Biocatalysis and Biotransformation

ISSN: 1024-2422 (Print) 1029-2446 (Online) Journal homepage: http://www.tandfonline.com/loi/ibab20

Thermoalkalophilic lipase from an extremely halophilic bacterial strain Bacillus atrophaeus FSHM2: Purification, biochemical characterization and application

Atefeh Ameri, Mojtaba Shakibaie, Mohammad Ali Faramarzi, Alieh Ameri, Sahar Amirpour-Rostami, Hamid Reza Rahimi & Hamid Forootanfar

To cite this article: Atefeh Ameri, Mojtaba Shakibaie, Mohammad Ali Faramarzi, Alieh Ameri, Sahar Amirpour-Rostami, Hamid Reza Rahimi & Hamid Forootanfar (2017): Thermoalkalophilic lipase from an extremely halophilic bacterial strain Bacillus atrophaeus FSHM2: Purification, biochemical characterization and application, Biocatalysis and Biotransformation, DOI: 10.1080/10242422.2017.1308494

To link to this article: http://dx.doi.org/10.1080/10242422.2017.1308494

BIOCATALYSIS AND BIOTRANSFORMATION, 2017
http://dx.doi.org/10.1080/10242422.2017.1308494

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Thermoalkalophilic lipase from an extremely halophilic bacterial strain *Bacillus atrophaeus* FSHM2: Purification, biochemical characterization and application

Atefeh Ameri[a], Mojtaba Shakibaie[a], Mohammad Ali Faramarzi[b], Alieh Ameri[c], Sahar Amirpour-Rostami[a], Hamid Reza Rahimi[d] and Hamid Forootanfar[e]

[a]Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran; [b]Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran; [c]Department of Medicinal Chemistry, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran; [d]Department of Toxicology and Pharmacology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran; [e]Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran

ABSTRACT

The present study was designed to isolate and identify an extremely halophilic lipase-producing bacterial strain, purify and characterize the related enzyme and evaluate its application for ethyl and methyl valerate synthesis. Among four halophilic isolates, the lipolytic ability of one isolate (identified as *Bacillus atrophaeus* FSHM2) was confirmed. The enzyme (designated as *Ba*L) was purified using three sequential steps of ethanol precipitation and dialysis, Q-Sepharose XL anion-exchange chromatography and SP Sepharose cation-exchange chromatography with a final yield of 9.9% and a purification factor of 31.8. The purified *Ba*L (Mw~85 kDa) was most active at 70 °C and pH 9 in the presence of 4 M NaCl and retained 58.7% of its initial activity after 150 min of incubation at 80 °C. The enzyme was inhibited by Cd^{2+} (35.6 ± 1.7%) but activated by Ca^{2+} (132.4 ± 2.2%). Evaluation of *Ba*L's stability in the presence of organic solvents showed that xylene (25%) enhanced the relative activity of the enzyme to 334.2 ± 0.6% after 1 h of incubation. The results of esterification studies using the purified *Ba*L revealed that maximum ethyl valerate (88.5%) and methyl valerate (67.5%) synthesis occurred in the organic solvent medium (xylene) after 48 h of incubation at 50 °C.

ARTICLE HISTORY
Received 7 May 2015
Revised 1 March 2017
Accepted 6 March 2017

KEYWORDS
Lipase; thermostability; *Bacillus atrophaeus*; purification; ethyl valerate

Introduction

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are biotechnologically valuable biocatalysts characterized by the ability to catalyse the hydrolysis of long-chain acylglycerols at the lipid–water interface and liberate diglycerides, monoglycerides, free fatty acids and glycerol (Guncheva and Zhiryakova 2011; Christopher et al. 2015). Besides their natural substrates, these serine hydrolases are also capable to catalyse the transesterification, esterification, acidolysis, aminolysis and alcoholysis of a wide range of acyl donors and nucleophiles (Khoobi et al. 2016; Dhake et al. 2013). The special properties of microbial-derived lipases including (i) activity toward organic solvents as a low-water-content environment, (ii) regio- and enantioselectivity, (iii) cost-effective production, (iv) good feasibility for immobilization and (v) susceptibility to express in different host microorganisms made these enzymes the second largest group of industrial biocatalysts after bacterial amylases (Hasan et al. 2006; Bora et al. 2013). Modifying oils and fats in food industries (to improve the texture and flavour of cheese and bread), wastewater treatment in pulp-, paper- and leather-manufacturing processes, as well as synthesis of biopolymers, biodiesel, flavouring compounds and enantio-specific pharmaceuticals are among the most important potential industrial and biotechnological applications of lipases (Hasan et al. 2006; Salihu and Alam 2015). As each biotechnological process may require specific properties, there is great interest in finding lipases harbouring unique characteristics such as a wide range of thermal and pH operational activity, as well as resistance to salt, heavy metals and organic solvents (Salihu and Alam 2015; Saengsanga et al. 2016).

CONTACT Mojtaba Shakibaie shakiba@kmu.ac.ir Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran; Hamid Forootanfar h_forootanfar@kmu.ac.ir Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran

Supplemental data for this article can be accessed here.

Due to the presence of the genus *Bacillus* in extreme habitats of the Earth-like volcanic water, desert soil and polar ice, there are many reports about isolating extremophilic lipases which originated from the genus *Bacillus* (Guncheva and Zhiryakova 2011; Samaei-Nouroozi et al. 2015). For example, Emtenani et al. (2013) obtained a thermo-alkaliphilic lipase (optimally works at pH 8 and 70 °C) from *B. subtilis* DR8806 that was isolated from a hot mineral spring. The purified lipolytic enzyme of *B. thermoleovorans* CCR11 (isolated from the "El Carrizal" hot springs in Veracruz, Mexico) worked optimally at 60 °C and pH 9–10 (Castro-Ochoa et al. 2005). Saengsanga et al. (2016) isolated an alkaline lipase (which exhibited maximal activity at pH 10) from *B. amyloliquefaciens* E1PA and successfully expressed it in an *E. coli* host. Characterization of the alkaline thermostable extracellular lipase (stable at 60 °C and pH 8 for 1 h) produced by *B. cereus* C7 (isolated from spoiled coconut) revealed its resistance towards a wide range of bile salts, chemical surfactants and heavy metals (Dutta and Ray 2009).

The main aim of the present study was the isolation and identification of a thermoalkalophilic bacterial strain capable of producing lipase from environmental samples followed by the evaluation of the factors affecting lipase production. The extracellularly produced lipase was then purified and characterized, and its ability to synthesize ethyl and methyl valerate was assessed in both solvent-free and organic solvent media.

