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Molecular cloning of a thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806: Expression and biochemical characterization

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ABSTRACT

A thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806 was functionally expressed as an N-terminal 6xHis-tagged recombinant enzyme in *Escherichia coli* BL21 using pET-28a(+) expression vector. Sequence analysis revealed an open reading frame of 639 bp encoding a 212-amino acid protein containing the well-conserved Ala-His-Ser-Met-Gly motif. One-step purification of the His-tagged recombinant lipase was achieved using Ni-NTA affinity chromatography with a specific activity of 1364 U/mg. The purified enzyme with an apparent molecular mass of 26.8 kDa demonstrated the maximum activity at 70 °C and pH 8.0 for hydrolysis of p-nitrophenylbutyrate as substrate. The enzyme activity was strongly inhibited by divalent ions of heavy metals such as Hg^{2+} and Cu^{2+}, while retained over 90% of the original activity in the presence of several reagents including DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), SDS (sodium dodecyl sulfate), urea, DMF (dimethylformamide), DTT (dithiothreitol), glycerol and Triton X-100. While being considerably stable in organic solvents, imidazolium-based ionic liquids (ILs) had stimulatory effects on the activity of purified lipase. Remarkable stabilization of enzyme at alkaline pH and in ionic liquids as well as its thermostability/thermoactivity are among the most fundamental characteristics which offer great potential for various biotechnological applications including detergent formulation, bioremediation processes and biotransformation in non-aqueous media.

1. Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a class of enzymes capable of hydrolyzing ester bonds in triglycerides at oil–water interface. Hydrolyzing the triglycerides, lipases also catalyze their synthesis from fatty acids and glycerol. Lipases, which are among the most widely used hydrolytic enzymes in industry, have become the object of particular attention for various biotechnological applications [1,2]. Thermostable lipases have garnered wide spread interest for potential application in the detergent, pharmaceutical, dairy, oil and fat industries due to their extreme stability at high temperatures and in organic solvents. In addition, operating bioprocesses at higher temperatures may result in an increase in diffusion rate and enhanced solubility of hydrophobic substrates as well as reduction in microbial contamination [3,4]. While harvesting a limited amount of enzymes from thermostable lipase producing bacteria, high expression level of protein has been achieved through cloning thermophilic genes into more appropriate mesophilic hosts [5]. Recently, ionic liquids (ILs) have gained much attention as an alternative to conventional organic solvents particularly in biocatalytic reactions. Owing to their unique properties including nonvolatility, thermal stability and ionic conductivity as well as nonflammability, ILs are considered as environmentally friendly green solvents [6]. The stimulatory effects of ILs on reactions involving lipases have been reported in previous studies [7,8]. A thermophilic lipolytic bacterium was previously isolated from Dig Rostam hot mineral spring in Iran. The strain was identified as *Bacillus subtilis* based on the 16S rDNA gene sequence (GenBank: JF309277) and has been deposited in Iranian Biological Resource Center under acquisition number of IBRC-M10742 [9]. The aim of the present study was to report the cloning, expression and purification of recombinant lipase from *Bacillus subtilis* DR8806, as well as biochemical properties of the purified enzyme.

2. Materials and methods

2.1. Bacterial strains and plasmids

A lipase producing strain *B. subtilis* DR8806 isolated from hot mineral spring was grown in nutrient broth at 37 °C. *E. coli* DH5α and BL21 (DE3) were used as cloning and expression hosts, respectively. The *E. coli* strains were cultivated in Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl) medium at 37 °C. *B. subtilis* DR8806 was served as the source of genomic DNA. The plasmid pTZ57R/T (Fermentas, Maryland, USA) was used for cloning and sequencing of the lipase gene. The

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target gene was cloned into pET-28a(+) expression vector (Novagen, USA) under the control of strong T7 promoter to allow the insert to be maintained and expressed in *E. coli* host. To screen the transformants, medium was supplemented with ampicillin or kanamycin at a final concentration of 100 μg/mL and 50 μg/mL, respectively.

2.2. Cloning and sequencing of the lipase gene

A pair of oligonucleotide primers was synthesized based on coding sequences available for the *B. subtilis* lipase gene. The sequence of the forward primer LipF was 5′-CGCGGATCCGCGATTATGAAATTTGTAAAAAGAAGG-3′ and that of the reverse primer LipR was 5′-CCCAAGCTTGGGTCATTAATTCGTATTCTGGCC-3′. Genomic DNA from *B. subtilis* DR8806 was prepared by the method developed by Sambrook and Russell [10]. The lipase gene was amplified from genomic DNA using a set of primers (LipF and LipR) with incorporated restriction enzymes *Bam*HI/*Hind*III (Takara, Dalian, China), allowing the directional in-frame ligation of the amplified fragment into pET-28a(+) vector. The purified amplicon was cloned into the T/A cloning vector pTZ57R/T, in accordance with the manufacturer's instructions. Competent cells of *E. coli* were prepared by using a conventional $CaCl_2$ method [10]. Transforming into the *E. coli* DH5α competent cells using heat shock method [10], the cells were cultivated on LB-agar medium containing ampicillin (100 μg/mL). The plasmid DNA was isolated with Fermentas plasmid DNA isolation kit (Fermentas, Maryland, USA) following the manufacturer's instructions. Colony PCR using specific primers, restriction enzyme analysis and sequencing after plasmid extraction were performed to confirm the presence of the target gene. The nucleotide sequence of *B. subtilis* DR8806 lipase gene was determined and submitted to GenBank database. The insert was subcloned in pET-28a(+) vector by digesting the pTZ57R/T cloning vector containing the lipase gene with *Bam*HI/*Hind*III followed by ligation to the previously digested expression vector using T4 DNA ligase (Takara, Dalian, China). The resulting expression construct was confirmed by lipase gene amplification and double restriction digestion. The recombinant pET-28a(+) vector was employed for the expression of the lipase gene in *E. coli* BL21 (DE3) competent cells. The aforementioned construct placed the insert in frame with N-terminal region coding for six His residues facilitating the protein purification process.

