International Journal of Biological Macromolecules 92 (2016) 96–104

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Lipase of *Bacillus stratosphericus* L1: Cloning, expression and characterization

Alisa Gricajeva[a,*], Vida Bendikienė[b], Lilija Kalėdienė[a]

[a] Department of Microbiology and Biotechnology, Faculty of Natural Science, Vilnius University, 21/27, M. K. Čiurlionio Street, LT-03101, Vilnius, Lithuania
[b] Department of Biochemistry and Molecular Biology, Faculty of Natural Science, Vilnius University, 21M.K. Čiurlionio Street, LT-03101, Vilnius, Lithuania

ARTICLE INFO

Article history:
Received 28 December 2015
Received in revised form 3 July 2016
Accepted 4 July 2016
Available online 5 July 2016

Keywords:
Bacillus stratosphericus
Lipase
Expression
Purification
Kinetics

ABSTRACT

Present work was undertaken to discover new lipolytic enzymes as well as novel bacterial strains for applications in biotechnology. One of the isolated strains identified as *Bacillus stratosphericus* L1 produced extracellular lipase (LipBST) which was cloned and expressed in *Escherichia coli*. Purified mature enzyme had a molecular mass of 19 kDa. Recombinant protein showed an activity of 6244.5 U/mg at pH 9, 35 °C. It was stable in the range of 35–55 °C and retained more than 60% activity after incubation for 4 h. LipBST was activated by organic solvents such as acetone and n-hexane. Lipase was inactivated by all investigated metal ions, inhibitors and detergents. LipBST was determined to be short-chain specific, but also hydrolyzed medium-, long-chain *p*-nitrophenyl and natural fatty substrates. The values of V_{max} and K_M for *p*-nitrophenyl butyrate, *p*-nitrophenyl caprylate, *p*-nitrophenyl decanoate were 1.1, 2.5, 0.1 mM min^{-1} and 5×10^{-2}, 3.4×10^{-2}, 194×10^{-2} mM, respectively. Biochemical characteristics of LipBST suggest a great potential for various biotechnological applications including detergent formulation, bioremediation and organic synthesis processes.

1. Introduction

Although sufficient amounts of lipases can be distinguished from plants, lately, due to their special biochemical properties, versatile catalytic performance, high yield, light obtaining and simplicity of gene manipulation, a significant advantage was gained by bacterial lipases. Their unique features consist of a wide range of substrate specifity, stability in extreme temperatures, pH, organic solvents, and ability to perform enantio-, chemo- and regioselective transformations [1,2]. Commercially important lipases are usually attained from microorganisms, which secrete them into extracellular medium. Such microbes can be found in different habitats like industrial waste, soil, fat food, etc. [3].

Lipases (EC 3.1.1.3) are one of the most stable enzymes which fall into the class of serine hydrolases and naturally catalyze hydrolysis of triacylglycerols [4]. Industrial reactions catalyzed by lipases occur under mild conditions with high degree of specifity reducing the production of undesirable products. Large industrial potential is attributed to lipases, which catalyze not only hydrolysis but also esterification, inter- and transesterification (acidolysis, alcoholysis) reactions in anhydrous reaction mixtures [5–7]. As a result, bacterial lipases represent a leading group of biocatalysts. Depending on the final product of the particular lipase catalyzed reactions, these enzymes are used in food, detergent, textile, cosmetics, paper, leather tanning, biofuel, pharmaceutical, agrochemical industries, bioremediation, production of surface active agents (surfactants) and preparing supplements rich in valuable fatty acids. Lipases can be as well used as biosensors [8–10].

In recent times, enzymes which have higher activities at low and moderate temperatures gained a significant interest as energy savers in different biotechnology fields such as production of fine chemicals, food, detergent and pharmaceutical industries [11]. In order to discover new possibilities of application of that kind of lipases in different temperature-sensitive processes and improve their use in existing fields, there is a need to expand the searches of these "technological tools", uncovering their new biochemical properties, molecular structure and bioactivities. In present report, highly promising lipolytic bacterium, identified as *B. stratosphericus* L1 was subjected for purification and analysis of its lipase which was active in a wide range of temperatures and pH.

Bacillus lipases belong to I.4 and I.5 subfamilies of true lipases [12] and are known to have a low homology of their molecular structure and weight (approximately 19 kDa) with other enzymes of the family. Conservative Gly-X-Ser-X-Gly (X – any amino acid) pentapeptide situated around the active site of the most of lipases is

* Corresponding author.
E-mail addresses: alisa.gricajeva@gmail.com, alisa.gricajeva@gf.vu.lt (A. Gricajeva).

http://dx.doi.org/10.1016/j.ijbiomac.2016.07.015
0141-8130/

Ala-X-Ser-X-Gly for the *Bacillus* lipases. Hydrophobic molecular lid domain is characteristic for the vast majority of know lipases and its "open form" is crucial for the enzyme activity. For this reason enzyme needs to undergo conformational changes which occur in the presence of interface, such as oil drops, and are called interfacial activation: the lid is dislocated and the enzyme shows its opened and active form. There is a considerable interest to achieve different lipases in their "open form" for the attainment of their higher activity, whereas *Bacillus* lipases are always in their active state and there is no need of interfacial activation since in their structure there is no lid domain covering their active site [5,13,14]. Therefore, due to extraordinary structural and biochemical features (activity in a wide pH range, stability in organic solvents, thermal stability) [2], *Bacillus* lipases are attractive in order to investigate them and adapt for a number of industrial processes.

In the present report, physical and chemical properties of the target lipase belonging to *Bacillus* subfamily were determined and possibilities of its usage in industrial biotechnology were discussed. For the achievement of higher yields of *B. stratosphericus* L1 lipase, enzyme was cloned, expressed in *E. coli* BL21 (DE3) and in two steps purified from the extracellular medium. Recombinant lipase (LipBST) of *B. stratosphericus* L1 was not only active and stable in a wide range of temperatures (10–65 °C) and pH, it also exhibited stability and was activated in the presence of different hydrophobic and hydrophilic organic solvents. Effect of different metal ions, detergents inhibitors and kinetic parameters were studied as well. Using TLC method, ability to hydrolyze not only synthetic *p*-nitrophenyl (*p*-NP) substrates but also natural oils and fats was evaluated.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Lipolytic bacteria were isolated from high-fat foods. Lipase production was screened on rhodamine B agar (RBA) plates with olive oil as a sole carbon source (pH 7). RBA plates were spot inoculated with a pure culture of each isolate and incubated at 30 °C and 60 °C for 2 days. Lipase producing bacteria were monitored by irradiating plates with UV light [15].

Most active bacteria were grown in 0.05% peptone and 0.03% yeast extract liquid medium (pH 7.5) for the obtainment of constitutively secreted lipases. Wild strain was grown at 30 °C temperature with 180 rpm shaking.

