Isolation, Cloning and Co-Expression of Lipase and Foldase Genes of *Burkholderia territorii* GP3 from Mount Papandayan Soil

Ludwinardo Putra¹, Griselda Herman Natadiputri², Anja Meryandini¹, and Antonius Suwanto^{1,2*}

¹Graduate School of Biotechnology, Bogor Agricultural University, Bogor 16680, Indonesia

²Biotechnology Research and Development, PT Wilmar Benih Indonesia, Bekasi 17530, Indonesia

Received: December 8, 2018

Revised: May 1, 2019

Accepted: May 1, 2019

First published online
May 14, 2019

*Corresponding author

Phone: +62-812-9973-680;

E-mail: asuwanto@indo.net.id

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2019 by
The Korean Society for Microbiology
and Biotechnology

Lipases are industrial enzymes that catalyze both triglyceride hydrolysis and ester synthesis. The overexpression of lipase genes is considered one of the best approaches to increase the enzymatic production for industrial applications. Subfamily I.2. lipases require a chaperone or foldase in order to become a fully-activated enzyme. The goal of this research was to isolate, clone, and co-express genes that encode lipase and foldase from *Burkholderia territorii* GP3, a lipolytic bacterial isolate obtained from Mount Papandayan soil via growth on Soil Extract Rhodamine Agar. Genes that encode for lipase (lipBT) and foldase (lifBT) were successfully cloned from this isolate and co-expressed in the *E. coli* BL21 background. The highest expression was shown in *E. coli* BL21 (DE3) pLysS, using pET15b expression vector. LipBT was particularly unique as it showed highest activity with optimum temperature of 80°C at pH 11.0. The optimum substrate for enzyme activity was C₁₀, which is highly stable in methanol solvent. The enzyme was strongly activated by Ca²⁺, Mg²⁺, and strongly inhibited by Fe²⁺ and Zn²⁺. In addition, the enzyme was stable and compatible in non-ionic surfactant, and was strongly incompatible in ionic surfactant.

Keywords: *Burkholderia territorii* GP3, *E. coli* BL21 (DE3) pLysS, foldase, lipase

Introduction

Lipases are enzymes that catalyze both the hydrolysis of triglycerides and synthesis of ester. This widely used enzyme group plays many roles in industrial applications, such as in the manufacturing of detergent formulation, biodiesel, and ester flavoring, as well as in bioremediation [1]. Lipases from Burkholderiaceae, such as *Burkholderia* and *Ralstonia*, are known for their potential for industrial applications, due to their high resistance to the solvent methanol and their high alkalinity [2]. By those characteristics, this lipase group could become a potential catalyst for biodiesel production by transesterification reaction [3].

Lipase-encoding genes are typically cloned and over-expressed for their applications in industry, particularly to ensure purity and high yield. There were several reports of lipase gene expression in different systems [2, 4, 5]. In particular, lipases from *Burkholderia* spp., which belong to subfamilies I.2, require a folding protein (foldase) in order

to be actively expressed. In members of this genus, the foldase-encoding gene (*lif*) was clustered with a lipase-encoding gene as an operon [6].

Two strategies for co-expression of lipase and foldase may be employed, including in vitro and in vivo folding. In in vitro folding, the lipase- and foldase-encoding genes are expressed in different hosts and vectors. Co-expression occurs as they are combined together in the same reaction, resulting in fully active lipase [7]. In in vivo folding, the lipase- and foldase-encoding genes are expressed in the same host, using either a two-plasmid or a one-plasmid system. The two-plasmid system involves the isolation and expression of both genes in separate vectors. On the other hand, the one-plasmid system requires only one plasmid for expression, as the lipase- and foldase-encoding genes are expressed together as one operon [8].

Genes that encode for lipase were successfully isolated from *Burkholderia* spp. and expressed in a different system, mostly in homologous hosts [2, 4]. Recently, the lipase-

encoding gene from *Burkholderia contaminans* was successfully expressed in a heterologous host for the first time [5]. The expression occurred in a two-plasmid system and thus far, there is no report for heterologous expression in a one-plasmid system. In this work, the co-expression of genes that encode for lipase and foldase genes from *Burkholderia territorii* GP3 was done in a one-plasmid system for heterologous expression. Moreover, we found that the expressed lipase demonstrates a unique characteristic, showing high activity even at 80°C, while other *Burkholderia*-derived lipases mostly showed their optimal activity at 70°C.