2.3. Expression of the lipase gene

A transformant of *E. coli* BL21 harboring the recombinant plasmid (pET-28a(+)-lip) was cultured in LB medium containing 50 μg/mL kanamycin and incubated overnight at 37 °C with 150 rpm shaking. The pre-culture was inoculated (1% v/v) into 200 mL fresh LB medium supplemented with kanamycin at 37 °C until the OD_{600} reached 0.5. To induce the expression of recombinant lipase, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 1 mM and the growing culture was incubated at 25 °C for a further 8 h-period. Following induction, the aliquots were harvested by centrifugation (12,000 g, 20 min, 4 °C) and the pellets were resuspended in 10 mL lysis buffer (NaH_2PO_4 50 mM NaCl, 0.3 M imidazole 10 mM and 1 mM PMSF, pH 8.0). Induced cells were disrupted by sonication for six 30 s burst with a 30 s cooling period between each burst, the cell-free extracts were obtained by centrifugation.

2.4. Purification of the recombinant lipase enzyme

The N-terminally-attached His-tag lipase was purified under native conditions using the immobilized metal ion affinity chromatography (IMAC) column (Qiagen, CA, USA). The crude enzyme preparation was loaded onto the Ni-nitrilotriacetate (Ni-NTA) column previously equilibrated with native binding buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0). The column was washed sequentially with native wash buffer containing 20 mM imidazole. Finally, the bound target proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) at a flow rate of 1 ml min^{-1}. The purification procedure was carried out at 4 °C.

2.5. Lipase assay and protein determination

Lipase activity was measured spectrophotometrically at 405 nm using 0.01 M p-nitrophenylbutyrate (pNPB) (Sigma–Aldrich, USA) as a substrate following incubation at 50 °C for 30 min and pH 8.0 (Tris-HCl buffer). One unit of lipase activity was defined as the amount of enzyme needed to liberate 1 μmol of p-nitrophenol per minute under standard assay conditions [2]. To determine the protein concentration, Bradford method was conducted using bovine serum albumin (Sigma–Aldrich, USA) as the standard [11]. The homogeneity of the purified enzyme and also the performance of affinity purification were analyzed on a 12% (w/v) SDS-PAGE polyacrylamide gel [12]. Protein bands were visualized by Coomassie brilliant blue R-250 staining. A protein standard (Vivantis, CA, USA) in the range of 10.5–175 kDa was used as molecular mass marker.

2.6. Enzyme characterization

2.6.1. Effect of pH on lipase activity and stability

To evaluate optimum pH of the recombinant enzyme, the lipolytic activity was assayed over a pH range from 2.0 to 10.0 using the following buffers (50 mM): glycine–HCl buffer pH 2.0–3.0, sodium acetate buffer pH 3.5–5.5, sodium phosphate buffer pH 6.0–7.5, Tris-HCl buffer pH 8.0–9.5, Na_2HPO_4–NaOH buffer pH 10. The pH stability of the lipase was determined after 60 min of pre-incubation at different pH values (2.0–10.0) at 50 °C. The residual lipolytic activity was determined under standard assay conditions. All enzyme assay experiments were conducted in triplicates.

2.6.2. Effect of temperature on lipase activity and stability

The effect of temperature on the activity of lipase was determined by monitoring the enzyme activity at various temperatures in the range of 30–80 °C for 30 min in Tris-HCl buffer, pH 8.0. The influence of temperature on lipase stability was analyzed by incubating the enzyme solution at above-mentioned temperatures for 60 min followed by the evaluation of the remaining activity according to standard assay method. To determine the enzyme half-life, the cloned lipase was kept at different temperatures ranging from 60 °C to 80 °C and the residual activity was assessed at 30 min intervals over a total period of 150 min.

2.6.3. Effect of metal ions on the purified lipase activity

The effects of various metal ions (Mg^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, K^+, Na^+, Fe^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Ba^{2+}, Cd^{2+} and Hg^{2+}) at 1 and 5 mM concentrations on the enzyme activity was assayed at 70 °C, pH 8.0 using p-nitrophenylbutyrate as substrate, upon pre-incubation of the purified lipase in each compound for 30 min. The enzyme activity of control sample (recombinant lipase without any metal ion) was taken as 100%. Lipase activity was measured as previously described.