For gene cloning and expression *E. coli* DH5α and BL21 (DE3) (Novagen) competent cells were used. *E. coli* DH5α harboring pTZ57R/T plasmid constructs (Thermo Fisher Scientific) were selected on Luria-Bertani (LB) medium (tryptone 1%, yeast extract 0.5%, NaCl 1%) supplemented with 100 mg/mL ampicillin (CALBIOCHEM®), 20 mg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal), 100 mM isopropyl-β-D-thiogalactopyronoside (IPTG) (Thermo Fisher Scientific) and 1% tributyrin (CALBIOCHEM®). *E. coli* DH5α or BL21 (DE3) harboring pET26(+)b plasmid constructs (Novagen) were selected on LB medium supplemented with 30 mg/mL kanamycin sulphate and 1% tributyrin. Recombinant cells were grown in 37 °C with 180 rpm shaking. LB and M9 (0.2% NH_4Cl, 0.6% KH_2PO_4, 1.2% $Na_2HPO_4 \cdot 7H_2O$, 2% glucose, 0.01% $MgSO_4$, 0.05% NaCl) supplemented with 30 mg/mL kanamycin were used for determination of optimal medium for the expression of recombinant lipase. Electrocompetent *E. coli* DH5α and BL21 (DE3) cells were prepared according to Sambrook and Russel [16].

2.2. Lipolytic activity assay

Bacterial isolates showing positive reaction on RBA plates were quantitatively tested for the lipase activity. Enzyme activity was estimated by spectrophotometric assay using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. 1 mL of 10 mM *p*-NPP in 2-propanol was infused with intensive mixing into 19 mL of 50 mM Briton-Robinson buffer, 0.1% gum arabic and 0.2% sodium deoxycholate (pH 8) solution [17].

Enzymatic reaction mixture was made of 900 μL substrate and 100 μL enzyme solutions. Enzyme concentration in assay mixture was kept at 100 nM. Substrate mixture was incubated at 30 °C for 10 min and the enzyme solution for 5 min. After incubation enzyme solution was added to substrate mixture, the absorption was measured at 410 nm at the moment of the beginning of the reaction (A_1) and after 5 min of incubation (A_2). General lipolytic activities were calculated using formula:

$$U = \frac{\Delta A \cdot Vt}{\varepsilon \cdot d \cdot Ve \cdot \Delta t}$$

$\Delta A - A_2$ (experimental) $- A_1$ (at the moment of the beginning of the enzymatic reaction);

V_t – total volume of the reaction mixture, mL;

ε – molar extinction of *p*-nitrophenyl at 410 nm wave light, 0.001477 mol l^{-1} mm^{-1};

d – width of the optical path, mm;

V_e – volume of the enzyme solution in the reaction mixture, mL;

Δt – time of the reaction, min.

Lipolytic activity unit (U) was defined as the amount of the enzyme which liberates 1 micromole of fatty acids per min under strict assay conditions. Lipase specific activity was expressed in units of activity per 1 mg of protein (U/mg).

In subsequent work the most active bacterial strain was used as a donor of lipase genes.

2.3. Identification and phylogenetic analysis of bacterial isolate

Genomic DNA was extracted with GeneJET Genomic DNA Purification Kit, according to the manufacturer (Thermo Fisher Scientific). 16 S rDNA was amplified by PCR using 27 F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1495 R (5′-CTA CGG CTA CCT TGT TAC GA-3′) universal primers (METABION). PCR conditions were used according to Kuisienė et al. [18]. Purified PCR products were sequenced at Vilnius University Institute of Biotechnology (Lithuania). Basic Local Alignment Search Tool (BLAST) [19] was used for database homology searches. Clustal W algorithm [20] was used for multiple sequence alignment. Evolutionary relatedness was analyzed with MEGA version 5.0 applying the Neighbor-Joining method with the bootstrap test (1000 replicates) [21].

2.4. Amplification and cloning of the lipase genes

Series of primers were designed based on conserved regions from reported lipases belonging to the *Bacillus* genus by aligning sequences submitted to National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Constructed primers were synthesized at METABION (Germany). Determination of the lipase genes was performed by PCR with the primer pairs shown in Table 1. PCR conditions were used as for the amplification of 16 S rDNA; optimal annealing temperatures and concentrations of primers were adjusted empirically. Amplified products were cloned into pTZ57R/T plasmid and transformed by electroporation into *E. coli* DH5α cells. Positive clones were selected by blue/white screening on LB plates supplemented with tributyrin. For further confirmation and analysis of cloned DNA, it was sequenced, aligned using BLAST. SignalP4 internet server tool (http://www.cbs.dtu.dk/

Table 1
Primer pairs constructed for lipase gene cloning.

Primer name	Primer sequence
F: lipBF	5'-ATGAAAGTGATGTTTG-3′
R: lipBR	5'-TTAATTCGTATTCTGTCC –3′
F: lipBPF	5'-ATGCTCTAACTATCCTCTGCA-3′
R: lipBPR	5'-TTAATATGGAATATCTCCATG-3′
F: lipBSF	5'-ATGAGCGAAAACATG-3′
R: lipBSR	5'-CTCGAGCCTGCTTTTTTCAC-3′
F: lipBSTF	5'-ATGAAAGTGATTCG-3′
R: lipBSTR	5'-ATTCGTATTCTGTCC-3′
F: lipBSIF	5'-ATGAGAGAAGGGCATTG-3′
R: lipBSIR	5'-TTACAACCGTAACCATCTG-3′
F: lipBST1F	5'-**CATATG**AAAGTGATTCG-3′
R: lipBST1R	5'**CTCGAG**ATTCGTATTCTGTCC-3′

Restriction targets are indicated in bold.

services/SignalP/) was used for the prediction of the putative signal peptide and its cleavage site.

2.5. Construction of the expression vector

Primers with restriction targets (Nde*I* in C- terminal and Xho*I* in N- terminal) were synthesized for the construction of the expression vector. After PCR, obtained target DNA fragments with restriction sites were cloned into the pTZ57R/T plasmid and subcloned into pET26(+)b expression vector. The latter construct was transformed into the *E. coli* BL21 (DE3) cells. Recombinant cells were screened on LB plates supplemented with 30 mg/mL kanamycin. The plasmid was subsequently sequenced for the confirmation of the appropriate insertion of the lipase gene into the vector.

2.6. Expression of the target gene

E. coli BL21 (DE3) harboring pET26(+)b plasmid with the insert were grown in LB medium supplemented with kanamycin until the OD_{600} reached 0.4, IPTG was added to a final 0.4 mM concentration. Every hour after induction (1–8 h), 1 mL of induced and uninduced cell samples were collected with their OD_{600} values adjusted to be equal 0.4. Cell debris was suspended in 100 μL of 4 x protein loading buffer (40% glycerol, 240 mM TRIS-HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue) for protein detection by SDS-PAGE [22]. Activity of the target protein in the other cell fractions (periplasmic, soluble and insoluble cytoplasmic) and optimization of induction conditions were performed as well.

2.7. Purification of recombinant lipase

Recombinant lipase was purified in two steps. Proteins were precipitated from *E. coli* BL21 (DE3) cell-free supernatant with ammonium sulphate at 60% saturation, dialyzed against 50 mM Briton-Robinson buffer (pH 8) and purified by immobilized metal

