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Partial Purification and Characterization of a Thermoalkalophilic Lipase Originated from *Bacillus atrophaeus* FSHM2 and its Application for Ester Synthesis

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A R T I C L E   I N F O

A B S T R A C T

A thermoalkalophilic lipase producing bacterial strain, identified as *Bacillus atrophaeus* FSHM2 using 16S rDNA sequencing analysis was isolated from salty soil and its lipase was partially purified and characterized. The obtained results revealed that glucose, hazelnut oil, urea and calcium ion positively affected the lipase production by increasing the lipolytic activity to 13582.5, 6270, 4442 and 5505 U L^{-1}, respectively compared to that of basal medium (4150 U L^{-1}). The partially purified lipase acted optimally at pH 9 and retained 88.2% of its initial activity after 1 h of incubation at 100°C. A two fold increase in the relative activity of the partially purified lipase was obtained in the presence of 4 M of NaCl. Application of the partially purified lipase for the synthesis of ethyl and methyl valerate in the organic solvent medium (xylene) resulted in 81.6 and 62.4% esterification, respectively, after 24 h of incubation.

Key words: Thermoalkalophilic lipase, *Bacillus atrophaeus*, partial purification, characterization, esterification

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a group of serine hydrolases which catalyze the hydrolysis, esterification, transesterification, acidolysis, aminolysis and alcoholysis of triacylglycerols or other water-insoluble esters into diacylglycerols, monoacylglycerols, Free Fatty Acids (FFA) and glycerol (Kumar *et al*., 2012; Patel *et al*., 2014). Compared to animal and plant-derived lipases, microbial lipases have received great attention owing to their ability to catalyze a wide variety of reactions in both aqueous and non-aqueous phases and relative cost-effectiveness of their production (Bouaziz *et al*., 2011; Andualema and Gessesse,

2012). These valuable biocatalysts have been widely used in various industries such as food (for example, hydrolysis of milk fat and cheese ripening in dairy foods), detergent, pulp and paper (papers with improved quality), fine chemicals (such as resolution of racemic acids and alcohols and regioselective acylations) and oleochemical manufacturing (Salameh and Wiegel, 2007; Sharma *et al*., 2011; Abdel-Fattah *et al*., 2012). Synthesis of biopolymers and biodiesel, resolution of enantio-specific pharmaceuticals and production of agro-chemicals and flavoring compounds are among other biotechnological applications of lipases (Soliman *et al*., 2007; Yu *et al*., 2010; Andualema and Gessesse, 2012).

Because of high demands for the lipases tolerant to extreme conditions, screening of the environments harboring halophilic and thermophilic lipase-producing microbial strains is still a main step for introducing lipolytic enzyme with remarkable properties (Daoud *et al*., 2013; Masomian *et al*., 2013). Extremophiles include microorganisms which are able to survive in harsh environmental conditions, such as extremely high or low temperatures, high concentration of salt, severe alkaline or acidic pH, low water activity and high pressure (Salameh and Wiegel, 2007; Moshfegh *et al*., 2013). There are many reports on the production of lipases originated from exteremophiles such as *Burkholderia ambifaria* YCJ01 (Yao *et al*., 2013), *Fervidobacterium nodosum* Rt17-B1 (Yu *et al*., 2010), *Staphylococcus xylosus* (Bouaziz *et al*., 2011), *Staphylococcus* sp. (Daoud *et al*., 2013) and *Aneurinibacillus thermoaerophilus* strain HZ. (Masomian *et al*., 2013). Isolates of *Bacillus* genera (*B. subtilis* DR8806 and *B. subtilis* NS8) have been found to produce lipases active at alkaline pH and in elevated temperature conditions (Olusesan *et al*., 2011; Emtenani *et al*., 2013). In the study conducted by Ghanem *et al*. (2000) an alkalophilic thermostable lipase (optimally worked at 60°C and pH 10.6) isolated from *Bacillus alcalophilus*. A thermostable lipase-producing bacterial strain *Geobacillus stearothermophilus* was isolated from desert soil sample and its related gene was cloned into *Escherichia coli* XL1-blue (Sifour *et al*., 2010).

In the present study, a thermoalkalophilic lipase-producing bacterial strain was isolated from hypersaline environments and its lipase was partially purified and characterized. In addition, the ability of the partially purified lipase for synthesis of methyl and ethyl valerate was evaluated in both solvent-free and organic solvent media.