Materials and methods

Chemicals

Brain heart infusion (BHI) broth, Rhodamine B and ethyl valerate were supplied by Merck Chemicals (Darmstadt, Germany). *p*-Nitrophenyl palmitate (*p*NPP), *p*-nitrophenol (*p*NP) and bovine serum albumin (BSA) were provided by Sigma-Aldrich (St. Louis, MO). SP Sepharose and Q-Sepharose were purchased from Pharmacia (Uppsala, Sweden). All other applied substances and solvents were of the highest purity available.

Sample collection, isolation and identification of halophilic bacterium capable to produce lipase

Fifteen salty and hypersaline soil samples were gathered from different sites of Dasht-e Lut (30°36′18″N, 59°04′04″E) near Kerman, Iran, where the land surface can reach maximum temperatures of 70.7 °C. Each 1 g of collected soil sample was re-suspended in sterile solution of NaCl (15% w/v, 20 mL) supplemented with Tween-80 (0.05% v/v). Next, the prepared soil suspension was filtered through Whatman No. 1 filter paper, and 0.5 mL of the resulting soil sample filtrate were streaked on a BHI agar dish supplemented with Rhodamine B (0.001% w/v), sodium chloride (15% w/v), and original olive oil (1% v/v). The prepared plates were subsequently incubated at 60 °C until the bacterial colonies with shiny orange fluorescence halos (an indicator of lipase activity) under ultraviolet light (350 nm) were developed (Castro-Ochoa et al. 2005; Hasan-Beikdashti et al. 2012). The most potent lipase-producing isolates were repeatedly subcultured to obtain an axenic culture of each isolate, which was then preserved in 20% (v/v) glycerol at −80 °C (Moshfegh et al. 2013).

The morphological and biochemical specifications of the selected bacterium were assessed based on the Bergey's Manual of Systematic Bacteriology (Vos et al. 2009). Furthermore, thermal and salt tolerance properties were demonstrated by culturing the selected isolate at various temperatures (5 − 75 °C) or in the BHI agar medium supplemented with various concentrations of NaCl (0 − 20% w/v), respectively. In addition, analysis of 16S rDNA gene sequencing was conducted after amplification of the related ribosomal gene segment with the primer pair of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as forward primer and 1525R (5′-AAGGAGGTGATCCAGCC-3′) as reverse primer followed by comparison of the obtained sequence using the BLAST software (Hasan-Beikdashti et al. 2012; Forootanfar et al. 2015).

Cultivation of the isolate and evaluation of factors affecting lipase production

To determine the growth curve and time course of lipase production the selected isolate was inserted into 500-mL Erlenmeyer flasks including 150 mL of basal medium composed of (g/L) mannose, 5; olive oil, 10; NaCl, 200; $(NH_4)_3PO_4$, 2; NaH_2PO_4, 2; $NaNO_3$, 2; and $FeSO_4.7H_2O$, 0.04 and incubated at 60 °C while slowly rotated for 72 h. Samples (1 mL) were taken at regular intervals and their OD_{600} was determined after removing the remaining olive oil (Gupta et al. 2007). Thereafter, the bacterial cells were separated by centrifugation (10,000 *g*, 10 min), and consequently, the lipolytic activity was assessed as elucidated in the following section.

The effects of carbon sources (fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, starch and sucrose all at final concentrations of 0.5% w/v),

nitrogen supplements containing yeast extract, tryptone, peptone, urea, ammonium chloride and ammonium sulphate (all at final concentrations of 0.5% w/v), lipase inducers including hazelnut oil, castor oil, coconut oil, sesame oil, sunflower oil, sweet oil, Tween-80 and Triton X-100 (all at final concentrations of 1% v/v), and metal cations of $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, KCl, $FeCl_3$ and $ZnSO_4.7H_2O$ (at final concentrations of 1 mM) on lipase production were assessed by individually adding each factor to the basal media (as mentioned earlier) followed by cultivating the isolate for 16 h and determining lipolytic activity.

Lipase assay and protein estimation

The previously described method of Hasan-Beikdashti et al. (2012) was applied to measure lipolytic activity. Briefly, 0.1 mL of the sample supernatant (as lipase solution) and 0.8 mL of phosphate buffer (0.1 M, pH 7.8) containing 0.4% of Triton X-100 and NaCl (1 M) were mixed with the lipase substrate (*p*NPP solution, 0.01 M) and the prepared mixture was consequently incubated at 60 °C for 30 min under mild shaking. Afterward, the absorbance of the reaction mixture was recorded at 410 nm using a Shimadzu double beam spectrophotometer (UV-1800, Shimadzu Corporation, Tokyo, Japan). The amount of enzyme that catalysed the formation of 1 μmol of *p*NP per min from *p*NPP was defined as one unit (U) of lipolytic activity. In order to determine the protein concentration, Bradford's colorimetric method was applied (Bradford 1976) using BSA as the standard.

Purification of the lipase

All purification steps were performed at 4 °C. First, the bacterial strain was cultivated in the basal medium (previously defined) for 16 h, and the lipase-containing culture broth was then prepared by two steps of removing the produced biomass using centrifugation (6000 *g*, for 10 min) followed by precipitation of the remaining fatty acids assisted by addition of a $CaCl_2$ solution (0.4 M) and subsequent centrifugation at 4 °C and 12,000 *g* for 20 min. The prepared cell-free culture broth was consequently brought to 80% saturation with pre-chilled ethanol, and the precipitated proteins were collected by centrifugation at 12,000 *g* for 25 min. The resulting precipitate was dissolved in Tris-HCl buffer (100 mM, pH 8) and dialysed against the same buffer. The concentrated sample was applied onto a Q-Sepharose XL column (16 mm × 130 mm) equilibrated with Tris-HCl buffer (50 mM, pH 8). The column was washed with a five-column volume of the same buffer at a flow rate of 120 mL/h to remove the unbound proteins and the bound proteins were eluted by increasing the NaCl concentration (0–500 mM) stepwise. Next, the previous step's pooled active fractions were loaded on the SP Sepharose column (16 mm ×130 mm) equilibrated using phosphate buffer (50 mM, pH 6.5). Thereafter, the column was washed with the same buffer and the bound proteins were eluted using NaCl gradient (0–500 mM) in the same buffer. After each purification step, the fractions obtained were analysed for lipolytic activity and protein content (A_{280}), and the specific activity, as well as the purification factor and yield, were calculated.

The subunit molecular mass of the purified lipase and the homogeneity of each purification step were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 10% gel based on the method described by Laemmli (1970). The N-terminal sequencing of the purified lipase was assessed by Seqlab Co. (Gottingen, Germany).