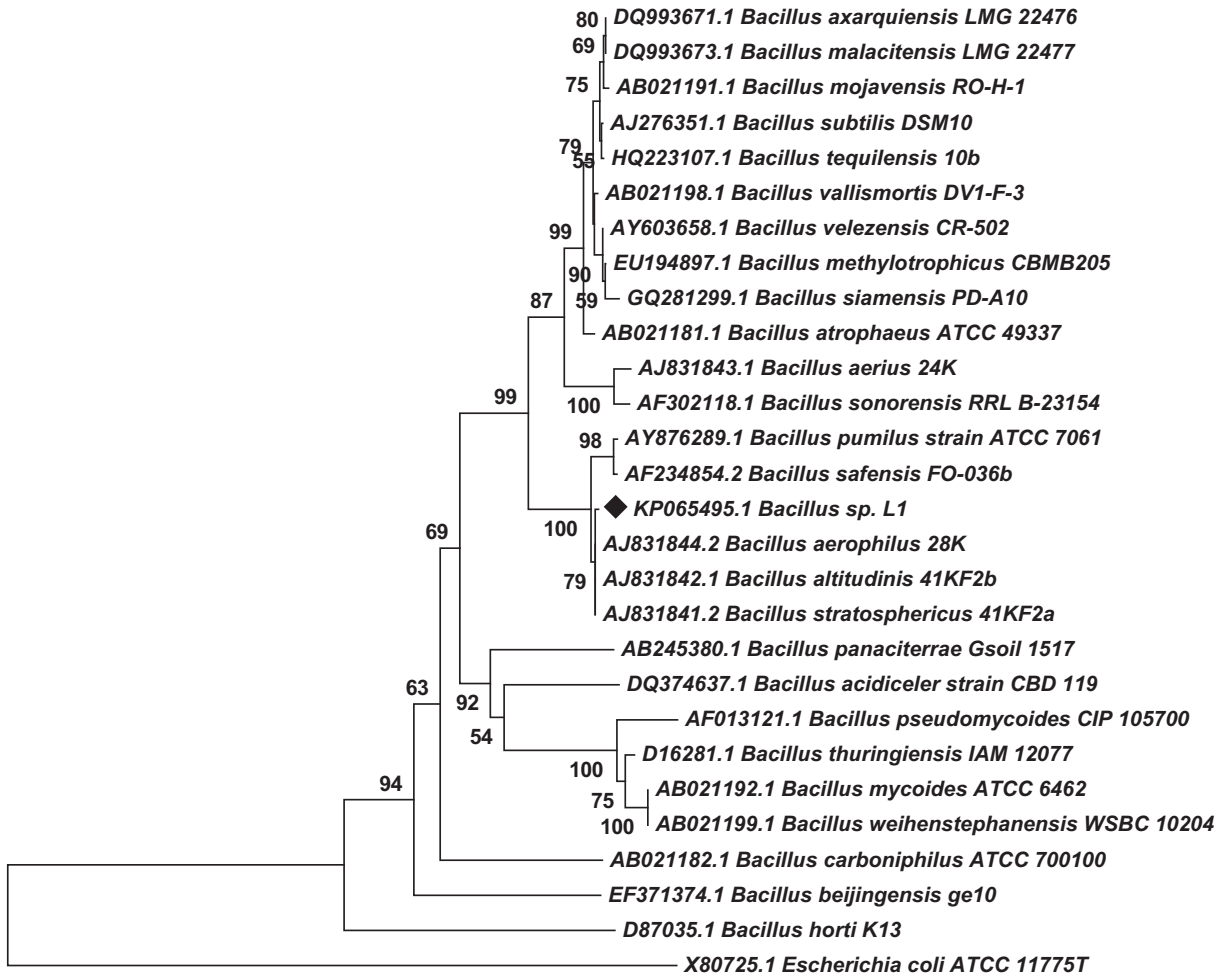

Fig. 1. Phylogenetic tree constructed on the basis of 16 S rRNA sequences. At the branch points bootstrap values are given. Each code written in the front of the species name indicates its accession number in the GenBank. Phylogenetic tree was reduced according to far phylogenetic relativeness of some *Bacillus* species type strains which did not affect the phylogenetic affinity of the studied strain.

affinity chromatography (IMAC). IMAC was performed according to manufacturer (Bio-Rad). Optimal concentration of imidazole for protein elution was determined experimentally by changing it from 250 mM to 500 mM. Elution fractions with the highest protein concentrations were dialyzed, analyzed by SDS-PAGE and zymography. Protein concentration was measured using Bradford method with BSA (bovine serum albumin) as a standard [23].

2.8. SDS-PAGE

For SDS-PAGE 5% concentrating and 12% stacking gels were used [24]. Resolved proteins were visualized by PageBlue Protein Staining Solution (Thermo Fisher Scientific) following standard procedures. Zymogram gel was washed three times for 15 min with renaturation buffer (50 mM Briton-Robinson buffer with 1% v/v triton X-100, pH 8.0) and three times for 10 min with 50 mM Briton-Robinson buffer, pH 8.0. Zymogram gel was transferred to sterile Petri dish and poured with 50 mM Briton-Robinson buffer with 1% v/v emulsified tributyrin and 1% w/v agar. Petri dishes with solidified medium were incubated at 35 °C temperature for 12–24 h until transparent area appeared.

2.9. Biochemical characteristics of the recombinant lipase

Temperature optima of recombinant lipase was determined by changing enzymatic reaction temperature in the range of 5–75 °C in 50 mM Briton-Robinson buffer, pH 8. Optimal pH was determined in Briton-Robinson buffer solutions with pH values ranging from 3 to 12. Thermal stability was measured by incubating the enzyme in different temperatures (25–65 °C) for 4 h. The effects of various metal ions (K^+, Li^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}; 5 mM), some inhibitors (EDTA, β-mercaptoethanol; 5 mM), detergents (Tween 40, Tween 60, Tween 80, Triton X-100, urea, SDS; 1%) and organic solvents (2-propanol, ethanol, methanol, acetone, n-hexane, DMSO; 50% v/v) on recombinant lipase activity were measured by incubating the enzyme with mentioned chemicals for 30 min at 35 °C, pH 9. Lipolytic activity measured in standard reaction conditions (35 °C, pH 9 and with *p*-NPB as a substrate) without chemical compounds added was considered as 100% activity.

Michaelis-Menten constant (K_M) and the maximum velocity of the reaction (V_{max}) were calculated using the linearization methods of Lineweaver-Burk. Turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_M) [25,26] were calculated as well; 20 mM stock solutions of *p*-NPB, *p*-NPC and *p*-NPD substrates which were subsequently diluted in 1 mL of reaction mixture up to 0.5 mM were used for the calculations. Concentration of the enzyme was 100 nM in reaction mixture. Conditions of the reaction were 35 °C, 50 mM Briton-Robinson buffer, pH 9.

Substrate specifity of recombinant lipase

Substrate specifity of the lipase was determined in optimal conditions (35 °C, 50 mM Briton-Robinson buffer, pH 9) with 10 mM *p*-nitrophenyl butyrate (*p*-NPB), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl decanoate (*p*-NPD), *p*-nitrophenyl laurate (*p*-NPL) and *p*-NPP. Substrates with a short-chain of carbon atoms (*p*-NPB and *p*-NPC) were prepared by dissolving them in 2-propanol, *p*-NPD, *p*-NPL, *p*-NPP were emulsified as described in Section 2.2 [27].

2.11. Thin-layer chromatography

Hydrolysis of natural fatty substrates (triolein, canola, flaxseed oils, and lard) by recombinant lipase was studied using thin-layer chromatography (TLC) method. Samples were prepared by mixing 0.4 mL of the lipid substrate with 4 mL of the enzyme solution with further incubation and stirring at 35 °C for 24 h. After incubation, samples were dissolved in hexane and diethyl ether (1:1) and 10 μL of each sample were applied on a TLC silica gel G-25 plates (MACHEREY-NAGEL). Control samples of triolein, canola, flaxseed oils, lard and oleic acid were prepared as the assayed samples without the addition of enzyme. After drying, TLC plates were put into petroleum ether, diethyl ether and acetic acid (80:20:2 [v/v/v]) solvent system for the detection of the hydrolysis products [28]. Chromatogram was visualized in iodine vapor saturated chambers.