MATERIALS AND METHODS

Chemicals: Both ρ-nitrophenol (ρ-NP) and ρ-nitrophenyl palmitate (ρ-NPP) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Rhodamine B, Brain Heart Infusion Broth (BHIB), peptone, yeast extract, valeric acid and agar were purchased from Merck Chemicals (Darmstadt, Germany). All other chemicals were of analytical grade.

Screening for lipase-producing halophilic microorganisms: Soil samples were collected from different locations of saline and hypersaline environments of Dasht-e Lut (30°36'18"N, 59°04'04"E), Kerman, Iran which is the hottest land surface (maximum temperature of 70.7°C). One gram of each soil sample was then added to 20 mL of 15% (w/v) NaCl sterile solution containing Tween 80 (0.05%, v/v) followed by shaking of samples and passing through the filter paper (Whatman No. 1). Thereafter, 500 µL of each obtained filtrate was spread on a BHI agar plate containing Rhodamine B (0.001%, w/v), olive oil (1%, v/v) and NaCl (15% w/v). The

culture plates were then incubated at 60°C and the colonies harboring orange fluorescence halos around them (under ultraviolet light of 350 nm) were regarded as the lipase producer (Hasan-Beikdashti *et al*., 2012; Daoud *et al*., 2013). The most potent lipase producing isolates were re-isolated for several times to confirm their purity and then conserved in glycerol 20% (v/v) at -80°C.

Identification of the selected isolate: Morphological and biochemical properties of the selected isolate were characterized according to the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). In addition, salt and thermal tolerance assays were performed by cultivating the selected isolate in the BHI agar medium containing different concentrations of NaCl (0-20%) and temperatures (5-75°C), respectively. Further identification of the selected isolate was done by 16S rDNA gene sequence analysis method after amplification of the desired gene using the forward primer 27F (5'-AGAG TTTGATCCTGGCTCAG-3') and the reverse primer 1525R (5'-AAGGAGGTGATCCAGCC-3') (Hasan-Beikdashti *et al*., 2012; Forootanfar *et al*., 2014). The amplified DNA fragment was consequently purified from 1% agarose gel and sent for automated sequencing (Bioneer, South Korea) using the above mentioned primers. In order to compare the sequence of amplified DNA with the sequences in GenBank, the Basic Local Alignment Tool (BLAST) was employed (Moshfegh *et al*., 2013; Forootanfar *et al*., 2014).

Media and culture conditions: The selected isolate was cultivated at 60°C and 120 rpm for 72 h in the 500 mL Erlenmyer flasks containing 150 mL of basal medium including 10 g L^{-1} olive oil, 5 mannose 200 g L^{-1} NaCl, 2 g L^{-1} $(NH_4)_3PO_4$, 2 g L^{-1} NaH_2PO_4, 2 g L^{-1} $NaNO_3$ and 0.04 g L^{-1} $FeSO_4.7H_2O$. Interval samples were taken and the OD_{600} was determined after decanting the remained olive oil (Hasan-Beikdashti *et al*., 2012). Lipase activity of the obtained supernatant (after removing bacterial cell using centrifugation at 10000 g for 10 min) was measured as described in the next section.

Assay for lipase activity and protein estimation: Activity of the lipase was determined spectrophotometrically using ρ-NPP as substrate according to the method described by Hasan-Beikdashti *et al*. (2012) with some modifications. The reaction mixture including 0.8 mL of 0.1 M phosphate buffer (pH 7.8) containing 0.4% of Triton X-100, 0.1 mL of the freshly prepared ρ-NPP solution (0.01 M) and 0.1 mL of taken supernatant sample was incubated at 60°C and 120 rpm for 30 min. The amount of liberated ρ-NP was then quantified by reading the absorbance of the sample at 410 nm by Shimadzu double beam spectrophotometer (UV-1800, Shimadzu Corporation, USA). One Unit (U) of the lipase activity was defined as the amount of the enzyme that caused the release of

1 μmol of ρ-NP per min from ρ-NPP substrate under the standard assay conditions. The protein concentration was estimated according to the method of Bradford (1976) using bovine serum albumin as the standard.

Effect of carbon and nitrogen sources, inducers and metal ions on lipase production: Influence of different carbon sources (glucose, mannitol, fructose, galactose, glycerol, maltose, lactose, sucrose and starch all at concentration of 0.5%), nitrogen sources including peptone, yeast extract, tryptone, urea, ammonium sulfate and ammonium chloride (final concentration of 0.5%), lipase inducers (sesame oil, sweet oil, sunflower oil, castor oil, coconut oil, hazelnut oil, Tween 80 and Triton X-100 at final concentration of 1%) and 1 mM of metal ions of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $FeCl_3$, KCl and $ZnSO_4.7H_2O$ on lipase production was evaluated by separately addition of each mentioned factor to basal medium and determination of lipase activity after 16 h incubation.