In this study, we isolated and cloned the lipase- and foldase-encoding genes from *Burkholderia territorii* GP3 and co-expressed both genes in the *Escherichia coli* BL21 background. The enzyme was further characterized to define its uniqueness for further industrial application.

Materials and Methods

Materials

The pGEM-T Easy and pET-15b vectors were purchased from Promega (USA) and Novagen (USA), respectively. *Escherichia coli* DH5 α and *E. coli* DH10 β were obtained from the lab culture collection and used as cloning hosts. The expression hosts, *E. coli* BL21 (DE3) (pLysS) and *E. coli* Origami B, were purchased from Takara (Japan). *E. coli* SHuffle B and SHuffle K were purchased from NEB (USA). All restriction enzymes and T4 DNA ligase used in this study were purchased from NEB. All p-nitrophenyl synthetic substrates were purchased from Sigma-Aldrich (USA).

Bacterial Isolation and Identification

Soil samples were collected from Mount Papandayan and enriched with livestock fat enrichment for 30 days. The soil was serially diluted, then incubated for 48–72 h at 30°C on Soil Extract Rhodamine Agar, which contained filtered-soil extract (500 g/l) as base media, bacto agar (1.5 g/l), PVA, olive oil (4%), Rhodamine B (0.1%). The medium was adjusted to pH = 3.0 using 0.2 M citrate-phosphate buffer. Lipolytic bacteria that showed a clear zone around their colonies were incubated for 24 h at 30°C on Luria Agar (yeast extract 5 g/l; NaCl 10 g/l, tryptone 10 g/l; bacto agar 15 g/l). The isolate was identified based on 16S rDNA using universal primer pairs for bacteria [9]. Sequence analysis was done using the 16S rRNA identification tools at www.ezbiocloud.net.

Amplification and Cloning of LipBT and LifBT

Genes that encode for lipase (*lipBT*) and foldase (*lifBT*) in *Burkholderia territorii* were jointly amplified as an operon using a modified primer pair [4]. The operon was cloned into pGEM-T Easy vector, using *E. coli* DH5 α as a host. Recombinant *E. coli* colonies were verified using colony PCR and further grown on

Fig. 1. Plasmid map of pET-15b+LipBT+LifBT recombinant vector.

The plasmid contains ampicillin resistance gene as selection marker, along with NdeI and NcoI restriction site.

Rhodamine Agar supplemented with ampicillin (100 μ g/ml). LipBT- and LifBT-encoding gene sequences were analyzed using Geneious 11.0.5 and VMD 1.9.3 [10, 11]. The operon was further cloned into pET15b with some modification at the multiple cloning site (pET-15b). The operon was cloned into pET-15b at the NdeI and NotI site (Fig. 1). Recombinant pET-15b was transformed to *E. coli* DH10 β using the heat shock method [12].

Amino Acid Analysis and Protein Modeling of LipBT

The amino acid analysis was carried out by Geneious 11.0.5. Alignment of LipBT and LifBT was achieved using BLAST. The secondary structure of each protein sample was predicted using the Predict Secondary Structure application from Geneious 11.0.5 [10]. Prediction of disulfide bonding of LipBT was carried out by DISULFIND [13]. The protein model of LipBT was acquired as PDB using SWISS-MODEL, and visualized by VMD [11, 14]. Quality assessment of the protein model was carried out by ProQ3 with the embedded CAD and LDDT scores [15].