2.12. Nucleotide sequence accession numbers

The sequences of 16 S rRNA and recombinant lipase (LipBST) genes are available in the GenBank (NCBI) under accession numbers KP065495.1 and KP331444.1, respectively.

2.13. Statistical analysis

Statistical analysis of the data was performed using Graph Pad Prism 6.0. All data was acknowledged as statistically significant since *P* values were calculated to be less than 0.05.

3. Results

3.1. Identification of the bacterial strain and cloning of its lipase genes

16 S rRNA gene sequence of the lipase producing strain showed highest similarity (an identity of 99%) to 16 S rDNA of *B. altitudinis, B. pumilus, B. stratosphericus* and *B. aerophilus*. Further phylogenetic analysis showed that the studied strain is more closely related to *B. aerophilus, B. altitudinis* and *B. stratosphericus* (Fig. 1).

Primers designed for lipase gene amplification revealed several PCR products of expected size. After cloning them into pTZ57R/T plasmid, selection of positive transformants on LB with tributyrin plates and sequencing, 3 lipase genes were detected giving a proof that the bacterial strain is expressing several lipases. These genes had striking similarity to *B. stratosphericus* triacylglycerol lipase, exported lipase acylhydrolyse and class 3 lipase with their EMI12622, EMI11992, EMI14902 accession numbers in the GenBank, respectively. Thus, although the 16 S rRNA gene sequencing did not give a precise species name, because of detected lipase genes, the lipolytic bacterial strain was assigned to be more closely related to *B. stratosphericus* species and was given name of *B. stratosphericus* L1. The lengths of the determined genes were 648, 816 and 1490 bp. On the basis of the results obtained from the native lipase analysis (data not given) and according to some published data [29,32] the 648 bp gene could code for a cold-adapted lipase. Since one of the objectives of the work was to isolate lipases which could be used in temperature sensitive processes, in further studies 648 bp gene was chosen for cloning, expression and characterization. Further cloning of 648 bp lipase gene, named *LipBST*, was performed using primers with restriction targets shown in Table 1. The presence of the lipase gene was proven by restriction digestion of the vector and insert with *NdeI* and *XhoI* restriction enzymes which liberated a product of the similar (~648 bp) size (data not shown). Full length 648 bp lipase gene which encoded for a protein consisting of 215 amino acids (22.89 kDa) had a putative signal peptide of 34 amino acids with cleavage site between Ala^{34} and Ala^{35}. According to SWISS-MODEL (http://swissmodel.expasy.org/) internet homology-modeling server and BLAST, LipBST had an identity of 79% to the similar crystalized protein – lipase A from *B. subtilis* (1I6W_A) [30]. Since one of the intentions of this work was to achieve expression of the protein into the extracellular medium,

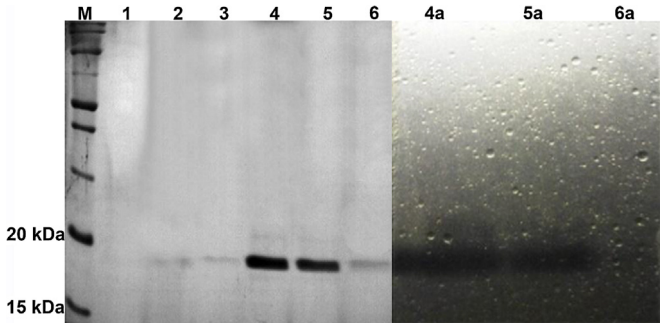

Fig. 2. SDS-PAGE (on the left) and zymogram of purified LipBST. 1, 2, 3–protein profiles achieved after washing of the IMAC column from nonspecifically bound proteins, 4, 5, 6–protein profiles after elution of target protein. In a zymogram visible clear zones of the tributyrin (4a, 5a, 6a) correspond to 4, 5, 6 protein bands on the level of 19 kDa molecular mass marker in SDS-PAGE.

before the subcloning of *LipBST*, pET26(+)b expression vector was modified by cutting off one of its feature sequences – a *pelB* signal sequence which is required for the periplasmic localization of the protein. *E. coli* BL21 (DE3) harboring pET26(+)b with the insert colony PCR with T7F/T7 R primers, restriction analysis with *NdeI* and *XhoI* restriction enzymes gave a product of expected size (data not shown). Sequencing of the latter construct did not reveal any open reading frame shifts.

3.2. Expression and purification

After the induction of recombinant *E. coli* BL21 (DE3) cells and determination of total protein content, expression of LipBST in the recipient cells was detected. In the electrophoresis gel at the level of 19 kDa molecular mass marker a protein band which matched the molecular mass of the mature LipBST was observed. It was determined that recombinant lipase is expressed into the extracellular medium, although, insignificant lipolytic activity was found in other fractions of the recombinant cells. Purification of LipBST was performed using cell-free supernatant.

Optimal medium for LipBST expression was found to be LB, since in this medium higher lipolytic activity than in M9 medium was measured. The optimal optical density of the *E. coli* BL21 (DE3) cells prepared for the induction was determined to be 0.4 in LB medium and 0.8–in M9. Final studied different inductor concentrations (0.1 mM, 0.2 mM, 0.4 mM) did not have significant effect on target protein expression, so the final minimal concentration of IPTG was 0.1 mM.

After precipitation of the recombinant LipBST from the extracellular medium, IMAC was performed with determined optimal 300 mM concentration of imidazole in elution buffer. Achieved fractions were analyzed by SDS-PAGE. In Fig. 2 SDS-PAGE gel and zymogram are shown.

3.3. Biochemical characterization of LipBST

Purified LipBST was determined to operate in a wide range of temperatures with maximum lipase activity reached at 35 °C. Almost the same (insignificantly lower) lipase activity was observed at 30 °C. Recombinant protein was not active at 5 °C, although, after raising the temperature LipBST maintained 37% of activity at 10 °C. More than 50% of maximum activity was observed at 20 °C. Raising temperature above 35 °C, enzyme activity declined and at 65 °C lipase was almost inactivated (Fig. 3A). LipBST retained remarkable thermal stability and showed more than 70% residual activity at all assayed range of temperatures (25–65 °C) for 4 h. At 25–55 °C at the second hour of incubation LipBST had a slight rise of activity (Table 2). At second and third hours of incubation in 35 °C, activation of LipBST was observed with residual activities of 126 ± 1% and 118 ± 6%, respectively.

Table 2
Effect of various temperatures on the stability of LipBST.

Temperature	Residual activity (%) of LipBST in time (h)			
	1	2	3	4
25 °C	93 ± 2	96 ± 1	89 ± 5	83 ± 7
35 °C	97 ± 4	**126 ± 1**	**118 ± 6**	95 ± 2
45 °C	79 ± 2	85 ± 2	78 ± 1	81 ± 2
55 °C	55 ± 1	71 ± 7	70 ± 1	77 ± 7
65 °C	0.36 ± 0.4	0	0	0

Specific activity of purified LipBST of 6244, 5 U/mg was considered as 100%. Effect of increasing activity is indicated in bold.

Table 3
Effect of various metal ions and some inhibitors on the activity of LipBST.