Lipase characterization

Influence of pH and temperature on activity and stability

The pH-activity profile of the purified lipase was demonstrated after performing lipase assay in the presence of a lipase substrate (*p*NPP) prepared in a 100 mM solution of the following buffers: citrate buffer (pH $3-5$), phosphate buffer (pH $6-8$) and carbonate buffer (pH $9-10$) all containing NaCl (1 M) under the previously mentioned assay conditions. The pH stability profile of the enzyme was evaluated by incubating the purified lipase at 60 °C for 300 min in the above-mentioned buffers (pH ranges of $3-11$), and the percentage of residual enzyme activities was determined.

In order to estimate the effect of temperature on the activity of the purified enzyme, the lipolytic activity was measured at temperatures of 4 °C, 20 °C, 30 °C, 37 °C, 45 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C. The temperature stability pattern of the purified lipase was demonstrated by pre-incubating the enzyme solution at temperatures of 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C and 80 °C for 300 min then measuring the residual lipase activity each 30 min (Cao et al. 2012; Daoud et al. 2013).

Effect of metal ions, enzyme inhibitors and surfactants on lipase activity

The effects of different metal ions including Mg^{2+}, K^{+}, Ca^{2+}, Cu^{2+}, Ag^{+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+} and Fe^{3+} (all

at final concentrations of 10 mM), enzyme inhibitors dithiothreitol (DTT, 0.5 mM) and EDTA (2.5 mM), as well as surfactants containing SDS (1 mM), Triton X-100 (1%), Tween-40 (1%), and Tween-80 (1%) on the activity of the purified lipase was determined by separately adding each factor into the assay mixture and measuring the residual lipase activity under standard assay conditions.

Effect of NaCl concentration on lipase activity and stability

Different concentrations of NaCl (1 – 5 M) were added to the standard reaction mixture (as described earlier) containing lipase, and the residual activity was measured to examine the influence of NaCl on the lipase activity. In addition, the salt stability of the purified lipase was assessed by pre-incubating the enzyme in the presence of different NaCl concentrations (1 – 5 M) for 300 min at 60 °C followed by determining the remaining lipolytic activity compared to the control (the purified lipase in the absence of NaCl).

Organic solvents stability of the purified lipase

The effect of organic solvents with different log *p* (Table 1) on the lipase stability was investigated by inserting each applied organic solvent into the enzyme solution to attain a final concentration of 25% (v/v) and incubating the prepared mixture for 1 h at 60 °C. The remaining organic solvents were then removed using centrifugation at 12,000 *g* for 5 min (4 °C), and the residual activity was subsequently measured as previously described.

Table 1. Stability of the purified lipase of *B. atrophaeus* FSHM2 towards organic solvents (25% v/v).

Organic solvent	Log *p*	Relative activity (%)
Control	–	100.0 ± 0.0
Methanol	−0.76	42.6 ± 1.2[a]
Ethanol	−0.24	83.7 ± 0.8[a]
n-Octanol	2.9	69.6 ± 0.5[a]
n-Butanol	0.89	117.8 ± 0.9[a]
n-Heptane	4.0	97.6 ± 1.6
Xylene	3.1	334.2 ± 0.6[a]
n-Hexane	3.5	153.2 ± 1.4[a]
Isoamylalcohol	1.3	121.4 ± 0.9[a]
Isopropyl alcohol	0.28	113.1 ± 1.3
Chloroform	2.0	7.7 ± 0.2[a]
1,4-Dioxane	−0.27	53.3 ± 0.5[a]
DMF	−1	53.4 ± 0.5[a]
Acetone	−0.23	23.4 ± 1.1[a]
Dichloromethane	1.25	100 ± 0.6
DMSO	−1.35	56.4 ± 0.7[a]

The reaction mixture was prepared by insertion of the purified lipase into each organic solvent solution (ratio of 3:1) followed by incubation at 60 °C for 1 h and subsequent measuring remained lipolytic activity. Experiments were performed in triplicates and significance ([a]) was attained after ANOVA with Dunnett's T3 *post hoc* test.

Lipase-mediated synthesis of methyl and ethyl valerate

Ester synthesis was performed in 50-mL stoppered conical flasks in aqueous and organo-aqueous medium based on the procedure reported by Khoobi et al. (2015). For the aqueous medium, the reaction mixture (final volume of 10 mL) containing valeric acid solution in methanol or ethanol (acid/alcohol molar ratios of 2:1, 1:1, 1:2, 1:3, 1:4 and 1:5) was prepared and the purified lipase of *B. atrophaeus* FSHM2 (10 U/mL, in Tris buffer 100 mM pH 9) was added. The reaction was subsequently incubated at 50 °C and 100 rpm for 1, 2, 4, 6, 12, 24, 36 and 48 h. In the solvent-containing medium, a solution including 4 mmol of methanol or ethanol and 1 mmol of valeric acid in anhydrous organic solvents of *n*-hexane or xylene (final volume of 10 mL) was prepared. Then, the purified lipase (10 U/mL, in Tris buffer 100 mM pH 9) was inserted into the prepared reaction mixture and allowed to stand with agitation at 50 °C on a rotary shaker (100 rpm) for 1, 2, 4, 6, 12, 24, 36 and 48 h. Afterwards, interval samples of 500 µL were periodically withdrawn, extracted using *n*-hexane and subjected to ester formation analyses using a gas chromatography apparatus (Agilent, 7890A) equipped with a flame ionization detector (FID, Agilent) and a capillary column (HP Innowax 30 m length × 0.32 µm i.d. × 0.5 µm thickness). The temperature range of 60 – 210 °C (with temperature rate of 10 °C/min) was used while the carrier gas nitrogen (flow rate of 30 mL/min), and the temperature of the injector and detector were set to 250 °C. Control experiments were carried out by inserting the inactivated lipase (using trichloro acetic acid 10%) into the reaction mixture. The ester yield was then calculated based on comparing the retention time and peak sample area with the standard (Kraai et al. 2008).

Statistical analyses

Three replicates of each above-mentioned experiment were conducted, and the obtained results were reported as mean ± SD. In order to calculate the statistical significance (probability values less than 0.05) between mean values, an independent sample *t*-test and one-way analysis of variance (ANOVA) with Dunnett's T3 *post hoc* test were applied using SPSS software (version 15.0, SPSS Inc., Chicago, IL).