Partial purification of lipase: After cultivation of the selected isolate in basal medium containing NaCl, 20% (w/v) and olive oil, 1% for 16 h, the produced biomass was removed by centrifugation (6000 g, for 10 min) and the remained fatty acids were precipitated by adding of $CaCl_2$ solution (0.4 M) and centrifugation at 4°C and 12000 g for 20 min. Thereafter, the pre-chilled ethanol was gradually added to the obtained cell-free culture broth under continuous stirring to reach 80 % (v/v) saturation followed by collecting the precipitate by centrifugation at 12000 g for 25 min. The precipitate was consequently dialyzed against phosphate buffer (0.1 M, pH 7.8) and analyzed for lipolytic activity and protein content. All the mentioned steps were performed at 4°C.

Characterization of the partially purified lipase
Effect of pH and temperature on activity and stability: Effect of pH on the lipolytic activity of the partially purified lipase was determined by measuring the concentration of ρ-NP liberated from ρ-NPP as a substrate prepared in the appropriate buffers (0.1 M) including citrate buffer (pH 3-5), phosphate buffer (pH 6-8) and carbonate buffer (pH 9-10) under standard assay conditions. In order to demonstrate the pH stability of the enzyme, the partially purified lipase was incubated at 4°C for 24 h in the above mentioned buffers (pH range of 3-11) and the remained lipase activity was measured.

The activity of the enzyme at different temperatures was determined by performing enzymatic assay at 4, 20, 30, 37, 45, 60, 70, 90 and 100°C. Effect of temperature on the stability of the partially purified lipase was evaluated by pre-incubating the enzyme sample at the above temperatures for 1 h and determining the residual activity (Cao *et al.*, 2012; Daoud *et al.*, 2013).

Effect of metal ions, EDTA, surfactants and salinity on the lipase activity: Effect of various metal ions, EDTA and

surfactants on the activity of the partially purified lipase was examined by separately pre-incubating the enzyme in the presence of $MgSO_4.7H_2O$ (10 mM), KNO_3 (10 mM), $CaCl_2.2H_2O$ (10 mM), $CuSO_4.5H_2O$ (10 mM), $AgNO_3$ (5 mM), $CoCl_2.6H_2O$ (5 mM), $CdCl_2$ (5 mM), $MnCl_2$ (5 mM), $ZnCl_2$ (5 mM), $FeCl_3$ (5 mM), Na_2EDTA (2.5 mM), Triton X-100 (1%), Tween 40 (1%), Tween 80 (1%) and SDS (1 mM) at 60°C and determining the residual lipolytic activity under enzymatic assay conditions. Furthermore, the salinity tolerance of the partially purified lipase was examined by determining the residual activity of the enzyme in the presence of NaCl (1-4 M).

Stability of the partially purified lipase in organic solvents: In order to investigate the influence of organic solvents on the stability of lipase, each of the applied organic solvents (ethanol, methanol, n-butanol, n-octanol, xylene, n-heptane, n-hexane, isopropyl alcohol, isoamyl alcohol, 1,4-dioxane, chloroform, acetone, dimethyl formamide, dimethyl sulfoxide and dichloromethane) were added to the enzyme solution to reach the final concentration of 25% (v/v) and then incubated at 60°C for 1 h. To eliminate the effect of hydrophobic organic solvents on the colorimetric assay of the enzyme activity, the organic solvents were removed by centrifugation (12000×g for 5 min) at 4°C before determining the residual activity.

Application of the partially purified lipase for the esterification of valeric acid: The partially purified lipase was used as the biocatalyst for the esterification of valeric acid in the presence of ethanol or methanol in both solvent-free and organic solvent (n-hexane, xylene, isopropyl alcohol and n-butanol) media according to the method described by Khoobi *et al.* (2015). In the case of the solvent-free system, the reaction mixture (final volume of 10 mL) was prepared by adding valeric acid solution in ethanol or methanol (acid/alcohol molar ratios of 2:1, 1:1, 1:2, 1:3, 1:4 and 1:5) to the partially purified lipase (10 U mL^{-1}, in phosphate buffer 0.1 M pH 9) followed by incubation at 50°C and 100 rpm for 1, 2, 4, 6, 12 and 24 h. For the non-aqueous medium, 4 mmol of ethanol or methanol and 1 mmol of valeric acid were dissolved in 10 mL of each applied anhydrous organic solvent (*n*-hexane, xylene, isopropyl alcohol and n-butanol) and the partially purified lipase was added to the reaction mixture and shaken at 50°C and 100 rpm for 1, 2, 4, 6, 12 and 24 h. Thereafter, the alkalimetric titration of the unreacted acid using 0.1 N of NaOH in the presence of phenolphthalein as an indicator was applied for determining the amount of the produced ester. The bioconversion percentage in the esterification reaction was then calculated from the consumed amount of valeric acid. Negative controls were designed by adding inactivated lipase to the reaction mixture.