Metal ions/ Inhibitors	Residual activity (%)	Metal ions/ Inhibitors	Residual activity (%)
Li^+	34 ± 6	Cd^{2+}	38 ± 6
K^+	42 ± 6	Ni^{2+}	16.2 ± 0.4
Ca^{2+}	30 ± 7	Cu^{2+}	3.7 ± 0.3
Mg^{2+}	29 ± 6	Zn^{2+}	9 ± 1
Co^{2+}	16 ± 2	Fe^{3+}	5 ± 1
Mn^{2+}	59 ± 2	EDTA	9 ± 1
Ba^{2+}	44 ± 7	β-mercaptoethanol	32 ± 1

Specific activity of purified LipBST of 6244, 5 U/mg was considered as 100%. EDTA – ethylenediaminetetraacetic acid.

Table 4
Effect of various detergents and organic solvents on the activity of LipBST.

Detergent	Residual activity (%)	Organic solvents	Residual activity (%)
Tween 40	14 ± 1	2-propanol	10 ± 2
Tween 60	11 ± 1	Ethanol	8 ± 1
Tween 80	10 ± 1	Methanol	59 ± 6
Triton X-100	14 ± 1	Acetone	**131 ± 2**
Urea	13 ± 4	n-hexane	**116 ± 1**
SDS	70 ± 6	DMSO	34 ± 4

Specific activity of purified LipBST of 6244, 5 U/mg was considered as 100%. Effect of increasing activity is indicated in bold. DMSO – dimethyl sulfoxide.

The purified LipBST activity dependence on hydrogen ion concentration showed that the recombinant enzyme was almost inactive at pH 3, 4 and 5. Starting from pH 6, LipBST activity improved and reached its optimum at the pH 9 (Fig. 3B). Higher than 80% activity LipBST showed at pH 8 and 10.

In order to detect LipBST substrate specifity, previously mentioned *p*-NP substrates were used. As indicated in the histogram (Fig. 3C) LipBST was specific to short-carbon-chain substrates with capability of hydrolyzing medium and long-chain substrates as well. Highest specific lipase activity measured in optimal conditions (35 °C, pH 9) with *p*-NPB substrate was 6244, 5 U/mg.

Effect of various metal ions had an inhibitory effect on the activity of LipBST. The enzyme was considerably unstable toward tested inhibitors (EDTA, β – mercaptoethanol) as well. Only after incubation with Mn^{2+} LipBST retained 59 ± 2% of activity. Results are shown in Table 3.

Studies of LipBST from *B. stratosphericus* L1 toward various detergents revealed that the enzyme is strongly inhibited by all detergents retaining only ~15% of activity. The only exception was the effect of SDS: LipBST retained 70 ± 6% of activity after incubation (30 min, 35 °C) of the enzyme in the presence of the latter chemical. The evaluation of the LipBST stability in different organic solvents was performed as well. It was revealed that LipBST was stable in acetone and n-hexane (131 ± 2% and 116 ± 1%, respec-

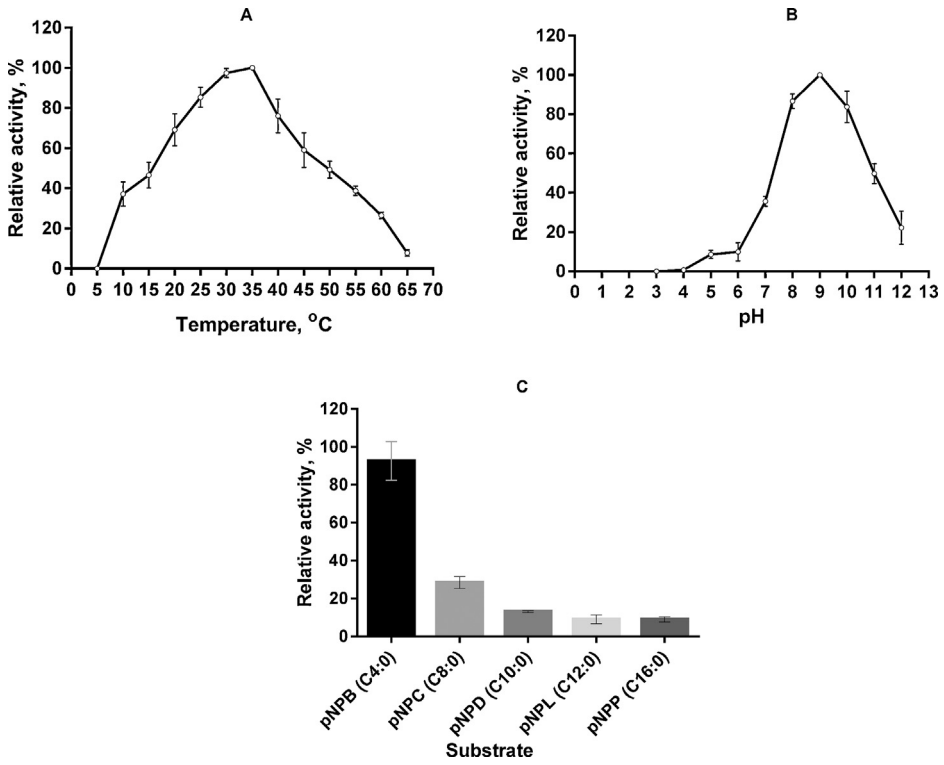

Fig. 3. Effect of temperature (A), pH (B) on the activity of LipBST and (C) its substrate specifity.

Table 5
Kinetic constants of LipBST for different substrates.

Substrate	V_{max} (mM min^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM^{-1} min^{-1})
p-NPB	1.1	5×10^{-2}	4.6×10^3	9.2×10^4
p-NPC	2.5	3.4×10^{-2}	9.9×10^3	30.0×10^4
p-NPD	0.1	194×10^{-2}	0.5×10^3	0.2×10^4

tively). Results obtained on studies of effect of various detergents and organic solvents are summarized in Table 4.

Values of the kinetic constants measured for LipBST are shown in Table 5. The lowest value of K_M (3.4×10^{-2} mM) and the highest values of k_{cat} and k_{cat}/K_M (9.9×10^3 $min^{-1}$and 30.0×10^4 mM^{-1} min^{-1}) were measured for *p*-NPC substrate.

3.4. Thin layer chromatography

In this study LipBST ability to perform the hydrolysis of chosen fatty substances (triolein, canola, flaxseed oils, and lard) was also defined. Thin-layer chromatogram of the hydrolysis products is shown in Fig. 4(A). LipBST was highly active toward canola and flaxseed oils. Gradually located hydrolysis products (diacylglicerols, fatty acids, esters) of the latter are shown in thin-layer chromatogram with the most distinctly visible canola oil hydrolysis products. Lard hydrolysis products appeared blurry in the chromatogram because unsaturated fatty acids that are found in the lard are poorly stained by iodine vapor. Among the lard hydrolysis products monoacylglycerols were also detected. Comparing the size of the test sample esters formed with the control samples which were unaffected by LipBST, quantitative changes were also seen and also proved that the hydrolysis of the lipids occurred. Fig. 4(B) depicts triolein, canola, flaxseed oils, and lard which were not dissolved in hexane and diethyl ether (the water fraction) chromatogram with the reference samples.

4. Discussion

In order to discover new and widen applications of known lipolytic enzymes, bacterial strain exhibiting high lipase activity was screened on RBA plates and selected for detailed investigation: identification, amplification of its lipase genes, their cloning and expression in heterologous *E. coli* BL21 (DE3) system. Purification and characterization of one of the detected low molecular mass lipases was carried out in detail.