Results and discussion

Isolation and identification of the selected lipase-producing bacterial strain

Screening of the thermo-halophilic bacterial strains (4 isolates acquired from 15 gathered samples) for lipase

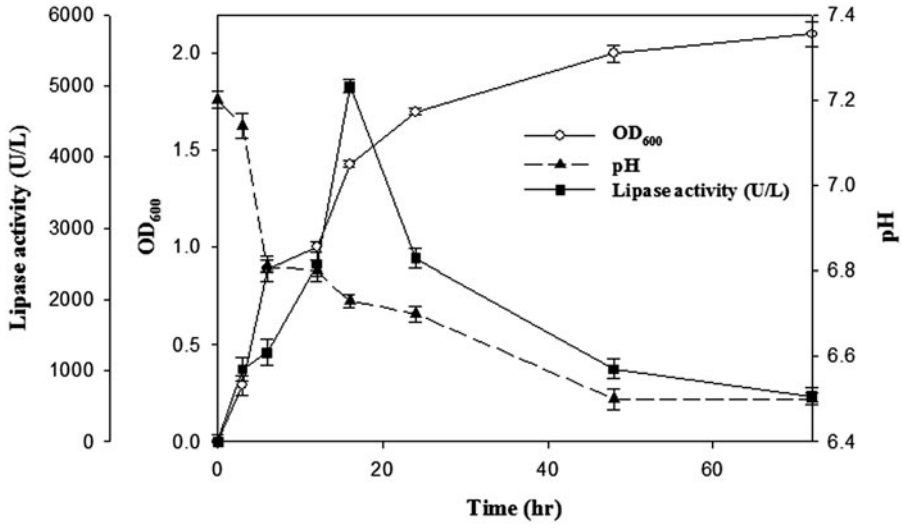

Figure 1. Time course of lipase production and growth curve of *B. atrophaeus* FSHM2 cultivated in a basal medium containing NaCl (20% w/v) and olive oil (1% v/v).

activity revealed that only one isolate (designated as M2) produced the related shiny fluorescence halos (under UV light, 350 nm) around their colonies when grown at 60 °C on the Rhodamine B containing BHI agar medium (supplemented with olive oil, 1% w/v and NaCl, 15% w/v) that indicated lipase production. The biochemical characterization results demonstrated that the selected microorganism was a Gram-positive, non-motile and rod-shaped bacterium that formed a white to pale yellow colony on the BHI agar medium and was capable to grow at a temperature range of 40–75 °C and in the presence of a high level of sodium chloride (2.5–20%). Isolate M2 represented positive reactions for oxidase and catalase but was not able to hydrolyse gelatin and starch. It was able to produce acid from maltose, sucrose, lactose, mannitol, glycerol, galactose, fructose and glucose. Comparison of the obtained sequence of the amplified 16S rDNA gene using the BLAST software revealed 99% similarity with *Bacillus atrophaeus*. The 1420 bp gene sequence was then submitted to GenBank (accession number, KF682367).

The time course of lipase production, alteration of OD_{600}, and pH changes in the culture broth of *B. atrophaeus* FSHM2 during a 72-h incubation period at 60 °C (Figure 1) indicated that maximal lipolytic activity (4995 U/L) occurred after 16 h of incubation where OD_{600} of culture broth reached 1.43.

The ability of *Bacillus* strains to secrete extremophilic lipases has been previously reported (Guncheva and Zhiryakova 2011). For example, Saengsanga et al. (2016) launched an alkaline lipase (optimal pH of 10) produced by *B. amyloliquefaciens* E1PA that was isolated from lipid-rich food waste. Dutta and Ray (2009) reported that the alkaline thermostable extracellular lipase of *B. cereus* C7 (isolated from spoiled coconut) was maximally produced after 24 h of cultivation.

Evaluation of factors affecting lipase production

Due to the critical role of the culture medium's chemical components on lipase productivity and the growth of lipase-producing microbial strains (Hasan-Beikdashti et al. 2012; Bora et al. 2013), the effects of carbon and nitrogen supplementation, metal ions and lipase inducers on lipase activity of *B. atrophaeus* FSHM2 were investigated in the present study. Glucose, hazelnut oil, and calcium ions positively affected lipase production by increasing production to 13,850 U/L, 6279 U/L and 5513 U/L in comparison with the basal medium (4987 U/L, $p \leq .05$). However, urea exhibited negative effect by decreasing lipase production to 4446 U/L compared to that of the basal medium (4987 U/L, $p \leq .05$).

Enzyme purification, molecular weight estimation and N-terminal sequencing

In the present study, the produced lipase of *B. atrophaeus* FSHM2 (designated as *Ba*L) was purified in three sequential steps. First, the cell-free culture broth was concentrated using ethanol precipitation (80%) and dialysis (39.8% recovery and 8.2 purification factor) and then two chromatographic steps employing anion (19.4% yield and purification factor of 20.7) and cation (9.9% recovery and 31.8 purification factor)-exchange media were successfully applied for lipase purification (Table 2). The homogeneity of each purification step is

Table 2. Purification steps for extracellular lipase produced by *B. atrophaeus* FSHM2.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (factor)
Centrifuged culture broth (crude)	173.0	94.3	1.8	100.0	1
0–80% ethanol precipitation and dialysis	68.9	4.7	14.7	39.8	8.2
Q-Sepharose XL anion-exchange chromatography	33.6	0.9	37.3	19.4	20.7
SP Sepharose cation-exchange chromatography	17.2	0.3	57.3	9.9	31.8

Table 3. Comparison of the N-terminal amino acid sequences of different *Bacillus* derived lipases.

Microorganism	*N*-terminal amino acid sequence[a]															Accession number
B. atrophaeus FSHM2	M	K	Q	R	V	N	P	D	L	L	Q	G	L	E	M	Present study
Bacillus atrophaeus	**M**	**K**	N	**R**	**V**	**N**	**P**	E	**L**	**L**	**Q**	**G**	**L**	**E**	**M**	GI:498487057
Bacillus atrophaeus	**M**	**K**	N	**R**	**V**	**N**	**P**	E	**L**	**L**	**Q**	**G**	**L**	**E**	**M**	GI:751416253
Bacillus atrophaeus UCMB-5137	**M**	**K**	N	**R**	**V**	**N**	**P**	E	**L**	**L**	**Q**	**G**	**L**	**E**	**M**	GI:830324800
Bacillus cereus	**M**	**K**	N	**R**	**V**	**N**	**P**	E	**L**	**L**	**Q**	**G**	**L**	**E**	**M**	GI:872530815
Bacillus cereus	**M**	**K**	N	**R**	**V**	**N**	**P**	E	**L**	**L**	**Q**	**G**	**L**	**E**	**M**	GI:983300457

[a]identical amino acids are shown as bold letter.

illustrated in Figure S1, and the molecular weight of the purified lipase was estimated to be approximately 85 kDa.

Olusesan et al. (2011) purified a thermostable lipase (molecular weight, 45 kDa) of the extremophilic *B. subtilis* NS8 using three sequential steps of ultrafiltration, anion-exchange chromatography (DEAE-Toyopearl), and size exclusion chromatography (Sephadex G-75) with the final purification factor of 500 and yield of 16%. The solvent-tolerant lipase (molecular weight, 69 kDa) of *B. sphaericus* MTCC 7542 was purified after two consecutive steps of ammonium sulphate precipitation (purification factor of 1.35 and yield of 74%) and DEAE–Sepharose anion-exchange chromatography (purification factor of 17.33 and yield of 5.7%) (Tamilarasan and Kumar 2012). A literature review revealed that the molecular weight of lipases originated from *Bacillus* strains usually vary from 19 kDa (*B. subtilis* 168) (Lesuisse et al. 1993) to 108 kDa (*Bacillus* sp. GK 8) (Dosanjh and Kaur 2002; Guncheva and Zhiryakova 2011).