Co-Expression of LipBT and LifBT

LipBT and LifBT were co-expressed in pGEM-T Easy and pET-15b plasmids in a variety of hosts, including *E. coli* DH5 α , *E. coli* DH10 β , *E. coli* BL21 (DE3) pLysS, *E. coli* Origami B, *E. coli* SHuffle B, and *E. coli* SHuffle K. Seed culture for each host was prepared on 10 ml Luria Broth supplemented with appropriate antibiotics (100 μ g/ml ampicillin or, 30 μ g/ml chloramphenicol) at 37°C and 200 rpm overnight. Seed culture (1%) was inoculated onto 50 ml Luria Broth supplemented with appropriate antibiotics and grown in the same conditions up to OD600 $\approx$ 0.5 and 0.25 mM IPTG was subsequently added into the culture, followed by further incubation at 37°C and 200 rpm for 16 h. Bacterial cells

were harvested by centrifugation at 7,000 ×g, 4°C for 10 min, and suspended in 5 ml Tris-HCl 0.1 M pH 8. The cells were lysed using Handy Sonic ur-21p sonicator for 15 min, and centrifuged at 11,000 ×g, 4°C for 15 min. The supernatant was collected as cell-free extract and used in further enzymatic assays.

Lipase activity was measured using spectrophotometry method based on hydrolysis using *p*-nitrophenyl ester as substrates [16]. Reaction mixture was composed of 940 µl Tris-HCl 0.1 M pH 8, 40 µl absolute ethanol, 10 µl *p*-nitrophenyl ester substrate, and 10 µl cell-free extract. The mixture was incubated at 40°C for 5 min. The concentration of the hydrolysis product, *p*-nitrophenol (pNP) was measured using a spectrophotometer at 405 nm. One unit of lipase was defined as the amount of lipases required to release 1 µmol of pNP under the specific condition mentioned.

Lipase Purification

Lipase produced by specific host carrying an expression vector with the highest lipase activity was partially purified using ammonium sulfate partial precipitation. The precipitation was done in five steps, resulting in five fractions of saturated ammonium sulfate: < 20%, 20–30%, 30–40%, 40–50%, and >50%. Each fraction was collected and dialyzed for 16 h. The lipase-specific activity of each fraction was quantified and compared as described above. The fraction showing the highest specific activity was used for characterization. Furthermore, SDS-PAGE on 10% (w/v) SDS polyacrylamide gels and zymography were used to determine the molecular masses of LipBT and LifBT. In this case, we did not perform heating before loading the samples for zymogram analysis. Zymogram was performed by incubating the gel in Tris-Cl pH 8 buffer for 20 min.

Characterization of LipBT

Purified LipBT activities in varying temperature and pH were evaluated using the lipase assay described above. The effect of temperature was determined within the range of 10–90°C. To test for LipBT thermostability, the protein was incubated at 10–80°C for 30 min in Tris-HCl 0.1 M, pH 8. The effect of pH was determined within the range of 3.0 to 13.0. For pH stability, LipBT was incubated at 40°C for 30 min at pH 3.0 to 13.0. The reactions were carried out on the following buffers (0.1M): citrate buffer (pH 3.0 to 5.0), phosphate buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to 13.0).

Substrate specificity of LipBT was determined towards *p*-nitrophenyl substrates with various ester chains, including *p*-nitrophenyl C₄, C₈, C₁₀, C₁₂, C₁₄, C₁₆, and C₁₈. Hydrolysis activity towards *p*-nitrophenyl C₄, C₈, C₁₀ was measured using spectrophotometry method. A different assay method was used for *p*-nitrophenyl C₁₂, C₁₄, C₁₆, and C₁₈. About 20 µl lipase was added to 880 µl of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate. After incubation at 30°C for 3 min, the reaction was started by addition of 100 µl substrate 8 mM (in isopropanol) and incubated at 30°C for 3 min. The reaction was stopped by addition of 0.5 ml of 3 M HCl. The

mixture was centrifuged at 12,000 ×g for 2 min, then 333 µl supernatant was mixed with 1 ml of 2 M NaOH. The OD was measured at 420 nm.