Identification of lipolytic bacterium involved its 16 S rDNA and lipase genes analysis. Sequencing and phylogenetic tree construction on the basis of 16 S rDNA showed that the strain in comparison with *Bacillus* ssp. type strains' 16 S rDNA partial sequences is more closely related to *B. stratosphericus* (*B. pumilus* group). However, it is known that bacteria related to *B. pumilus* group cannot be distinguished from species such as *B. safensis*, *B. stratosphericus*, *B. altitudinis* and *B. aerophilus* only by 16 S rDNA [31]. For that reason, lipase genes which were found in the chromosomal DNA of the examined strain allowed to approve and assign it to *B. stratosphericus* species (L1). Although there are numerous reports on bacteria producing lipolytic enzymes, this is the first report describing one of the lipases produced by *B. stratosphericus* species.

Since *B. stratosphericus* L1 is capable of producing at least 3 lipases it is important to know on what factors expression of different lipase genes depends. Eggert et al. reported that *B. subtilis* lipA and lipB expression is differentially regulated on transcriptional level and depends on external pH and amino acids present in the medium [32]. However more recent research is needed in order to provide more information and identify other factors involved in the regulation process.

Expression of the low molecular mass lipase named LipBST was achieved in soluble form and at high levels. It was secreted to the extracellular medium of the heterologous host. Consequently target protein was purified from the supernatant of the cultural fluid. Mature lipase was purified in two steps (precipitation by ammo-

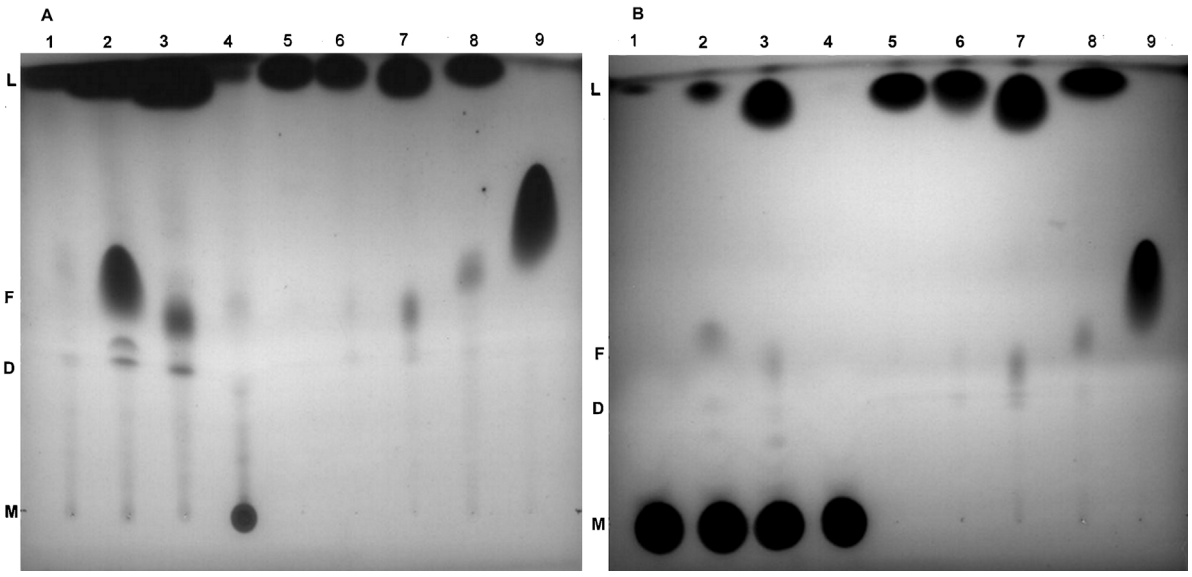

Fig. 4. (A). Recombinant LipBST specifity toward natural lipid substrates: 1–hydrolysis of triolein, 2–canola oil, 3–flaxseed oil, 4–lard; 5, 6, 7, 8–triolein, canola, flaxseed oils and lard hydrolysis control tracks, 9–oleic acid control. (B). Control chromatogram of fatty substrates undissolved in hexane and diethyl ether (aqueous layers of the samples): 1–9 corresponds to the numbers of Fig. 4 (A). L – lipids, F – fatty acids, D – diacylglicerols, M – monoacylglycerols.

nium sulphate and IMAC) and, as it was expected and determined by SDS-PAGE, had a molecular mass of 19 kDa. Such lipases are known to have lowest homology comparing with other bacterial lipases of known structure. They also lack the molecular lid domain and therefore do not need interfacial activation [2].

Temperature and pH are one of the most important factors in mediation enzyme-catalyzed reaction rates. Therefore, one of the primary prerequisites in the study of enzymes' best function is determination of its optimal temperature and pH. In this study, LipBST lipase of *B. stratosphericus* L1 (a mesophile) which had operating optimum at 30–35 °C, was active in a wide range of temperatures (10–65 °C). LipBST was catalytically active at temperatures below 30 °C: it retained 37%-82% of activity in 10–25 °C range of temperatures comparing with maximum activity reached at 30–35 °C. Activity of LipBST at temperatures higher than 35 °C was lower comparing with those just described. The fall of enzyme activity in the temperature range of 40–65 °C was sharper than in temperature range below 30 °C. Hence, LipBST distinguished feature of cold-loving enzyme active in low and moderate temperatures [33–35] as well as stability in higher temperatures (Table 2). Activity of enzymes at lower temperatures is governed by the nature of protein structures and conformations, particularly higher flexibility of the enzyme with reduced number of stabilizing interactions [11]. Contrary, thermophilic enzymes are characterized by a higher stability with a numerous factors enabling it: stabilization by metals, increase in surface charge, presence of many proline residues, salt, disulfide bridges and hydrogen bonds [36]. According to the temperature characteristics, LipBST could be used in an ecofriendly industrial fields where production does not require heat. It could be applied in the temperature sensitive synthesis of organic fine chemicals, for bioremediation and bioconversion processes as well as in various processes where stability in higher temperatures is required.

LipBST was identified as an alkaline lipase as it showed optimal activity at pH 9. Enzyme retained approximately 90% of activity at pH 8 and 10. This may be attributed to ionization of certain amino acids in polypeptide that may favor activity and stability of the enzyme at these pH values. Alkaline lipases are important in detergent industries. LipBST was less than 10% active in the acidic range of pH (pH < 6) and more than 30% active in the neutral pH. In pH range higher than 10 LipBST had ~50% (pH 11) and ~30% (pH 12) of activity. While most *Bacillus* lipases are stable in neutral to moderately alkaline pH, a number of the enzymes have a significantly higher pH optimum. Lipases exhibiting activity in higher than pH 9 values can be sufficient for effective industrial processes. Only few *Bacillus* lipases are known to have pH optimum in acidic range. Such lipases are required in leather industry [36].

Various metal ions can have stimulating as well as suppressing effect on the activity of lipases. Often divalent metal ions like Ca^{2+} are important for the activity of the lipases, especially thermostable, whereas these cations usually stabilize structure of the enzyme and more rarely participate directly in the catalysis [37]. For the *Bacillus* lipases, in general, Ca^{2+} ions are needed for the activation of the enzyme. These ions can have not only positive structural effect; they also form insoluble Ca-salts with fatty acids which are released during hydrolysis, thus, lowering product inhibition. Other metal ions can activate, have no effect or inhibit activity of *Bacillus* lipases [36]. In the present report, all studied metal ions had inhibitory effect on LipBST, especially Cu^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+} ions, suggesting that they alert LipBST conformation negatively. Highest residual activities of $59 \pm 2\%$, $44 \pm 7\%$, $42 \pm 6\%$ LipBST retained after incubation with Mn^{2+}, Ba^{2+} and K^{+} ions. Ca^{2+} inhibited the activity of LipBST to $30 \pm 7\%$.

