 °C (Kim et al. 2002), amd *B. subtilis* lipase 168 retained 100 % activity after 30 min at 40 °C (Lesuisse et al. 1993). In this study, lipase Lip_{BP} kept 52 % residue activity at 60 °C for 8 h. These results indicated that the recombinant Lip_{BP} is thermotolerant and 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 their influence on the enzyme activity. All the tested metal ions (1 mM) were found to inhibit the enzyme activity. Many lipases have the Ca^{2+}-binding motif which plays an important role in enzyme activity as well as in thermostability (Ma et al. 2006; Dutta and Ray 2009). However, Lip_{BP} had no such Ca^{2+}-binding motif around the catalytic triad (Ser^{111}, Asp^{167}, and His^{190}), and this suggested that the Ca^{2+} ion could not bind to the protein molecule (Fig. s-2 in supplementary materials). As shown in Fig. 6, this enzyme showed Ca^{2+}-independent catalytic activity. Meanwhile, many lipases were stimulated by Ca^{2+}, such as *B. cereus* C7 lipase (1 mM, 200 %) (Dutta and Ray 2009), *B. subtilis* lipase (10 mM, 116 %) (Ma et al. 2006), and *Pseudomonas xuorescens* lipase. On the other hand, the activity of *B. pumilus* lipase B26 (Ca^{2+}-independent lipase) was almost constant at a wide range of Ca^{2+} concentrations (Kim et al. 2002). The protein structure in which Lip_{BP} does not contain calcium binding sites may provide the reason (Fig. 6b). These Ca^{2+}-independent lipases show potential applications in industrial uses. For example, in laundry uses, the advantages has been reflected in the full hydrolytic activity even in the presence of a Ca^{2+} chelating component, whereas other lipases might lose most of their lipolytic activity in such conditions.

In this research, Lip_{BP} 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. Shokri et al. (2014) found that only SDS and EDTA had significant effects on *Pseudomonas* sp. lipase activity. The CALB (lipase from *Candida antarctica* ZJB09193) activities were enhanced in the presence of Tween-20 and sorbitol, and inhibited by SDS and Tween-80 (Liu et al. 2012). Also, we chose many organic solvents to measure the value of the enzyme as a biocatalyst in organic solvents. Most of the organic solvents showed inhibition or no effect to this enzyme. However, the lipase Lip_{BP} was activated by 10 % (v/v) n-hexane (115 %). According to the reported enzymes, n-hexane slightly enhanced the *Bacillus sphaericus* 205y lipase activity (Rahman et al. 2003) and *B. megaterium* lipase activity (Lima et al. 2004).

As the gene lip_{BP} was derived from *Bacillus* sp., we used the *B. subtilis* expression vector to express foreign proteins and achieved recombinant protein secretion expression in *B. subtilis* WB800N with the *amyQ* signal sequence. The recombinant protein expression level was very low (0.15 mg/ml) in this study. On the other hand, *B. subtilis* is classified as being generally recognized as a safe (GRAS) organism due to its lack of pathogenicity and absence of endotoxins. Further advantages of *B. subtilis* are its direct secretion of proteins into the fermentation supernatant by the protein secretion machineries, its capacity for genetic manipulation, easy handling and short processing times (Schallmey et al. 2004). The advantage of secretion of the target protein can be seen in a natural separation of the product from cell components, simplifying downstream processing, as well as in the provision of better refolding conditions compared to the reducing conditions in the cell cytoplasm. These abilities have been largely successful in producing correctly folded and soluble heterologous proteins (Tosato and Bruschi 2004), and make *B. subtilis* have

potential applications as a gene expression host (Schallmey et al. 2004). As we all know, the amount of recombinant proteins is dependent on four major factors: efficiency of transcription, mRNA stability, efficiency of translation, and stability of the protein. This may be due to incorrect folding of the target protein, or extracellular protease secreted by *B. subtilis* host cell which recognize and degrade heterologous proteins.

In this study, we obtained the Ca^{2+}-independent lipase gene (lip_{BP}) from the strain *B. pumilus* Nws-bp1 isolated from food factory sewage, and the gene was functionally expressed in *B. subtilis.* The enzyme properties, such as the small molecular weight (181-aa, 19.22 kDa without the signal peptide), alkaline-adapted/Ca^{2+}-independent lipase, and secretory expression in *B. subtilis*, show the potential value in further research. Also, the detailed report of Lip_{BP} enzymatic properties and the recombinant proteins expression in *B. subtilis* will reveal the potential value in industrial applications. Future investigations will focus on the application of this enzyme in enantioseparation and studies on structure–function relationships. In addition, strategies for optimizing heterologous Lip_{BP} expression in *B. subtilis* will be studied.

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