The effect of various organic solvents, metal ions and surfactants on LipBT stability was evaluated by incubating purified lipase at 40°C for 30 min in the presence of the following substances: organic solvent, methanol, ethanol, butanol, isopropanol, acetonitrile, and *n*-hexane (50%); metal ions, CaCl₂, MgCl₂, CuCl₂, ZnCl₂, and FeCl₂ (0.1M); surfactants, DMSO, Triton X-100, Tween-80, and sodium dodecylsulfonate (SDS) (1%). As for control, the lipase was incubated in buffer Tris-HCl pH 8.0. Following that, lipase activities were measured at 80°C, pH 11.0 for 5 min.

Results and Discussions

Bacterial Isolation and Identification

Soil Extract Agar is a bacterial isolation medium that contains limited nutrients, micro-nutrients that cannot be found in other nutrient-rich media [17]. In this study, we used a combination of Rhodamine Agar and Soil Extract Agar as the base medium, instead of Luria Bertani. Such modification may increase the possibilities of growing lipolytic bacteria that are previously non-culturable. Five bacterial colonies were successfully isolated on Soil Extract Rhodamine Agar. Among them, isolate GP3 was the only one that showed lipolytic activity. Upon growth on Rhodamine Agar for approximately 5 days, the color of GP3 colonies changed from orange to green, and then later turned gray. In contrast, the color of bacterial colonies on In Luria Agar shifted from yellow to purple. Such a shift in colony pigmentation was not observed on Soil Extract Rhodamine Agar, in which the bacterial colonies remained pink throughout the incubation period. Aside from the possibility of pigment degradation due to bacterial death, such pigmentation variability might be caused by difference in the media composition and growth conditions, such as pH and temperature [18].

Based on the 16S rDNA analysis, isolate GP3 shared the highest similarity with *Burkholderia territorii* LMG25818 (99.69%), followed by *Burkholderia cepacia* ATCC 25416 (99.61%), *Burkholderia anthina* R-4183 (99.61%), *Burkholderia seminalis* R-24196 (99.53%), and *Burkholderia metallica* R-16017 (99.45%). *Burkholderia territorii* LMG25818 was originally isolated from soil in Papua New Guinea soil and considered as a novel bacterium [19]. While it possessed lipolytic activity, to date there is no report on *B. territorii* lipase-encoding gene isolation and expression from the species. Thus far, only the lipase-encoding genes from *Burkholderia contaminans* and *Burkholderia cepacia* have been successfully isolated and expressed [4, 5].

Fig. 2. Amino acid alignment and secondary protein structure prediction of LipBT with related lipase from *Burkholderia contaminans* LTEB11, *Burkholderia cepacia* G63, and *Burkholderia cepacia* ATCC 25416.

The purple bars represented α -helix structure, and the yellow arrows represent β -sheet. The diagram describes how amino acid sequence showed differences in the secondary structure formation.

Cloning and Amino Acid Analysis of LipBT

The *B. territorii* GP3 lipase operon is 2,312 bp in length, and is composed of the 1,092 bp α/β hydrolase-encoding gene (*lipBT*), and the 1,035 bp of the lipase foldase-encoding gene (*lifBT*) (Fig. 1). Both genes were regulated by the T7 constitutive promoter from pGEM-T Easy. Both of *lipBT*

Fig. 3. Predicted protein structure of LipBT.

(A) 3D structure of LipBT. α -helix and β -sheet structure are marked in purple and yellow respectively; (B) predicted catalytic triad (Ser-Asp-His) of LipBT. Ser₁₃₁ (ER 131:CA), Asp₃₀₈ (SP 308:CA), and His₃₃₀ (US 330:CA) are marked as purple, green, and red, respectively.

and *lifBT* were closely related to the lipase- and foldase-encoding genes in *B. cepacia* INT3-BP177 (99.0%), *B. cenocepacia* ZYB002 (98.6%), *Burkholderia* sp. SIB005 (98.6%), *B. cepacia* FDAARGOS_388 (98.2%), and *B. cepacia* ATCC25416 (98.2%). The amino acid identity among LipBT and corresponding proteins in *B. cepacia* ATCC25416, *B. contaminans* LTEB11, and *B. cepacia* G63 was 97.0%, 97.0%, and 91.5%, respectively. As for *LifBT*, it showed the same identity percentage.