As presented in Table 3, the N-terminal amino acid sequence of *Ba*L was MKQRVNPDLLQGLEM, which showed 86.7% similarity to *B. atrophaeus* and *B. cereus* lipases.

BaL characterization

Influence of pH and temperature on BaL activity and stability

According to the relative activity profile of the *Ba*L (Figure 2) at different pH levels, the optimum pH of the enzyme was 9 where the maximum stability of the enzyme was also observed at this pH. The lipolytic activity was gradually decreased by decreasing the pH from 9 to 3 (15.8%) (Figure 2). Determining the pH stability of the *Ba*L (Figure 2) revealed that the enzyme

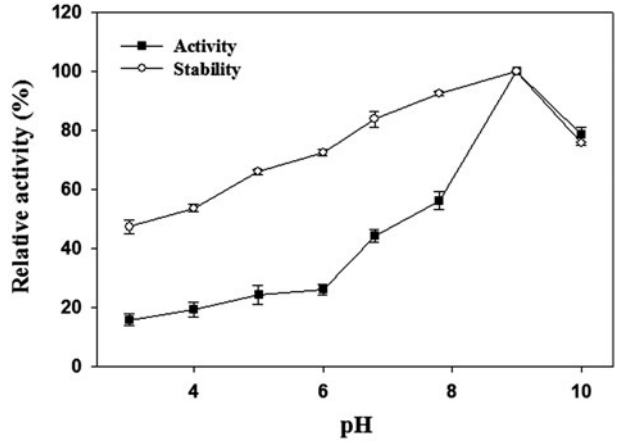

Figure 2. Influences of pH on the activity (■) and stability (○) of the purified lipase of *B. atrophaeus* FSHM2.

lost 7.5% and 24.4% of its maximal activity after 5 h of incubation at pH 7.8 and pH 10, respectively. Similar results were reported by Chen et al. (2007) who observed that more than 70% of the original activity of the purified lipase of *B. cereus* C71 (optimum pH of 9) was retained after 3 h of incubation over a pH range of 8.5–10. In general, the optimum pH of most *Bacillus*-originated lipases falls on the alkaline side (Guncheva and Zhiryakova 2011).

After demonstrating lipase activity at various temperatures, it was determined that the *Ba*L showed maximal activity at 70 °C (Figure 3(a)) and altering the temperature to 80 °C, 90 °C and 100 °C decreased the lipolytic activity to 88.2%, 71.5% and 43.2% of the maximum, respectively. At 37 °C and 45 °C, the enzyme showed approximately half of its maximal activity (Figure 3(a)). The stability curve of the *Ba*L (Figure 3(b)) revealed that the enzyme lost 51.3% and 58.3% of its maximum activity after 150 min of incubation at 80 °C and 75 °C, respectively. However, the *Ba*L retained 92.5% of its original activity after 300 min of incubation at 55 °C (Figure 3(b)). Lee et al. (1999)

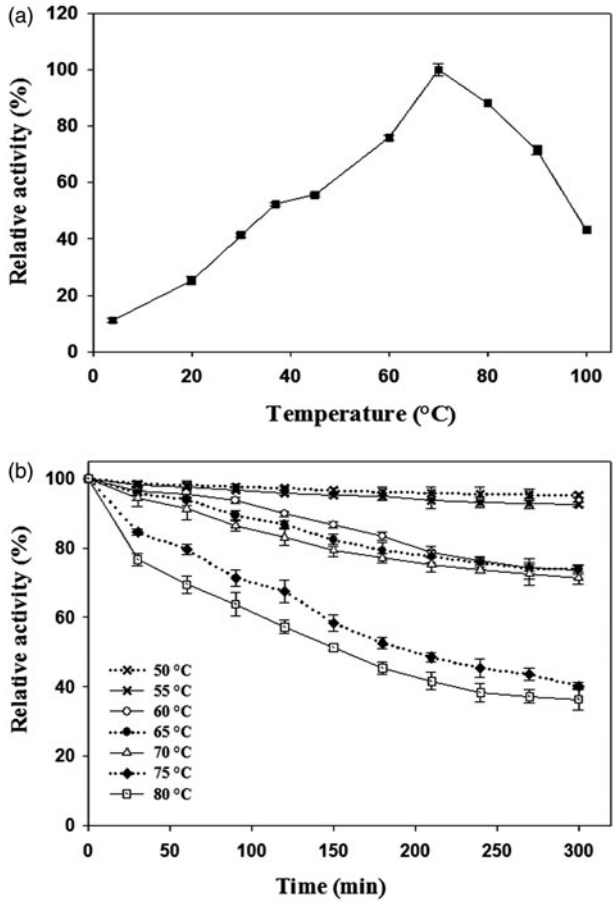

Figure 3. (a) Temperature-activity profile and (b) temperature stability curve of the purified lipase of *B. atrophaeus* FSHM2.

reported that the purified lipase of *B. thermoleovorans* ID-1 showed maximal activity at 70–75 °C and retained 50% of its maximal activity after 30 min of incubation at 70 °C. Bradoo et al. (1999) observed that 50% of the initial activity of the thermotolerant lipase from *B. stearothermophilus* SB-1 remained after 15 min of incubation at 100 °C.

Effect of metal ions, enzyme inhibitors, detergents and salt concentration on BaL activity

As presented in Table 4, except for calcium, which enhanced the relative activity of the *Ba*L to 132.4 ± 2.2% (p value <.05), all of the other heavy metal ions tested significantly declined the *Ba*L activity (Table 4). Chen et al. (2007) reported that the purified lipase of *B. cereus* C71 was inhibited by all the heavy metal ions tested. In general, the positive effect of Ca^{2+} on the hydrolysis process of the lipases, especially lipases originating from the *Bacillus* genus, are ascribed to (i) the structural role of this divalent cation and (ii) suppression of the inhibitory activity of the formed fatty acids (released during enzymatic hydrolysis) due to the formation of insoluble Ca-salts of the

Table 4. Obtained results of relative activity of the purified lipase from *B. atrophaeus* FSHM2 in the presence of metal ions, enzyme inhibitors and detergents.