2.6.4. Effect of organic solvents and ionic liquids on lipase performance

The effect of organic solvents on lipase activity was determined following pre-incubation of enzyme for 30 min at 25 °C under 150 rpm shaking in the presence of methanol, acetone, hexane, heptane, toluene, ethanol, chloroform, isopropanol, diethyl alcohol, butanol and isoamyl alcohol at concentrations of 10% v/v and 20% v/v. The incubation was conducted in closed screw cap tubes with silicone rubber gasket in order to prevent evaporation of the enzyme reaction.

The influence of imidazolium-based ionic liquids (ILs) on lipase activity was investigated after a 30 min-preincubation of the purified enzyme with different concentrations of ILs (ranging from 2 to 10% v/v) at 50 °C and pH 8.0. The remaining activity was analyzed by spectrophotometric method under the assay condition. The lipolytic activity was determined by triplicate experiments.

2.6.5. Influence of various effectors on enzyme activity

The effect of a variety of chemical reagents (1 and 5 mM) on the enzyme activity was investigated by pre-incubating the lipase for 30 min at 70 °C in 50 mM Tris-HCl buffer (pH 8.0) containing following chemical agents; oxidizing agents: ammonium persulfate, potassium iodide and H_2O_2, reducing agents: ascorbic acid and β-mercaptoethanol, chelating agents: sodium citrate and EDTA (ethylenediaminetetraacetic acid), detergents: SDS, CTAB (cetyltrimethylammonium bromide) and Triton X-100, additives: PEG 4000 (polyethylene glycol) and glycerol, inhibitors: PMSF (phenyl methyl sulfonyl fluoride), DTT, DMF, urea, DTNB, sodium fluoride, mercuric chloride and phenanthroline. The activity of the enzyme without additives was assumed as 100%.

2.6.6. Effect of commercial detergents on enzyme stability

The stability of the recombinant lipase in commercial enzyme-containing powder/liquid detergents was investigated. The solid detergents utilized in this study were as follows: Barf (Paxan, Iran), Vash (Henkel, Germany), Softlan (Pakshoo, Iran), Persil (Henkel, Germany) washing powder and handwash powder, Pril (Henkel, Germany), Shoma (TolyPers, Iran), Finish (Reckitt Benckiser, Canada) and Darya (TolyPers, Iran). The liquid detergents tested were Goli (Paxan, Iran), Persil (Henkel, Germany), Ave (Pakshoo, Iran) and Ganj (RaminGostar, Iran). Dissolving in tap water, the solid detergents were prepared at a final concentration of 5 mg/L, while a 100-fold dilution of liquid detergents was performed to simulate washing conditions. To inactivate the endogenous enzymes in aforementioned detergents, diluted detergent preparations were pre-incubated for 30 min at 80 °C before the addition of recombinant lipase. The enzyme was added to powder/liquid detergent solution following incubation at 70 °C for 60 min, followed by activity measurement. To allow further comparison, the effect of commercial detergents on the stability of a commercial lipase (porcine pancreatic lipase, PPL) was also studied under the same experimental conditions.

2.6.7. Determination of substrate specificity

Lipase substrate specificity was analyzed using the spectrophotometric assay, using 0.01 M p-nitrophenyl acetate (C2), butyrate (C4) and palmitate (C16) dissolved in ethanol as substrates.

3. Results and discussion

3.1. Cloning and sequence analysis of the lipase gene

The sequence of the lipase gene expressed in-frame with an N-terminal region coding for six His residues has been deposited in

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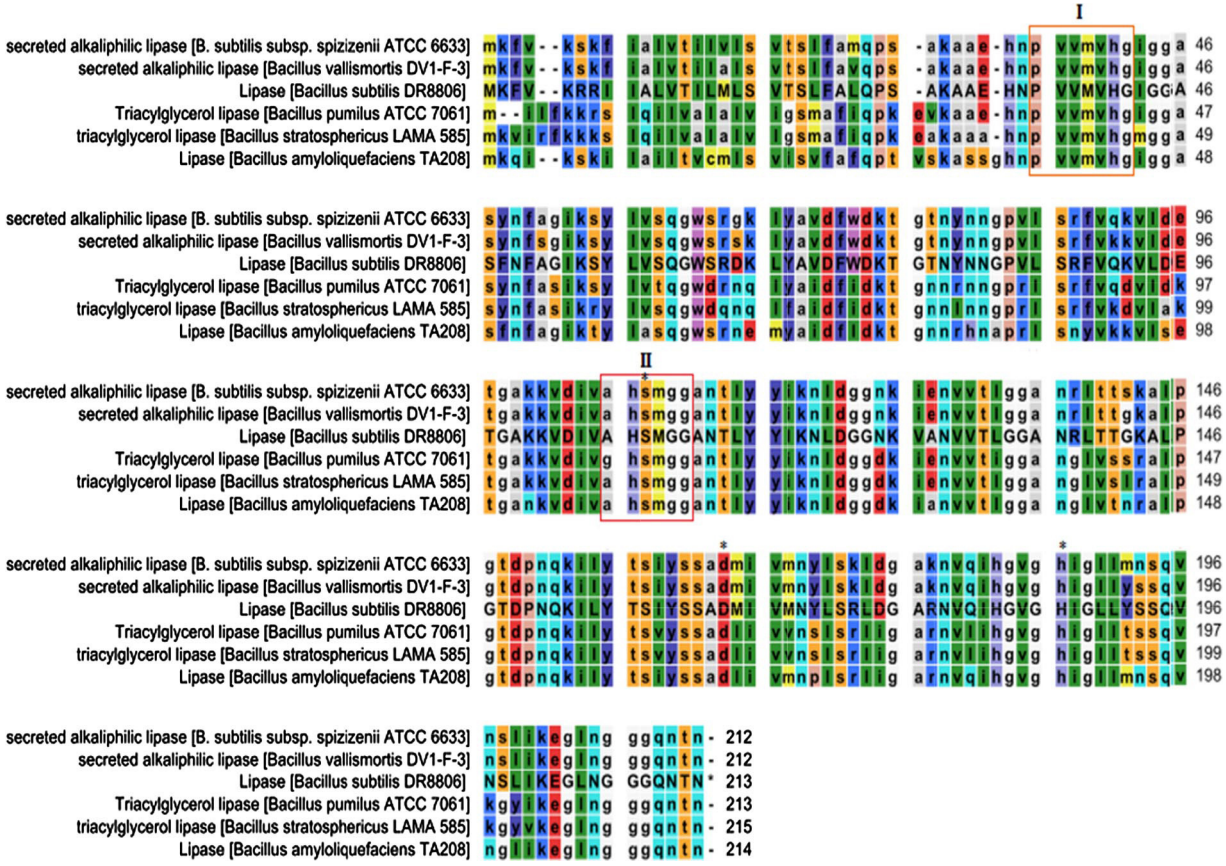