Interestingly, LipBST was almost completely inhibited with EDTA. This fact intersects with previously discussed results, since none of the tested metal ions activated or helped to maintain stable LipBST activity without its decrease. LipBST activity diminished in the presence of β-mercaptoethanol as well but not as strongly as with EDTA. β – mercaptoethanol is responsible for the disruption of the disulfide bonds which can be present and stabilize the tertiary structure of the enzyme.

Response of the lipases to the presence of surfactants depends on their concentration and can variate. In general, with a few tested *Bacillus* lipases it can be concluded that these enzymes are tolerant to the presence of detergents as their activity is not affected or they are even activated by them. Surfactants increase the water-lipid area, stabilize the open conformation of the enzyme (these factors are not applied for the lipases lacking the lid domain), increase substrate solubility and prevent lipases from aggregation what leads to higher activity [36]. LipBST was strongly inhibited by all tested

surfactants with the only exception of ionic detergent SDS. In the presence of SDS, LipBST retained 70 ± 6% of activity comparing with the control. This may be attributed to the ability of the detergent to prevent enzyme aggregation by weakening hydrophobic interactions and ameliorating substrate accessibility to the enzymes' active center [38]. Negative effect of the surfactants on LipBST was probably due to their ability to block substrate entrance to the active center of the enzyme. Slight LipBST activity decrease in the presence of SDS seems to be unique phenomenon since usually SDS inhibits the enzyme 100% [39,40]. Lipases stable in different detergents can be applied in detergent-making industries as additives.

Enzymatic reactions in organic solvents are an emerging area of research and regarded as an extra valuable for the achievement of various industrial products. Screening of solvent-tolerant bacteria is usually cheaper than modifying known enzymes. Usually thermophilic lipases exhibit tolerance toward different organic solvents but, nevertheless, lipases which are active in low and moderate temperatures are being attempted to isolate and are under demand as well [41]. For that reason, LipBST was subjected for the study of stability in different organic solvents. LipBST was found to be not only stable in both, hydrophobic and hydrophilic organic solvents but also was activated by some of them. LipBST was activated by n-hexane (116 ± 1%) and acetone (131 ± 2%). In the presence of methanol LipBST retained residual activity of 59 ± 6%. In the most of the transesterification reactions for biosynthesis of new esters hexane is the most favorable solvent. Good stability in polar solvents is also related with their usage in manufacturing of flavor esters and production of biodiesel where one of the reactants is usually methanol [36,39]. Hydrophobic solvents are regarded as more superior in the enzyme-solvent interactions, mainly by maintaining the solvation carcass of the protein. Hydrophilic solvents are known to be more destabilizing to lipases as they remove water molecules off the protein surface. This can explain inactivation of LipBST by 2-propanol and ethanol. LipBST activation by acetone is rather rare feature of the lipases. Such enzymes can be used in oleochemistry industry. Recently, lipases were reported to be under demand for ecofriendly catalysis of ascorbyl oleate [42]. Stability of lipases in organic solvents correlates with distribution of surface charged residues and surface property of the enzyme. However, the mechanism of enzyme interactions and adaptation toward different organic solvents is yet poorly understood and needs more research [39].

As the substrate specifity assay showed, LipBST was highly active toward short-chain *p*-NPB substrate. In order to understand catalytic properties and clarify enzyme preferences toward different *p*-NP substrates, kinetics of the LipBST activity in reactions with *p*-NPB ($C_{4:0}$), *p*-NPC ($C_{8:0}$) and *p*-NPD ($C_{10:0}$) were studied. A meaningful set of the maximum velocity (V_{max}) and Michaelis-Menten constants (K_M) for the different substrates were determined [26]. Better substrate for LipBST appeared to be *p*-NPC: concentration at which enzymatic reaction reached its half-maximum rate with this substrate was 1.5 times lower than measured with *p*-NPB. Catalytic efficiency (second-order rate constant) was 3.26 times higher where *p*-NPC was used as a substrate. The lowest affinity LipBST had toward *p*-NPD (Table 5). Such substrate specifity of a LipBST could be explained based on the structural feature of the enzyme – the absence of the molecular lid. The surface of the enzyme more easily accommodates substrates with fatty acid chains not longer than eight carbon atoms. Substrates which have longer carbon atom chains do not fit and stick out of the protein surface and are not efficiently hydrolyzed [30].

Moreover, LipBST hydrolyzed natural fatty substrates (animal fats and oils). Applications of the lipases not only for the obtainment of new esters but also for the hydrolysis of lipids for the release of valuable fatty acids are of a great importance. Fatty acids are used in manufacturing a variety of high value products and obtainment of polyunsaturated fatty acids (PUFAs) which can have beneficial effect on human health. Some valuable fatty acids can be preferably released from different lipids by lipases [10,43,44].

Further work will cover the study of two other lipases found in *B. stratosphericus* L1, their activity modulation, determination of their properties and possibilities of application in biotechnology.

Acknowledgement

This work was supported by a grant "Biocatalysts selection and development for biogas production and utilization of biomass conversion processes control. BIOKONVERSA. Nr. VPI-3.1-ŠMM-10-V.

References

[1] R. Sangeetha, I. Arulpandi, A. Geetha, Bacterial lipases as potential industrial biocatalysts: an overview, Res. J. Microbiol. 6 (2011) 1–24.
[2] R. Gupta, N. Gupta, P. Rathi, Bacterial lipases: an overview of production, purification and biochemical properties, Appl. Microbiol. Biotechnol. 64 (2004) 763–778.
[3] R. Kumar, A. Sharma, A. Kumar, D. Singh, Lipase from *Bacillus pumilus* RK31: production, purification and some properties, World Appl. Sci. J. 16 (2012) 940–948.
[4] A. Ebrahimpour, R.N. Rahman, M. Basri, A.B. Salleh, High level expression and characterization of a novel thermostable, organic solvent tolerant 1,3-regioselective lipase from *Geobacillus* sp. strain ARM, Bioresour. Technol. 102 (2011) 6972–6981.
[5] J.C.J. Quilles, R.R. Brito, J.P. Borges, C.C. Aragon, G. Fernandez-Lorente, D.A. Bocchini-Martins, E. Gomes, R. da Silva, M. Boscolo, J.M. Guisan, Modulation of the activity and selectivity of the immobilized lipases by surfactants and solvents, Biochem. Eng. J. 93 (2015) 275–280.
[6] P.Y. Stergiou, A. Foukis, M. Filippou, M. Koukouritaki, M. Parapouli, L.G. Theodorou, E. Hatziloukas, A. Afendra, A. Pandey, E.M. Papamichael, Advances in lipase-catalyzed esterification reactions, Biotechnol. Adv. 31 (2013) 1846–1859.
[7] B.D. Ribeiro, A. Machado de Castro, M.A. Coelho, D.M. Freire, Production and use of lipases in bioenergy: a review from the feedstocks to biodiesel production, Enzyme Res. 2011 (2011) 1–16.
[8] E. Sirisha, N. Rajasekar, M.L. Narasu, Isolation and optimization of lipase producing bacteria from oil contaminated soils, Adv. Biol. Res. 4 (2010) 249–252.
[9] N. Verma, S. Thakur, A.K. Bhatt, Microbial lipases: industrial applications and properties, Int. Res. J. Biol. Sci. 1 (2012) 88–92.
[10] T.R. Moharana, A.R. Byreddy, M. Puri, C. Barrow, N.M. Rao, Selective enrichment of omega-3 fatty acids in oils by phospholipase A1, PLoS One (2016), http://dx.doi.org/10.1371/journal.pone.0151370 (available online 16 March).
[11] G. Rabbani, E. Ahmad, M.V. Khan, M.T. Ashraf, R. Bhat, R.H. Khan, Impact of structural stability of cold adapted *Candida antarctica* lipase B (CalB): in relation to pH, chemical and thermal denaturation, RSC Adv. 5 (2015) 20115–20131.
[12] A. Messaoudi, H. Belguith, I. Gram, B.J. Hamida, Classification of EC 3.1.1.3 bacterial true lipases using phylogenetic analysis, Afr. J. Biotechnol. 9 (2010) 8243–8246.
[13] J. Mukherjee, M.N. Gupta, Molecular bioimprinting of lipases with surfactants and its functional consequences in low water media, Int. J. Biol. Macromol. 81 (2015) 544–551.
[14] T. Eggert, G. Pencreac'h, I. Douchet, R. Verger, K.J. Jaeger, A novel extracellular esterase from *Bacillus subtilis* and its conversion to monoacylglycerols hydrolase, Eur. J. Biochem. 267 (2000) 6459–6469.
[15] G. Kouker, K.J. Jaeger, Specific and sensitive plate assay for bacterial lipases, Appl. Environ. Microbiol. 1 (1987) 211–213.
[16] J. Sambrook, D.W. Russell, Molecular Cloning: a Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, New York, 2011.
[17] V. Bendikienė, B. Surinėnaitė, B. Juodka, M. Safarikova, Insights into catalytic action mechanism of *Pseudomonas mendocina* 3121-1 lipase, Enzyme Microb. Technol. 34 (2004) 572–577.
[18] N. Kuisienė, J. Raugalas, M. Stuknytė, D. Čitavičius, Identification of the genus *Geobacillus* using genus-specific primers, based on the 16S-23S rRNA gene internal transcribed spacer, FEMS Microbiol. Lett. 277 (2007) 165–172.
[19] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.
[20] J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[21] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (2011) 2731–2739.