The protein modeling was constructed, along with the model consistency. The model with a CAD score of 0.865 and LDDT score of 0.732 (which indicates the right folding of protein) was chosen for analysis [15]. LipBT consisted of 364 amino acids, including 40 amino acids that were predicted as signal peptide. It carried the conserved pentapeptide G-X1-S-X2-G, as X1 and X2 stands for His and Glu, respectively (Fig. 2). Its catalytic triads were identified as Ser131, Asp308, and His330, located in the coil structure of the protein (Fig. 3). Two cysteine residues, Cys234 and Cys314, were predicted to form disulfide bonding, resulting in formation of a disulfide bridge, as in *Burkholderia* lipases [13]. Amino acid alignment among LipBT and the other *Burkholderia* lipases revealed the presence of 17 non-conserved amino acid residues. As these amino acids cause the difference in the length and formation of secondary structures, there is no evidence that these could determine the characteristics of the lipase activity.

Co-Expression and Partial Purification of LipBT

LipBT and *LifBT* were co-expressed as an operon in both

Table 1. Lipase activity of LipBT expressed in various vectors and *Escherichia coli* hosts.

Vectors	Hosts	Lipase activity (U/mg)
pGEM-T Easy	DH5 α	0.08 $\pm$ 0.01
pET-15b	DH10 β	0.11 $\pm$ 0.02
	BL21 (DE3) pLysS	6.73 $\pm$ 0.24
	Origami B	0.41 $\pm$ 0.05
	SHuffle B	0.19 $\pm$ 0.02
	SHuffle K	0.16 $\pm$ 0.01

The hydrolytic activity was measured at 40°C, pH 8.0 for 5 min.

pGEM-T Easy and pET-15b. *E. coli* DH5 α was used as host for pGEM-T Easy recombinant vector, while *E. coli* DH10 β , BL21 (DE3) pLysS, Origami B, SHuffle B, and SHuffle K were used as host for recombinant pET-15b. Lipase activity was measured from cell-free extracts of these host and vector combinations. *E. coli* BL21(DE3) pLysS expressing pET-15b+LipBT+LifBT showed the highest lipase activity (6.73 U/mg) (Table 1). Both SHuffle B and SHuffle K have the similar activity, while Origami B is slightly higher. In comparison, lipase activities derived from both the pGEM-T Easy and pET-15b background were similar. Our data indicate that expression host selection has more effect on increasing lipase activity than vector selection. In this case, *E. coli* BL21 (DE3) pLysS is the best expression host. Lysozyme S, encoded in the pLysS plasmid, can decrease basal protein expression, thus leading to higher activity of lipase [20]. Thus, lipase produced in the *E. coli* BL21 (DE3) pLysS and pET-15b background was used for further purification and characterization.

LipBT was partially purified using ammonium sulfate precipitation. The process consisted of four steps and resulted in five fractions of purified lipase (<20%, 20–30%, 30–40%, 40–50%, and >50%). Fraction 20–30% showed the highest specific activity (15.88 U/mg), while fraction <20%

Table 2. Specific activity of ammonium sulfate precipitation fractions for LipBT purification.

Fraction	Lipase activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)
<20%	0.19	0.20	0.85
20–30%	16.45	1.04	15.88
30–40%	12.51	1.17	10.66
40–50%	8.31	1.48	5.61
>50%	0.08	0.31	0.26

The hydrolytic activity was measured at 40°C, pH 8.0 for 5 min.

Fig. 4. SDS-PAGE and zymography of LipBT.

Lane M: low-range ladder; lane 1: pET-15b(-); lane 2: purified LipBT (20–30% fraction); lane 3: zymogram of purified LipBT.

and >50% showed the lowest activity (Table 2). SDS-PAGE results showed that the molecular mass of LipBT was around 30 kDa (Fig. 4).