Fig. 1. Comparison of the amino acid sequences of various lipases originated from genus *Bacillus*. The amino acids of catalytic triad (His, Ser and Glu) are marked with asterisks. Oxyanion hole (box I) and conserved pentapeptide (box II) are illustrated.

the GenBank under the accession number KC262177. The sequence of the putative lipase gene contains a complete ORF of length 639 bp which encodes a 212 amino acid precursor with a molecular mass of 22.8 kDa and a theoretical pI of 9.72. The putative signal peptide cleavage site was predicted to be located between Ala-31 and Ala-32 using SignalP 4.0 software [13]. Lipases typically have a Ser-Asp-His catalytic triad in which the active site serine is located within the middle of the conserved pentapeptide Gly-X-Ser-X-Gly [14]. The catalytic site of recombinant lipase comprises the triad of Ser^{110}, Glu^{166} and His^{189}. According to multiple alignment of *Bacillus* lipase sequences obtained from GenBank, *B. subtilis* DR8806 lipase sequence exhibited the conserved consensus motif initiated with Ala (Ala-His-Ser-Met-Gly) instead of Gly. The alignment of various sequences of thermostable *Bacillus* lipases revealed a high degree of sequence similarity at both DNA and amino acid levels (Fig. 1).

3.2. Overexpression and purification of the recombinant lipase

Competent *E. coli* BL21 (DE3) cells were transformed with appropriate recombinant pET-28a(+) expression system encoding the lipase gene with the His-tag sequence in an N-terminal configuration. The insertion of target fragment into expression vector in the correct direction and reading frame was firstly confirmed by lipase amplification using specific linker primers. Cloning of lipase gene into pET-28a(+) vector was also confirmed by double digestion. The expression of lipase was carried out by the induction of IPTG-inducible bacteriophage T7 promoter using 1 mM IPTG. The enzyme was expressed as an amino-terminal 6x-His tag to assist the protein purification using Ni-NTA column. One-step IMAC-purification of the recombinant lipase resulted in a homogenous protein as represented by SDS-PAGE (Fig. 2). With the insertion of a 3.8 kDa extension region belonging to expression vector, the predicted molecular mass for deduced protein sequence of recombinant lipase was estimated to be approximately 26.8 kDa. Although thermophilic bacteria could be ideal candidates as thermostable lipase producers, only a minute amount of enzyme can be obtained which may not be suitable for industrial application [3]. As shown in Table 1, the fusion enzyme was ultimately purified three-fold with a yield of 57% resulting in an increased lipase specific activity from 456.3 (crude enzyme) to 1364 U/mg (purified recombinant lipase).

3.3. pH profile and stability

The effect of pH on enzyme activity and stability was examined in the pH range of 2.0–10.0. Illustrated in Fig. 3, the enzyme was active in a broad range of pH (pH 5.0–10.0) with an optimum pH of 8.0 in 50 mM Tris-HCl buffer. In spite of being highly active within a broad pH range, a sharp decline in lipase activity was monitored at pH below 5.0, at which almost none or low activity was observed. The high enzyme activity at alkaline pH was probably due to Ala-Gly (first Gly-residue) substitution in the consensus sequence Gly-X-Ser-X-Gly [15]. The optimum pH of *B. subtilis* DR8806 lipase was identical to that of other thermophilic lipases from *B. subtilis* EH 37 [16], *Bacillus sphaericus* 205y [17], *Bacillus* sp. L2 [18] and a lipolytic enzyme from *B. subtilis* DR8806 [19]. The pH stability of enzyme showed that it was stable (>90% of lipase activity) at pH values ranging from 8.0 to 10.0 upon a 60-min treatment at 50 °C in Tris-HCl buffer. The enzyme was fairly stable at pH 5 with 57% residual activity. Nevertheless, the lipase stability decreased sharply when the pH was below 5.0. Lipases which are stable