Metal ions	Final concentration	Relative activity (%)
Control	–	100
Mg^{2+}	10 mM	74.4 ± 1.4[a]
K^{+}	10 mM	86.7 ± 0.8[a]
Cu^{2+}	10 mM	50.3 ± 1.1[a]
Ca^{2+}	10 mM	132.4 ± 2.2[a]
Ag^{+}	10 mM	59.6 ± 1.4[a]
Co^{2+}	10 mM	52.1 ± 0.9[a]
Cd^{2+}	10 mM	35.6 ± 1.7[a]
Mn^{2+}	10 mM	77.3 ± 0.8[a]
Zn^{2+}	10 mM	93.8 ± 2.3
Fe^{3+}	10 mM	53.5 ± 1.5[a]
Enzyme inhibitors		
EDTA	2.5 mM	71.4 ± 1.1[a]
DTT	0.5 mM	73.4 ± 2.2[a]
Detergents		
Triton X-100	1%	47.3 ± 1.6[a]
Tween-80	1%	42.1 ± 1.3[a]
Tween-40	1%	8.6 ± 0.9[a]
SDS	1 mM	2.3 ± 1.5[a]

[a]ANOVA analysis with Dunnett's T3 *post hoc* test was applied to determine the significant values (p value <.05).

mentioned fatty acids (Guncheva and Zhiryakova 2011).

EDTA (as chelating agent) was able to inhibit the *Ba*L activity (Table 4) which was in accordance with the results obtained by Kanwar et al. (2006) who described the negative effect of EDTA on the activity of the lipase originating by *B. coagulans* MTCC-6375 (78%). In contrast, the activity of *B. pumilus* B26 lipase was not affected in the presence of EDTA (Kim et al. 2002). The negative effect of DTT as a reducing agent on the activity of the *Ba*L (Table 4) was similar to the previously reported results of Sabri et al. (2009) who determined 22.5% of decreased activity of purified lipase originating from *Bacillus* sp. L2.

The non-ionic surfactants containing Triton X-100, Tween-80 and Tween-40, as well as the anionic surfactant of SDS significantly decreased the *Ba*L activity to 47.3 ± 1.6%, 42.1 ± 1.3%, 8.6 ± 0.9% and 2.3 ± 1.5, respectively (Table 4). In contrast, Chen et al. (2007) achieved 160%, 141% and 128% of relative activity of *B. cereus* C71 lipase in the presence of Triton X-100 (0.5%), Tween-80 (0.5%) and Tween-20 (0.5%), respectively.

As shown in the salt tolerance profile of the *Ba*L (Figure 4), the relative activity and stability of the enzyme were gradually increased by elevating the salt concentration and maximum relative lipolytic activity was observed with a 4 M concentration of NaCl (Figure 4). However, the purified lipase was less active (31.4%) and less stable (16.7%) in the absence of NaCl compared with those of 4 M NaCl (p value <.05, Figure 4). At a higher salt concentration (5 M), both the relative activity (79.8%) and stability (63.2%) of the

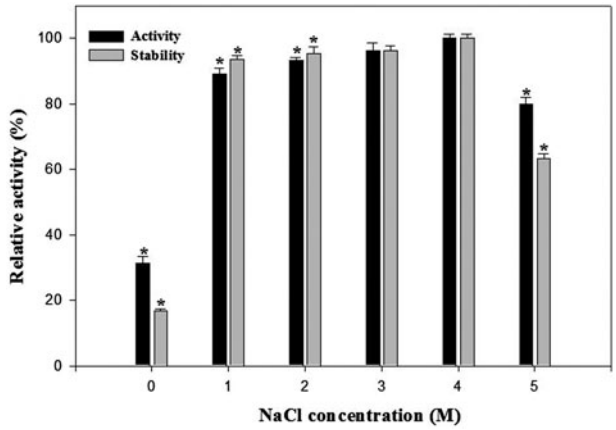

Figure 4. Influence of NaCl concentrations on the activity and stability of the purified lipase. Lipase activity was measured using *p*NPP as a lipase substrate containing different NaCl concentrations, while enzyme stability was demonstrated after 5 h of incubation of the enzyme in the presence of NaCl and measuring of residual activity. *Significance (p value $<.05$) was determined after the ANOVA analysis of the attained results ($n = 3$) compared with those of control (enzyme reaction mixture in the presence of 4 M NaCl).

*Ba*L significantly dropped compared to the 4 M NaCl concentration (p value $<.05$) (Figure 4). A similar pattern occurred when the reaction mixture of lipase assay was prepared in the absence of NaCl (p value $<.05$) (Figure 4). It seems that the presence of salt up to the critical concentration of 4 M is beneficial for the hydrolytic activity of the *Ba*L. Similar results were achieved by Balaji and Jayaraman (2014) where a halophilic lipase producer (*Bacillus* sp. VITL8) was isolated from oil-contaminated areas. They found that more than 55% of the original activity of the produced lipase remained at 60 °C and pH 10 in the presence of 3 M NaCl (Balaji and Jayaraman 2014).

Stability of the BaL in the presence of organic solvents

Due to the importance of organic solvent stability of enzymes in various industrial processes the stability of the *Ba*L in the presence of fourteen common organic solvents harbouring different log p values (−1.35 to 4.0) was also evaluated in the present study. As shown in Table 1, the relative activity of the *Ba*L was significantly enhanced in the presence of *n*-butanol (117.8 ± 0.9%), xylene (334.2 ± 0.6%), *n*-hexane (153.2 ± 1.4%), isoamyl alcohol (121.4 ± 0.9%) and isopropyl alcohol (113.1 ± 1.3%), while other applied solvents declined the residual *Ba*L activity after 1 h of incubation at 60 °C (Table 1). Bradoo et al. (1999) reported that the relative activity of the produced lipase *B. atrophaeus* was negatively affected by the

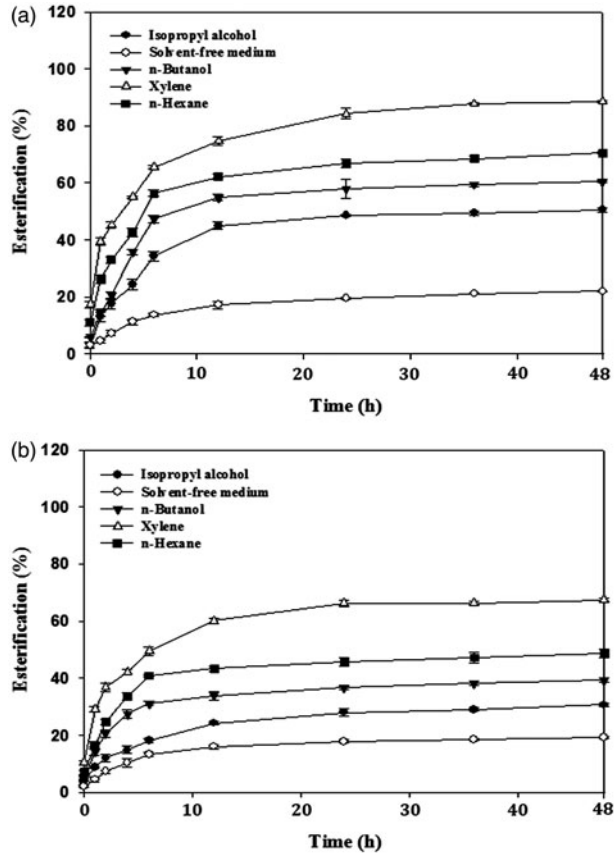

Figure 5. Lipase-catalysed synthesis of (a) ethyl valerate and (b) methyl valerate in solvent-free and organic solvent-containing media. Data are shown as mean ± SD ($n = 3$).