Characterization of LipBT

LipBT was characterized based on spectrophotometry and analyzed as relative or residual activity [21]. The optimal temperature of LipBT was 80°C (Fig. 5), in contrast to lipases from other *Burkholderia* spp. that showed optimal activity at temperatures around 40–70°C [4, 5, 22, 23]. LipBT was thermally stable at 10–40°C, in which high residual activity of the enzyme was observed following 30 min incubation at said temperature (>80%). Similarly, other *Burkholderia* spp. lipases showed low stability at higher temperature (>50°C). It was suggested that the thermal stability of this enzyme is hypothesized to be mainly determined by the formation of a disulfide bond among its cysteine residues [24].

LipBT was most optimal at pH 11.0, and it was stable at pH 5–11 (Fig. 6). Most *Burkholderia* lipases are considered as alkaline enzymes, since they are highly active and stable in an alkaline environment (pH>8.0) [25]. For example, *Burkholderia cepacia* ATCC 25416 and *Burkholderia contaminans* LTEB11 lipases showed optimum activity at pH 11.0 and 8.0, respectively [4, 5]. To date, there has not yet been any report on lipases that work in acidic conditions from *Burkholderia* spp. As an alkaline enzyme, LipBT may potentially be used in biodiesel production and as a detergent additive [26].

Fig. 5. Effect of temperature on the activity and stability of LipBT.

(A) Optimum temperature of LipBT at pH 8.0. The 100% relative activity represented an enzyme activity of 19.35 U/mg; (B) Thermostability of LipBT after incubation for 30 min at pH 8.0 in various temperatures. The thermostability was measured at 80°C, pH 8.0. The 100% relative activity represented an enzyme activity of 18.22 U/mg.

Various *p*-nitrophenyl compounds with various ester chain lengths (C_4 - C_{18}) were employed to assess the substrate specificity of LipBT (Table 3). LipBT shows the highest activity for C_{10} and the lowest activity for C_4 . The same characteristic was reported in other *Burkholderia* lipases, in which they showed preferences for substrates containing long ester chains [4]. To our knowledge, there has not yet been any report for *Burkholderia* lipase with optimum activity against substrates with short ester chains. The structure and motifs on oxyanion holes have played a crucial role in determining substrate preferences [27].

LipBT was highly stable in the presence of alcohol solvents, such as methanol, ethanol, isopropanol, butanol, and acetonitrile (Table 4). In the presence of alkaline

Fig. 6. Effect of pH on the activity and stability of LipBT.

(A) Optimum pH of LipBT at 80°C. The 100% relative activity represented an enzyme activity of 22.41 U/mg; (B) The pH stability of LipBT after incubation for 30 min at 40°C in various pH. The pH stability was measured at 80°C, pH 11. The 100% residual activity represented an enzyme activity of 20.67 U/mg.

solvents, LipBT was highly stable and slightly activated, and its residual activity was >100. Most *Burkholderia* lipases are highly stable in methanol, which makes them potential catalyzers for transesterification in biodiesel production [2].

The effect of metal ions on LipBT activity was examined

Table 3. Substrate specificity of LipBT at 80°C, pH 11.0.

<i>p</i> -Nitrophenyl substrate	Relative activity (%)
C_4	28.12
C_8	77.57
C_{10}	100.00
C_{12}	92.96
C_{14}	79.15
C_{16}	86.75
C_{18}	54.81

The 100% relative activity represented an enzyme activity of 21.59 U/mg.

Table 4. Effect of organic solvents on LipBT stability after incubation for 30 min at 40°C, pH 8.0.

Solvent	Residual activity (%)
Control	100.0
Methanol	94.9
Ethanol	82.8
Butanol	78.0
Isopropanol	77.8
Acetonitrile	85.0
n-Hexane	120.1

Enzymatic activity was measured at 80°C, pH 11.0. The 100% residual activity represented an enzyme activity of 20.59 U/mg.

by incubating purified enzyme in various ions. Ca^{2+} , Mg^{2+} , and Mn^{2+} caused slight activation on LipBT, while Zn^{2+} and Fe^{2+} showed strong inhibitory effect on the enzyme (Table 5). It was reported that Ca^{2+} increased the thermal stability of lipase in *Chromohalobacter japonicas* BK-AB18 [28]. As Ca^{2+} helps in the formation of disulfide bond, it is possible to increase the thermal stability of LipBT with the addition of this ion [29].