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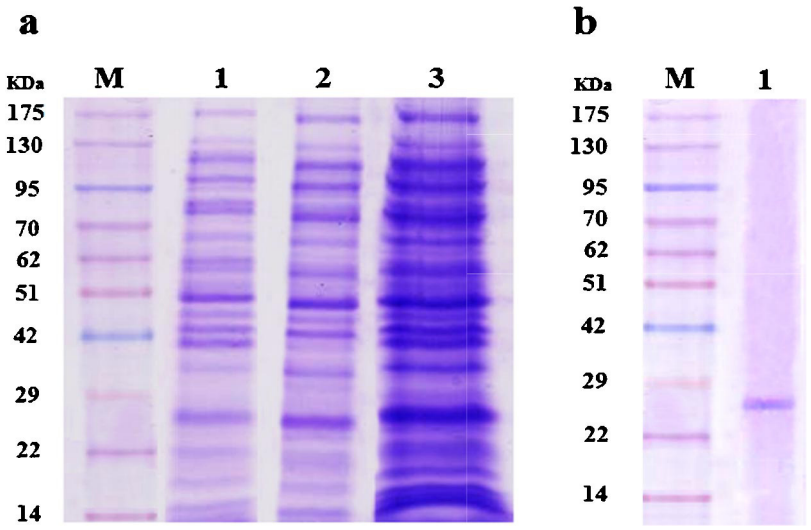

Fig. 2. SDS-PAGE analysis of recombinant α-lipase. (a) M marker, lanes 1–3: cell fractions at 2 h (lane 1), 4 h (lane 2) and 8 h (lane 3) post induction with 1 mM IPTG, (b) M marker, lane 1 purified recombinant lipase using Ni-NTA affinity chromatography.

Table 1
Purification steps of His-tagged recombinant lipase using Ni-NTA column.

Purification step	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude	456.3	100	45,557	1	100
Ni-NTA column	1364	19	26,000	3	57

under alkaline conditions are considered as promising candidates for removable of fat stains in detergent formulation [20].

3.4. Effect of temperature on enzyme activity and stability

The effect of temperature on lipase activity was measured at different temperatures ranging from 30 °C to 80 °C at 5 °C intervals for 30 min (Fig. 4). The temperature profile of lipase is of great importance for high temperature operational bioprocesses, since the enzyme was active over a wide range of temperatures (30–80 °C), with an optimum activity at 70 °C. The optimum temperature of enzyme was in agreement with other thermostable lipases from *Bacillus* sp. L2 [18] and *Geobacillus* sp. T1 [3]. An esterase from *Bacillus subtilis* DR8806 showed an optimal temperature at 50 °C as a mesophilic enzyme [19]. The recombinant enzyme retained 90% and 87.5% of its initial activity after incubation at 75 °C and 80 °C for 1 h, respectively. A recombinant lipase from *Bacillus thermoleovorans* ID-1 with an optimum temperature of 75 °C retained 50% and 30% of its initial activity after 1-h incubation at 60 °C and 70 °C, respectively. The results indicated that the enzyme might be valuable in lipid processing industry, which requires the enzyme to be stable above 50 °C [2]. The half-life of the purified lipase was calculated to be 145 min at its optimum temperature. The enzyme retained 50% of its original activity after 92 min incubation at 80 °C, pH 8.0. An extra-cellular lipase produced by *Bacillus licheniformis* MTCC 6824 showed a half-life ($t_{1/2}$) of 48 min at 60 °C [21]. The half-life of *Bacillus stearothermophilus* MC 7 lipase was found to be 30 min at 70 °C [22]. Considering its appropriate properties, the recombinant alkalothermophilic enzyme will play a dominant role

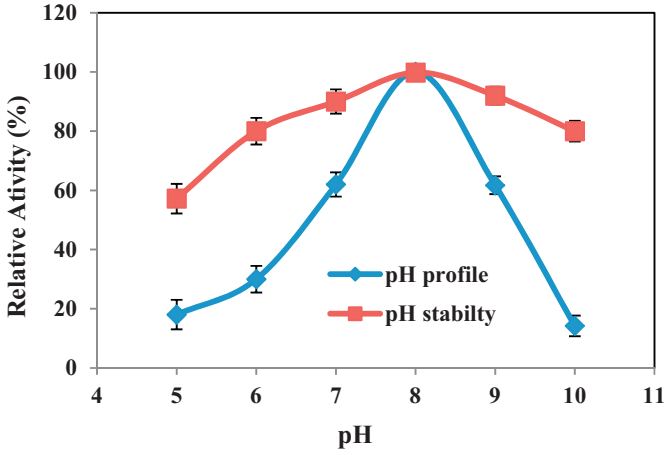

Fig. 3. Effect of pH on the activity and stability of *B. subtilis* DR8806 recombinant lipase. Effect of pH on lipase activity was investigated by assaying its activity at various pH values. The enzyme stability was determined by incubating the enzyme at 50 °C for 60 min at different pH values. Each data point depicts the mean of three independent assays.