presence of all studied organic solvents except for diethyl ether that increased the lipase activity to 120%. Benzene (30%) and hexane (30%) increased the residual activity of the purified lipase of *Bacillus* sp. Lip2 by 207% and 201%, respectively (Nawani and Kaur 2007). Guncheva and Zhiryakova (2011), who reviewed the catalytic properties of the lipases originating from the genus *Bacillus*, described that these biocatalysts are very stable in the presence of organic solvents. This stability was especially notable with nonpolar solvents due to their ability to shift the equilibrium of lipase conformation from closed to open form and modify the substrates and products solubility in the reaction medium (Guncheva and Zhiryakova, 2011).

Application of BaL for esterification

The results of the *Ba*L application for synthesis of ethyl valerate (Figure 5(a)) and methyl valerate (Figure 5(b)) clearly indicated that the organo-aqueous solvent medium is more appropriate for lipase-assisted esterification reaction compared to the aqueous medium. Among the four applied organic

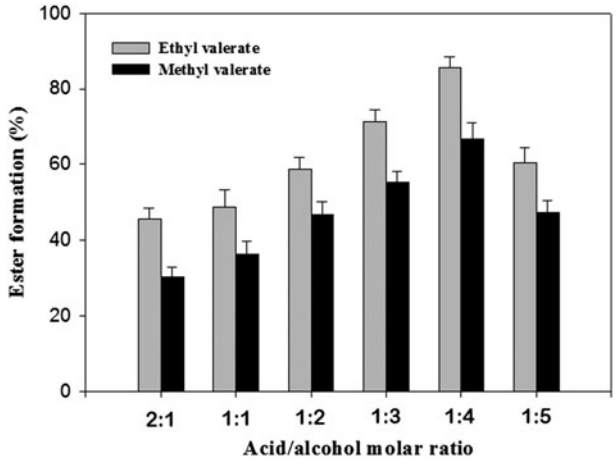

Figure 6. Influence of molar ratio of valeric acid/ethyl or methyl alcohol on esterification percentage assisted by the purified lipase of *B. atrophaeus* FSHM2 in a xylene containing medium at 50 °C.

solvents, xylene was the most efficient one which increased the esterification percentages of ethyl valerate and methyl valerate to 88.5% and 67.5%, respectively, after 48 h of incubation in the presence of *Ba*L (Figure 5(a,b)). In contrast, the solvent-free system yielded esterification percentages of 19.3% and 22% for methyl valerate and ethyl valerate, respectively (Figure 5(a,b)) under the same conditions. Analysis of the reaction mixture containing inactivated lipase (as a negative abiotic control) revealed an esterification percentage of 4%. In the study of Kumar et al. (2006), who applied the purified lipase *B. coagulans* BTS-3 for esterification of ethanol and propionic acid, it was observed that the esterification reaction was optimal in the presence of *n*-hexane at 55 °C. In general, the presence of an optimum amount of water is essential for lipase-catalysed esterification reactions due to water's critical role as a lubricant for mobility of the enzyme in catalytic action (Sivaramakrishnan and Muthukumar 2014). On the other hand, excessive water can shift the esterification equilibrium towards hydrolysis of the produced ester (Dhake et al. 2013). As the molar ratio of acid/alcohol is another parameter affecting esterification reaction yield, (Khoobi et al. 2015), the present study also evaluated this factor. The results (Figure 6) revealed that the highest esterification percentages of both ethyl valerate (85.6%) and methyl valerate (66.8%) occurred at the molar ratio of 1:4 (v/v) acid to alcohol. Kumar et al. (2013), who applied the immobilized lipase *Bacillus* sp. DVL2 for the production of ethyl oleate, determined that the molar ratio of 1:1 (v/v) oleic acid and ethanol was the most appropriate ratio to achieve the highest ester formation (63%).

Conclusions

*Ba*L, a thermoalkalophilic and extracellular lipase (optimum temperature of 70 °C and pH 9), was successfully purified from a cell-free culture broth of an extremely halophilic bacterial strain (*B. atrophaeus* FSHM2) using three consecutive purification steps. Approximately 92.5% of the *Ba*L initial activity was retained after incubating the purified lipase at 55 °C for 5 h. The relative activity of the *Ba*L (approximate M_w of 85 kDa) was considerably enhanced in the presence of NaCl (4 M), Ca^{2+} (10 mM) and the organic solvent xylene (25%). Ethyl valerate (conversion, 88.5%) and methyl valerate (conversion, 67.5%) were synthesized after *Ba*L-catalysed esterification in the xylene containing medium for 48 h.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

This work was financially supported by the grant 94/1691 from Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran.

References

Balaji L, Jayaraman G. 2014. Metal ion activated lipase from halotolerant *Bacillus* sp. VITL8 displays broader operational range. Int J Biol Macromol 67:380–386.

Bora L, Gohain D, Das R. 2013. Recent advances in production and biotechnological applications of thermostable and alkaline bacterial lipases. J Chem Technol Biotechnol 88:1959–1970.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Bradoo S, Saxena RK, Gupta R. 1999. Two acidothermotolerant lipases from new variants of *Bacillus* spp. World J Microbiol Biotechnol 15:87–91.

Cao Y, Zhuang Y, Yao C, Wu B, He B. 2012. Purification and characterization of an organic solvent-stable lipase from *Pseudomonas stutzeri* LC2-8 and its application for efficient resolution of (R, S)-1-phenylethanol. Biochem Eng J 64:55–60.

Castro-Ochoa LD, Rodriguez-Gomez C, Valerio-Alfaro G, Ros RO. 2005. Screening, purification and characterization of the thermoalkalophilic lipase produced by *Bacillus thermoleovorans* CCR11. Enzyme Microb Technol 37:648–654.

Chen S, Qian L, Shi B. 2007. Purification and properties of enantioselective lipase from a newly isolated *Bacillus cereus* C71. Process Biochem 42:988–994.