Statistical analyses: All the mentioned experiments were performed in triplicate and the obtained data were expressed

as Mean±SD. SPSS software (ver.15) for Windows (SPSS Inc., Chicago) was used for the statistical analysis. Differences between the groups were determined using one-way Analysis of Variance (ANOVA) and p-values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Isolation and identification of the selected lipolytic isolate: Among the four isolated bacterial colonies (obtained from 15 collected samples), the production of lipase by isolate M2 was verified on the BHI agar medium containing olive oil (1%), NaCl (15% w/v) and fluorescent dye Rhodamine B at 60°C by developing an orange-colored fluorescent halos (when exposed to UV light, 350 nm) around the lipase producing bacterial strain (Fig. 1a). The biochemical characteristics of the isolate M2, a non-motile, gram-positive rod-shaped bacterium, are summarized in Table 1. The BLAST results of the 16S rDNA amplified gene (1420 bp) revealed 99% identity to *Bacillus atrophaeus*. The gene sequence was deposited in GenBank under the accession number of KF682367.

Screening oil contaminated soils, industrial effluents and salty habitats for lipase producing microbial strains has been introduced as the main step to obtain lipases harboring noticeable biochemical properties (Abdel-Fattah *et al*., 2012; Masomian *et al*., 2013). For example, Patel *et al*. (2014) isolated an organic solvent-stable *Pseudomonas* sp. DMVR46 from oil spilling sites near the industrial area able to produce alkaline lipase. Daoud *et al*. (2013) obtained a lipolytic halotolerant bacterium *Staphylococcus cohnii* strain CJ3 after screening saline and hypersaline environments, like salted animal skin, salt lake, marine sediments and effluents, from a fish processing industry. In the present study, after screening for lipase producing halophilic bacterial strain, one lipolytic isolate able to grow in the media containing 15% NaCl was separated from hypersaline lake and identified as *B. atrophaeus* FSHM2.

Lipase production: The growth curve and lipase productivity profiles of *B. atrophaeus* FSHM2 in basal medium containing NaCl (20%, w/v) and olive oil (1%) during 72 h of incubation

Table 1: Biochemical characteristics of isolate M2

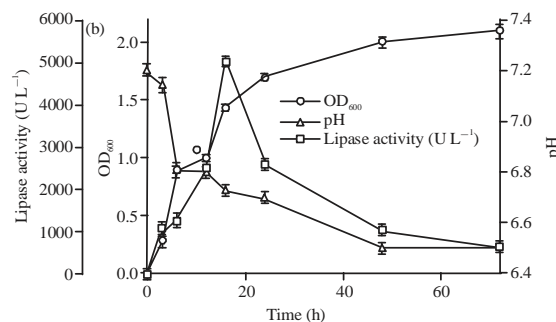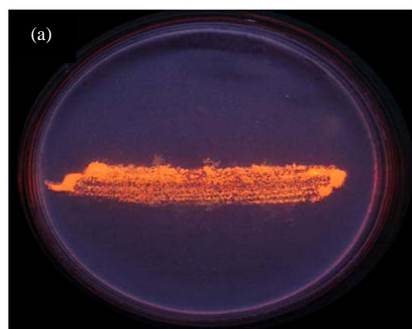| Characteristics	Results
Catalase production	+
Oxidase activity	+
Voges-Proskauer test	+
Methyl red test	-
Acid from	
D-Glucose	+
Maltose	+
D-Galactose	+
Sucrose	+
Lactose	+
Fructose	+
Mannitol	+
Glycerol	+
Hydrolysis of	
Casein	+
Gelatin	-
Starch	-
Utilization of citrate	+
Nitrate reduced to nitrite	+
Formation of	
Indole	+
Dihydroxyacetone	+
H_2S	+
Growth in NaCl (%)	
2.5	+
5	+
7.5	+
10	++
15	++
20	++
Growth at (°C)	
5	-
30	+
40	++
50	++
60	++
65	++
70	++
75	++