LipBT showed high stability in the presence of non-ionic surfactants (DMSO, Triton X-100, and Tween-80) (Table 6). SDS, an ionic surfactant, caused strong inhibitory effect on LipBT, in which the enzyme only retained 29% residual activity after 30 min of incubation in the presence of SDS. It is important to determine lipase stability towards various surfactants to assess whether or not the enzyme may be compatibly used as an additive in detergents [30].

In conclusion, we isolated, cloned, and co-expressed genes that encode for family I.2 lipase and its specific foldase from *Burkholderia territorii* GP3, a bacterium isolated from Mount Papandayan soil. The amino acid sequences of the lipase LipBT were closely related to those

Table 5. Effect of metal ions on LipBT activity after incubation for 30 min at 40°C, pH 8.0.

Metal ion	Relative activity (%)
Control	100.0
Ca^{2+}	120.6
Fe^{2+}	25.3
Mg^{2+}	114.9
Zn^{2+}	62.5
Mn^{2+}	103.7
Cu^{2+}	95.9

Enzymatic activity was measured at 80°C, pH 11.0. The 100% relative activity represented an enzyme activity of 20.48 U/mg.

Table 6. Effect of surfactants on LipBT activity after incubation for 30 min at 40°C, pH 8.0.

Surfactant	Relative activity (%)
Control	100.0
Tween-80	103.8
Triton X-100	111.9
DMSO	100.3
SDS	29.2

Enzymatic activity was measured at 80°C, pH 11.0. The 100% relative activity represented an enzyme activity of 19.88 U/mg.

of *B. contaminans* LTEB and *B. cepacia* ATCC 25416 with 97.0% similarity. LipBT was unique compared to the other *Burkholderia* lipases, as it showed optimum activity at 80°C and pH 11.0. In general, LipBT may potentially be used as a catalyst in biodiesel and detergent formulation.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- Hasan F, Shah AA, Hamed A. 2006. Industrial application of microbial lipases. *Enzyme. Microbiol. Technol.* **39**: 235-251.
- Yang J, Guo D, Yan Y. 2007. Cloning, expression and characterization of a novel thermal stable and short-chain alcohol tolerant lipase from *Burkholderia cepacia* strain G63. *J. Mol. Catal. B.* **45**: 91-96.
- Sebastian J, Muraleedharan C, Santhiagu A. 2016. A comparative study between chemical and enzymatic transesterification of high free fatty acid contained rubber seed oil for biodiesel production. *Cog. Eng.* **3**: 1-12.
- Wang X, Yu X, Xu Y. 2009. Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from *Burkholderia cepacia* ATCC 25416. *Enzyme. Microb. Technol.* **45**: 94-102.
- Alnoch RC, Stefanello AA, Martini VP, Richter JL, Mateo C, de Souza EM, et al. 2018. Co-expression, purification and characterization of the lipase and foldase of *Burkholderia contaminans* LTEB11. *Int. J. Biol. Macromol.* **116**: 1222-1231.
- Arpigny JE, Jaeger KE. 1999. Bacterial lipolytic enzymes: classification and properties. *J. Biochem.* **343**(1): 177-183.
- Quyen DT, Schmidt-Dannert C, Schmid RD. 1999. High-level formation of active *Pseudomonas cepacia* lipase after heterologous expression of the encoding gene and its modified foldase in *Escherichia coli* and rapid in vitro refolding. *Appl. Environ. Microbiol.* **65**: 787-794.