Christopher LP, Zambare VP, Zambare A, Kumar H, Malek L. 2015. A thermo-alkaline lipase froma new thermophile

Geobacillus thermodenitrificans AV-5 with potential application in biodiesel production. J Chem Technol Biotechnol 90:2007–2016.

Daoud L, Kamoun J, Ali MB, Jallouli R, Bradai R, Mechichi T, Gargouri Y, Ali YB, Aloulou A. 2013. Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase. Int J Biol Macromol 57:232–237.

Dhake KP, Thakare DD, Bhanage BM. 2013. Lipase: a potential biocatalyst for the synthesis of valuable flavour and fragrance ester compounds. Flavour Frag J 28:71–83.

Dosanjh NS, Kaur J. 2002. Biochemical analysis of a native and proteolytic fragment of a high-molecular-weight thermostable lipase from a mesophilic *Bacillus* sp. Protein Expr Purif 24:71–75.

Dutta S, Ray L. 2009. Production and characterization of an alkaline thermostable crude lipase from an isolated strain of *Bacillus cereus* C7. Appl Biochem Biotechnol 159:142–154.

Emtenani S, Asoodeh A, Emtenani S. 2013. Molecular cloning of a thermo-alkaliphilic lipase from *Bacillus subtilis* DR8806: Expression and biochemical characterization. Process Biochem 48:1679–1685.

Forootanfar H, Amirpour-Rostami S, Jafari M, Forootanfar A, Yousefizadeh Z, Shakibaie M. 2015. Microbial-assisted synthesis and evaluation the cytotoxic effect of tellurium nanorods. Mater Sci Eng C Mater Biol Appl 49:183–189.

Guncheva M, Zhiryakova D. 2011. Catalytic properties and potential applications of *Bacillus* lipases. J Mol Catal B: Enzym 68:1–21.

Gupta N, Sahai V, Gupta R. 2007. Alkaline lipase from a novel strain *Burkholderia multivorans*: statistical medium optimization and production in a bioreactor. Process Biochem 42:518–526.

Hasan F, Shah AA, Hameed A. 2006. Industrial applications of microbial lipases. Enzyme Microb Technol 39:235–251.

Hasan-Beikdashti M, Forootanfar H, Safiarian MS, Ameri A, Ghahremani MH, Khoshayand MR, Faramarzi MA. 2012. Optimization of culture conditions for production of lipase by a newly isolated bacterium *Stenotrophomonas maltophilia*. J Taiwan Inst Chem Engrs 43:670–677.

Kanwar SS, Ghazi IA, Chimni SS, Joshi GK, Rao GV, Kaushal RK, Gupta R, Punj V. 2006. Purification and properties of a novel extra-cellular thermotolerant metallolipase of *Bacillus coagulans* MTCC-6375 isolate. Protein Expr Purif 46:421–428.

Khoobi M, Khalilvand-Sedagheh M, Ramazani A, Asadgol Z, Forootanfar H, Faramarzi MA. 2016. Synthesis of polyethyleneimine (PEI) and β-cyclodextrin grafted PEI nanocomposites with magnetic cores for lipase immobilization and esterification. J Chem Technol Biotechnol 91:375–384.

Khoobi M, Motevalizadeh SF, Asadgol Z, Forootanfar H, Shafiee A, Faramarzi MA. 2015. Polyethyleneimine-modified superparamagnetic Fe_3O_4 nanoparticles for lipase immobilization: characterization and application. Mater Chem Phys 149–150:77–86.

Kim HK, Choi HJ, Kim MH, Sohn CB, Oh TK. 2002. Expression and characterization of Ca(2+)-independent lipase from Bacillus pumilus B26. Biochim Biophys Acta 1583:205–212.

Kraai GN, Winkelman JGM, Vries JG, Heeres HJ. 2008. Kinetic studies on the *Rhizomucor miehei* lipase catalyzed esterification reaction of oleic acid with 1-butanol in a biphasic system. Biochem Eng J 41:87–94.

Kumar D, Nagar S, Bhushn I, Kumar L, Parshad R, Gupta VK. 2013. Covalent immobilization of organic solvent tolerant lipase on aluminum oxide pellets and its potential application in esterification reaction. J Mol Catal B Enzym 87:51–61.

Kumar S, Pahujani S, Ola RP, Kanwar SS, Gupta R. 2006. Enhanced thermostability of silica-immobilized lipase from *Bacillus coagulans* BTS-3 and synthesis of ethyl propionate. Acta Microbiol Immunol Hung 53:219–231.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee DW, Koh YS, Kim KJ, Kim BC, Choi HJ, Kim DS, Suhartono MT, Pyun YR. 1999. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 179:393–400.

Lesuisse E, Schanck K, Colson C. 1993. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 216:155–160.

Moshfegh M, Shahverdi AR, Zarrini G, Faramarzi MA. 2013. Biochemical characterization of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xinjiangense*. Extremophiles 17:677–687.

Nawani N, Kaur J. 2007. Studies on lipolytic isoenzymes from a thermophilic *Bacillus* sp.: Production, purification and biochemical characterization. Enzyme Microb Technol 40:881–887.

Olusesan AT, Azura LK, Forghani B, Bakar FA, Mohamed AKS, Radu S, Manap MYA, Saari N. 2011. Purification, characterization and thermal inactivation kinetics of a non-regioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS 8. New Biotechnol 28:738–745.

Sabri S, Rahman RNZRA, Leow TC, Basri M, Salleh AB. 2009. Secretory expression and characterization of a highly Ca^{2+}-activated thermostable L2 lipase. Protein Expr Purif 68:161–166.

Saengsanga T, Siripornadulsil W, Siripornadulsil S. 2016. Molecular and enzymatic characterization of alkaline lipase from Bacillus amyloliquefaciens E1PA isolated from lipid-rich food waste. Enzyme Microb Technol 82:23–33.

Salihu A, Alam MZ. 2015. Solvent tolerant lipases: a review. Process Biochem 50:86–96.

Samaei-Nouroozi A, Rezaei S, Khoshnevis N, Doosti M, Hajihoseini R, Khoshayand MR, Faramarzi MA. 2015. Medium-based optimization of an organic solvent-tolerant extracellular lipase from the isolated halophilic *Alkalibacillus salilacus*. Extremophiles 19:933–947.

Sivaramakrishnan R, Muthukumar K. 2014. Direct transesterification of *Oedogonium* sp. oil be using immobilized isolated novel *Bacillus* sp. lipase. J Biosci Bioeng 117:86–91.

Tamilarasan K, Kumar D. 2012. Purification and characterization of solvent tolerant lipase from *Bacillus sphaericus* MTCC 7542. Biocatal Agric Biotechnol 1:309–313.

Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KA, Whitman W. 2009. Bergey's manual of systematic bacteriology. 2nd ed. London, New York: Springer Dordrecht Heidelberg.