[22] R. Gudiukaitė, A. Gegeckas, D. Kazlauskas, D. Čitavičius, Influence of N- and/or C-terminal regions on activity expression, characteristics and structure of lipase from *Geobacillus* sp. 95, Extremophiles 18 (2014) 131–145.
[23] M.M. Bradford, Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[25] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 3 (1934) 658–666.
[26] G. Rabbani, E. Ahmad, N. Zaidi, S. Fatima, R.H. Khan, pH-induced molten globule state of *Rhizopus niveus* lipase is more resistant against thermal and chemical denaturation than its native state, Cell Biochem. Biophys. 62 (2012) 487–499.
[27] M. Sifour, H.M. Saeed, T.I. Zaghloul, M.M. Berekaa, Y.R. Abdel-Fattah, Purification and properties of a lipase from thermophilic *Geobacillus stearothermophilus* strain-5, Int. J. Biol. Chem. 4 (2010) 203–212.
[28] S. Kazlauskas, V. Kiriliauskaitė, L. Kalėdienė, V. Bendikienė, High performance protein-coated microcrystals of *Rhizomucor miehei* lipase: preparation and application for organic synthesis, Appl. Biochem. Biotechnol. 176 (2015) 321–332.
[29] A.R. Wi, S.J. Jeon, S. Kim, H.J. Park, D. Kim, S.J. Han, J.H. Yim, H.W. Kim, *Bacillus* sp. W130-35 isolated from a tidal mud flat, Biotechnol. Lett. 36 (2014) 1295–1302.
[30] G. van Pouderoyen, T. Eggert, K.J. Jaeger, B.W. Dijkstra, The crystal structure of Bacillus subtilis lipase: a minimal α/β hydrolase fold enzyme, J. Mol. Biol. 309 (2001) 215–226.
[31] Y. Liu, Q. Lai, C. Dong, F. Sun, L. Wang, G. Li, Z. Shao, Phylogenetic diversity of the Bacillus pumilus group and the marine ecotype revealed by multilocus Sequence Analysis, PLoS One (2013), http://dx.doi.org/10.1371/journal.pone.0080097 (available online 11 November).
[32] T. Eggert, U. Brockmeier, M.J. Dröge, W.J. Quax, K.E. Jaeger, Extracellular lipases from *Bacillus subtilis*: regulation of gene expression and enzyme activity by amino acid supply and external pH, FEMS Microbiol. Lett. 225 (2003) 319–324.
[33] R. Litantra, S. Lobionda, J.H. Yim, H.K. Kim, Expression and biochemical characterization of cold-adapted lipases from antarctic *Bacillus pumilus* strains, J. Microbiol. Biotechnol. 23 (2013) 1221–1228.
[34] N. Rashid, Y. Shimada, S. Ezaki, H. Atomi, T. Imanaka, Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A, Appl. Environ. Microb. 67 (2001) 4064–4069.
[35] G. Feller, G. Gerday, Psychrophilic enzymes: hot topics in cold adaptation, Nat. Rev. Microbiol. 1 (2003) 200–208.
[36] M. Guncheva, D. Zhyriakova, Catalytic properties and potential applications of *Bacillus* lipases, J. Mol. Catal. B: Enzym. 68 (2011) 1–21.
[37] M. Nagar, S.K. Dwivedi, D. Shrivastava, A review on industrial application in microbial lipases, Int. J. Pharm. Res. Sci. 2 (2013) 631–641.
[38] G. Kaur, A. Singh, R. Sharma, V. Sharma, S. Verma, P. Sharma, Cloning, expression, purification and characterization of lipase from *Bacillus licheniformis*, isolated from hot spring of Himachal Pradesh, India, 3 Biotech. http://link.springer.com/article/10.1007%2Fs13205-016-0369-y (available online 23 September 2015).
[39] N.H. Kamarudin, R.N. Rahman, M.S. Ali, T.C. Leow, M. Basri, A.B. Salleh, A new cold-adapted, organic solvent stable lipase from mesophilic *Staphylococcus epidermidis* AT2, Protein J. 33 (2014) 296–307.
[40] M.A. Salameh, J. Wiegel, Effects of detergents on activity, thermostability and aggregation of two alkalithermophilic lipases from *Thermosintropha lipolytica*, Open Biochem. J. 4 (2010) 22–28.
[41] A. Kumar, K. Dhar, S.S. Kanwar, P.K. Arora, Lipase catalysis in organic solvents: advantages and applications, Biol. Proced. Online (2016), http://dx.doi.org/10.1186/s12575-016-0033-2 (available online 13 January).
[42] M. Stojanović, D. Veličković, A. Dimitrijević, N. Milosavić, Z. Knežević-Jugović, D. Bezbradica, Lipase-catalyzed synthesis of ascorbyl oleate in acetone: optimization of reaction conditions and lipase reusability, J. Oleo Sci. 62 (2013) 591–603.
[43] F. Hasan, A.A. Shah, A. Hameed, Methods for detection and characterization of lipases: a comprehensive review, Biotechnol. Adv. 27 (2009) 782–798.
[44] K. Stránský, M. Zarevúcka, Z. Kejík, Z. Wimmer, M. Macková, K. Demnerová, Substrate specifity, regioselectivity and hydrolytic activity of lipases activated from *Geotrichum* sp, Biochem. Eng. J. 34 (2007) 209–216.