8. Martini VP, Glogauer A, Muller-Santos M, Iulek J, de Souza EM, Mitchell DA, *et al.* 2014. First co-expression of a lipase and its specific foldase obtained by metagenomics. *Microb. Cell Fact.* **13**: 171.
9. Marchesi JR, *et al.* 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* **64**: 795-799.
10. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, *et al.* 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647-1649.
11. Humphrey W, Dalke A, Schulten K. 1996. VMD: Visual Molecular Dynamics. *J. Mol. Graph.* **14**: 33-38.
12. Sambrook J, Green RM. 2012. Molecular cloning: A Laboratory Manual 4th Edition. New York(US): Cold Spring Harbor.
13. Ceroni A, Passerini A, Vullo A, and Frasconi P. 2006. DISULFIND: a Disulfide Bonding State and Cysteine Connectivity Prediction Server. *Nucleic Acids Res.* **34**: 177-181.
14. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, *et al.* 2018. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* **46**: 296-303.
15. Uziela K, Hurtado DM, Shu N, Wallner B, Elofsson A. 2017. ProQ3D: Improved model quality assessments using Deep Learning. *Bioinformatics* **33**: 1578-1580.
16. Kim YO, Khosasih V, Nam BH, Lee SJ, Suwanto A, Kim HK. 2012. Gene cloning and catalytic characterization of cold adapted lipase of *Photobacterium* sp. MA1-3 isolated from blood clam. *J. Biosci. Bioeng.* **114**: 589-595.
17. Hamaki T, Suzuki M, Fudou R, Jojima Y, Kajiura T, Tabuchi A, *et al.* 2005. Isolation of novel bacteria and actinomycetes using soil-extract agar medium. *J. Biosci. Bioeng.* **99**: 485-492.
18. Basu S, Bose C, Ojha N, Das N, Das J, Pal M, Khurana S. 2015. Evolution of bacterial and fungal growth media. *Bioinformation* **11**: 182-184.
19. De Smet B, Mayo M, Peeters C, Zlosnik JE, Spilker T, Hird TJ, *et al.* 2015. *Burkholderia stagnalis* sp. nov. And *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. *Int. J. Syst. Evol. Microbiol.* **65**: 2265-71.
20. Bane SE, Velasquez JE, Robinson AS. 2006. Expression and purification of milligram levels of inactive G-protein coupled receptors in *E. coli*. *Protein Expr. Purif.* **52**: 348-355.
21. Arifin, Ranlym A, Kim SJ, Yim JH, Suwanto A, Kim HK. 2013. Isolation and biochemical characterization of *Bacillus pumilus* lipases from the Antarctic. *J. Microbiol. Biotechnol.* **23**: 661-667.
22. Takeda Y, Aono R, Doukyu N. 2006. Purification, characterization, and molecular cloning of organic-solvent-tolerant cholesterol esterase from cyclohexanetolerant *Burkholderia cepacia* strain ST-200. *Extremophiles* **10**: 269-277.
23. Gupta R, Gupta N, Rath P. 2004. Bacterial lipases: an overview of production, purification, and biochemical properties. *Appl. Microbiol. Biotechnol.* **64**: 763-781.
24. Liu T, Wang Y, Luo X, Li J, Reed SA, Xiao H, *et al.* 2016. Enhancing protein stability with extended disulfide bonds. *Proc. Natl. Acad. Sci. USA* **113**: 5910-5915.
25. Svendsen A, Borch K, Barfoed M, Nielsen TB, Gormsen E, Patkar SA. 1995. Biochemical properties of cloned lipases from the *Pseudomonas* family. *Biochim. Biophys. Acta* **1259**: 9-17.
26. Seitz EW. 1974. Industrial applications of microbial lipases—a review. *J. Am. Oil. Chem. Soc.* **51**: 12-16.
27. Casas-Godoy L, Duquesne S, Bordes F, Sandoval G, Marty A. 2012. *Lipases and Phospholipases: Methods and Protocols*, pp. 4-11. Springer Science Business Media, New York.
28. Hertadi R, Widhyastuti H. 2015. Effect of Ca²⁺ to the activity and stability of lipase isolated from *Chromohalobacter japonicas* BK-AB18. *Procedia Chem.* **16**: 306-313.
29. Liebeton K, Zacharias A, Jaeger KE. 2001. Disulfide bound in *Pseudomonas aeruginosa* lipase stabilizes the structure but is not required for interaction with its foldase. *J. Bacteriol.* **183**: 597-603.
30. Cherif S, Mnif S, Hadrich F, Abdelkafi S, Sayadi S. 2011. A new highly alkaline lipase: an ideal choice for application in detergent formulations. *Lipids Health Dis.* **10**: 221.