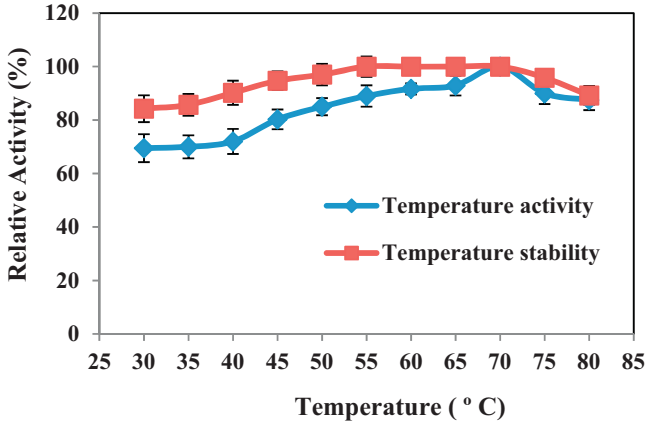

Fig. 4. Effect of temperature on the enzyme activity and stability. The purified lipase was assayed at different temperatures (30–80 °C). For the stability test, the enzyme was incubated for 60 min at various temperatures, pH 8.0. The lipolytic activity was determined by triplicate experiments.

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Table 2
Effect of metal ions on enzyme activity[a].

Metal salts	Relative activity (%) 1 mM	5 mM
Control	100.0	100.0
$MgCl_2$	54	51
$CuCl_2$	36	20
$MnCl_2$	53	69
$CaCl_2$	52	44
KCl	45	50
NaCl	39	57
$FeCl_2$	33	46
$ZnCl_2$	87	47
$CoCl_2$	100	43
$PbCl_2$	54	42
$BaCl_2$	78	54
$CdCl_2$	45	64
$HgCl_2$	12	0

[a] The lipase activity was monitored under the standard assay conditions following incubation with various metal ions at 1 and 5 mM concentrations. The percent lipase activity for control was taken as 100%.

Table 3
Effect of organic solvents on the purified recombinant lipase[a].

Organic solvent	Relative activity (%) 10% v/v	20% v/v
Control	100	100
Methanol	96	92
Acetone	90	92
Hexane	83	98
Heptane	85	108
Toluene	94	85
Ethanol	75	92
Chloroform	102	101
Isopropanol	88	90
Diethyl alcohol	96	91
Butanol	93	96
Isoamyl alcohol	97	92

[a] The enzyme was pre-incubated at 25 °C for 30 min with different organic solvents at 10% and 20% v/v prior to lipase assay. A sample reaction without organic solvent was taken as control.

in industrial applications including biopolymer/biodiesel synthesis as well as the production of pharmaceuticals, cosmetic, flavor and commercial laundry detergents [20].

3.5. Effect of metal ions on lipase activity

The effect of metal ions on the activity of the cloned lipase is represented in Table 2. The influence of metal ions could be attributed to the change in the solubility and behavior of ionized fatty acids upon complex formation with metal ions and the direct inhibition of enzyme catalytic function [22]. The results demonstrated that the enzyme was dramatically inactivated by Hg^{2+} and Cu^{2+}, suggesting the ability of these ions to alter enzyme conformation and direct inhibition of the catalytic site. Pb^{2+}, Zn^{2+} and Ba^{2+} ions led to a moderate reduction in enzyme activity at 5 mM concentration. The lipase enzyme was also inactivated by 50% in the presence of KCl, while retained almost 100% activity after being exposed to $CoCl_2$ at a concentration of 1 mM. A lipase from *B. stearothermophilus* MC 7 was inhibited by divalent ions of heavy metals, completely by Cu^{2+} and strongly by Fe^{2+} and Zn^{2+} [22]. The lipolytic activity of OST-lipase from *B. sphaericus* 205y was stimulated in the presence of Ca^{2+} and Mg^{2+} and inhibited to the extent of 90% and 94% when $ZnSO_4$ and $FeCl_3$ were used, respectively [17]. Partial inhibition of BP-6 LipA activity was caused by 10 mM concentrations of Fe^{3+}, Cu^{2+}, Pb^{2+} and Zn^{2+}, whereas Hg^{2+} caused a significant inhibition of the recombinant enzyme [23].

3.6. Effect of organic solvents and ionic liquids on lipase

The influence of various organic solvents at concentrations of 10% and 20% v/v on lipase activity was assessed under standard conditions (Table 3). The recombinant enzyme was remarkably stable toward organic solvents, retaining more than 95% of its activity in the presence of isoamyl alcohol, methanol, hexane, butanol and diethyl alcohol. A relative activity of 102% was apparent in the presence of chloroform. The lipolytic activity was enhanced at higher concentrations of heptane showing stimulatory effect of the solvent on the enzyme. Other solvents did not promote the lipolytic activity, however, high lipolytic activity was observed at lower concentrations of the organic solvents. The exposure of BF-3 lipase to organic solvents elucidated that the enzyme was activated by methanol, ethanol and toluene (10% and 20% v/v) whereas the stability of enzyme was drastically reduced with increasing the concentration of organic solvents [24]. Moreover, the activity of an alkaline metallolipase from *B. licheniformis* MTCC 6824 was found to be considerably decreased in chloroform, acetonitrile and ethyl acetate [21]. Organic solvent-tolerant lipases have gained focus as potentially valuable enzymes in non-aqueous biocatalysis. Thus, the stability of recombinant enzyme toward organic solvents is of great significance particularly for synthesis of esters using lipolytic enzymes.

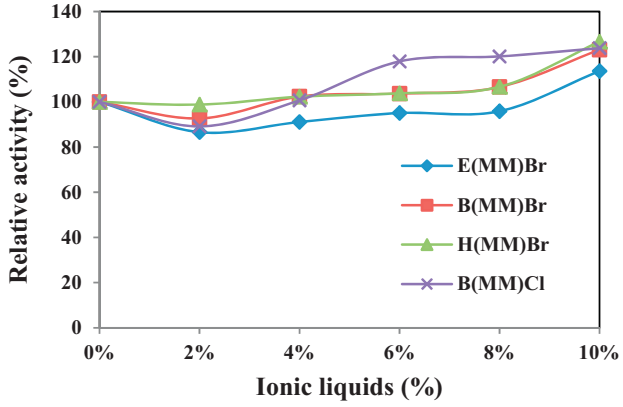

Fig. 5. Influence of various imidazolium-based ionic liquids with 2–10% (v/v) concentrations on the activity of recombinant lipase from *B. subtilis* DR8806. Values presented are the means of triplicate analyses.

Exhibiting the features of both organic and ionic compounds, ILs emerged as remarkably interesting non-aqueous media for numerous enzymatic reactions such as alcoholysis, ammoniolysis and perhydrolysis [6]. As displayed in Fig. 5, the recombinant enzyme was also investigated for its lipolytic activity against different 1-alkyl-3-methylimidazolium-based ILs including 1-ethyl-3-methylimidazolium bromide ([EMIM][Br]), 1-*n*-butyl-3-methylimidazolium bromide ([BMIM][Br]), 1-hexyl-3-methylimidazoliumbromide ([HMIM][Br]) and 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]). In agreement with previously reported studies, ILs had promoting influence on lipase activity [7,8]. Besides, the enzyme activity was improved with the increase in the concentration of the ionic liquid. 1-Butyl-3-methylimidazolium chloride ([BMIM][Cl]) exhibited the highest promoting effect among all investigated ILs in the present study. The stimulatory impact of Br-containing ILs on enzyme activity was as follows [HMIM][Br] > [BMIM][Br] > [EMIM][Br]. Similar to previous reports [7,8], the lipase activity was enhanced with the increase in alkyl chain length of the imidazolium ring of ILs. The effect of the elongation of alkyl side chain on the hydrolytic activity seems to be in correlation with the hydrophobic nature of the alkyl group, improving the ability of IL to obstruct the lipase non-polar active site [7]. Lipase catalyzed-reactions have benefited most from the exploitation of ILs [6]. The results indicated that the

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Table 4
Effect of various chemical reagents on recombinant lipase activity[a].

Reagent molecule	Relative activity (%)	
Oxidizing agents	1 mM	5 mM
Ammonium persulfate	86	80
Potassium iodide	81	99
Reducing agents		
Ascorbic acid	70	55
β-Mercaptoethanol	77	106
Chelating agents		
Sodium citrate	39	46
EDTA	88	82
Detergents	10% (w/v)	20% (w/v)
SDS	79	93
CTAB	100	100
Triton X-100	100	98
Additives	1% (w/v)	2% (w/v)
PEG 4000	94	87
Glycerol	106	95
Inhibitors	1 mM	5 mM
DMF	87	95
Urea	88	99
PMSF	87	79
DTT	90	94
DTNB	102	90
Sodium fluoride	63	0
Mercuric chloride	79	61
Phenanthroline	82	72

[a] The recombinant lipase was incubated with different effectors for 30 min under optimal conditions. The residual activity was measured under the standard enzyme assay condition.

cloned lipase had greater activity and stability in ionic liquids than in traditional organic solvents.

3.7. Effect of additives, inhibitors, oxidizing and reducing agents on lipolytic activity

As illustrated in Table 4, the purified lipase exhibited high stability toward a variety of known reagents, retaining more than 90% of its activity after pre-incubation with DTNB, SDS, urea, DMF, DTT, glycerol and Triton X-100. The presence of oxidizing agents such as ammonium persulfate and potassium iodide in the reaction mixture resulted in a moderate decrease in lipase hydrolytic activity. The decreased enzyme activity might be due to partial oxidation of His residue in catalytic triad. The enzyme was slightly affected when treated with reducing agents; 30% and 23% decrease in the activity of recombinant lipase were observed in the presence of ascorbic acid and β-mercaptoethanol, respectively. Partial inactivation of the lipase in the presence of PMSF probably was observed due to modification of an essential serine residue in the enzyme catalytic site. CTAB and Triton X-100 detergents had no effect on the purified lipase activity. Addition of 1% v/v glycerol and 1 mM DTNB to the enzyme mixture gradually enhanced the lipolytic activity in comparison to the control. Sodium fluoride showed moderate inhibitory effect (37%) on lipase activity at 1 mM concentration, but caused a complete block of activity at 5 mM concentration. The lipase was moderately stable toward EDTA, exhibiting only a 13% decrease in its initial activity after 30 min pre-incubation. Contradictory results have been published in literatures regarding the effect of different additives on lipase activity. The enzyme activity of *Bacillus cereus* BF-3lipase was drastically diminished in the presence of SDS and PMSF [24]. Additionally, the aforementioned agents strongly inhibited a recombinant lipase from thermophilic *Bacillus* sp. L2 at 1 and 10 mM concentrations. Decreasing the lipase activity in the presence of a chelating agent could be as a result of its influence on the interfacial area between the substrate and lipase and also the necessity of metal ions for preserving the enzyme stability and activity [18]. Accordingly, the capability of *B. subtilis* DR8806 to withstand relatively harsh washing conditions (alkaline pH, elevated temperatures as well as surfactant/additive resistance) is a prerequisite of industrial enzymes used in detergent preparation.

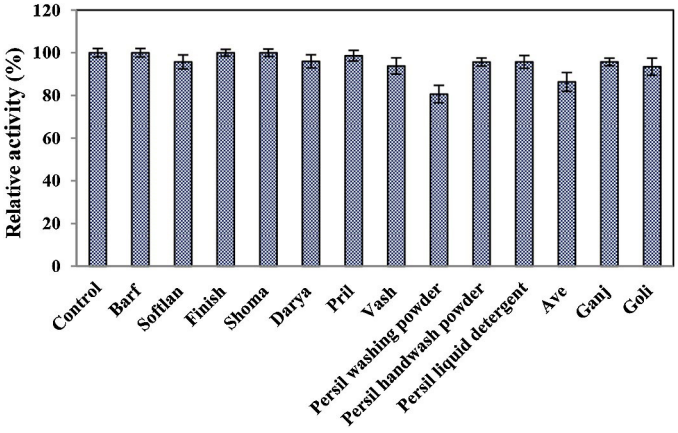

Fig. 6. Effect of different commercial solid/liquid detergents on lipase activity. The enzyme was first treated with detergent and the residual activity was measured under assay conditions. The activity of control samples in the absence of detergents was taken as 100%. Values presented are the means of triplicate analyses.

3.8. Stability of enzyme toward commercial solid/liquid detergents

The compatibility of recombinant lipase with solid/liquid household laundry and automatic dishwashing detergents was investigated following pre-incubation of the purified enzyme with a broad range of commercial detergents. As represented in Fig. 6, the enzyme was extremely stable in the presence of powder and liquid detergents, remaining 100% of its activity toward Shoma, Barf and Finish and more than 95% in the presence of Pril, Persil, Softlan and Ganj after 1 h incubation at 70 °C. Interestingly, the recombinant lipase was more stable than the porcine pancreatic lipase (PPL), which was completely inhibited by commercial detergents used including Pril, Sotlan, Barf, Ave and Sotlan (data not shown). Additionally, similar to the recombinant lipase, PPL was totally stable toward Finish and Persil solid detergents. The stability of the recombinant lipase against commercial laundry detergents makes it a potential candidate in household detergent formulation.

3.9. Substrate specificity

The enzyme specificity was studied toward p-nitrophenylesters of different alkyl chain lengths (C2, C4 and C16). The highest hydrolytic activity was obtained for p-nitrophenylbutyrate, indicating a clear preference of the enzyme for short acyl chain lengths. Moreover, p-nitrophenylacetate was hydrolyzed quite well, whereas p-nitrophenoylpalmitate with longer chain length (C16) was converted slightly. Enzymes belonging to subfamily I.4 of lipases are the small alkaline proteins with preference toward short-chain substrates [25]. Similar results have been reported for *Bacillus* sp. BP-6 LipA and a thermophilic lipase from *B. thermoleovorans* ID-1 [2,15], although other reported lipases have shown preference for esters with longer fatty acids when assayed against p-nitrophenyl derivatives [22,24].

4. Conclusion

In retrospect, a thermo-alkaliphilic lipase gene from *B. Subtilis* DR8806 was successfully cloned into pET-28a(+) vector. The success of extracellular expression of an alkalophilic organic lipase and the simple purification method developed promises many

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biotechnological advantages. The pH profile of recombinant lipase of *B. subtilis* DR8806 is similar to other reported extracellular esterase previously purified from this isolate, but it shows some differences in properties with respect to temperature activity and stability, metal ions, stability toward surfactants and organic solvents. The results clearly demonstrated that the enzyme was not only stable but also moderately activated in aqueous solutions of imidazolium-based ionic liquids. Therefore, the replacement of organic solvents with ILs, which are considered as recyclable clean solvents or catalysts of green chemistry, may lead to significant development of lipase hydrolytic activity. Owing to high stability at elevated temperatures, a broad range of pH and resistant against organic solvents/ionic liquids, the recombinant lipase can potentially be applied in the fields of detergents, oil and fat, dairy, and pharmaceutical industries.

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