Fig. 1(a-b): (a) Positive colony of lipase producing isolate (*Bacillus atrophaeus* FSHM2) on BHI agar plate containing Rhodamine B after exposure to UV light and (b) growth and lipase productivity profiles of *Bacillus atrophaeus* FSHM2 during 72 h of incubation in basal medium supplemented with olive oil (1%) and NaCl (20%)

at 60°C are shown in Fig. 1b. The maximum lipase production was observed after 16 h of incubation (4995 U L^{-1}), at which the optical density of the liquid culture at 600 nm was 1.43, followed by a gradual decease to 637 U L^{-1} after 72 h (Fig. 1b). The same pattern of lipolytic activity profile was reported by Li *et al*. (2013) in which the maximum lipase productivity (2400 U L^{-1}) of *Stenotrophomonas maltophilia* CGMCC 4254 was achieved after 24 h of incubation (OD$_{600}$, 1.9) followed by a decrease to about 1100 U L^{-1}. They attributed this observation to the proteolytic degradation of the produced lipase (Li *et al*., 2013). The lipase activity of *Staphylococcus cohnii* strain CJ3 was maximally (~7500 U L^{-1}) occurred after 24 h of incubation (OD$_{600}$, 2.5) in the presence of NaCl (0.9 M) (Daoud *et al*., 2013).

Effect of carbon and nitrogen sources, lipase inducers and metal ions on lipase production: As presented in Fig. 2a the greatest increase in lipase production was observed in the culture medium containing glucose (13852.5 U L^{-1}), sucrose (6222.5 U L^{-1}) and maltose (5162.5 U L^{-1}), respectively, compared to that of the basal medium (4150 U L^{-1}). In contrast, other carbon sources including galactose, mannitol, lactose and starch significantly decreased the lipase productivity of *B. atrophaeus* FSHM2 by 4097.5, 3762.5, 2865, 1517.5, 1325 and 1200 U L^{-1}, respectively compared to the basal medium (4150 U L^{-1}) (Fig. 2a). The obtained results of the present study were in agreement with the findings of Kumar *et al*. (2012), who achieved higher activity of lipase in the culture medium supplemented with glucose. The same results were reported by Sangeetha *et al*. (2008) where the lipase activity of *B. pumilus* SG2 was enhanced in the presence of glucose. However, the negative effect of glucose on lipase production was described by Hasan-Beikdashti *et al*. (2012), who determined reduction in lipase productivity of *Stenotrophomonas maltophilia* in the presence of glucose. They attributed the suppressive role of glucose to its catabolite repression effect on lipase as the most famous inducible enzyme (Hasan-Beikdashti *et al*., 2012). As obtained in the present study, sucrose positively influenced the production of *Bacillus atrophaeus* FSHM2 lipase (Fig. 2a), which was in accordance with the study by Sangeetha *et al*. (2008), who reported the same effect of sucrose on the lipase productivity of *B. pumilus* SG2. In the case of starch the lipolytic activity of *B. atrophaeus* FSHM2 was decreased (Fig. 2a), which was in agreement with the study by Kulkarni and Gadre (2002), who reported the suppressing effect of starch on the lipase activity of *Pseudomonas fluorescens* NS2W.

As presented in Fig. 2b, the supplementation of cultivation medium by organic nitrogen sources like peptone, yeast extract and tryptone resulted in a significantly inhibitory effect on the lipase production of *B. atrophaeus* FSHM2. On the contrary,

in most cases, lipase production was positively influenced by the application of nitrogen sources. For example, Abdel-Fattah *et al*. (2012) showed that, after adding the optimized concentration of peptone (6.359 g L^{-1}) to the culture medium of *Geobacillus thermodenitrificans*, the lipase activity was enhanced by 592.59 U min^{-1} mL^{-1}. Bora and Bora (2012) attributed this observation to the release of NH$_4^+$ ions from peptone or tryptone, which stimulated the growth and simultaneously its protease inhibiting nature at low concentration. Among the inorganic nitrogen sources applied in the present study (urea, ammonium sulphate and ammonium chloride), none of them exhibited any positive effects on the lipase activity of *B. atrophaeus* FSHM2 (Fig. 2b). The same results were observed by Salihu *et al*. (2011), who reported a significant reduction in the lipolytic activity of *Candida cylindracea* after the insertion of NH$_4$Cl to the culture medium. In the study by Bora and Bora (2012), it was reported that various inorganic nitrogen sources including ammonium sulphate and urea enhanced lipase production of *Bacillus* sp. LBN2 in the range of 10-17 U mL^{-1}.

As shown in Fig. 2c, which illustrates the influence of five vegetable oils, two nonionic surfactants (Tween 80 and Triton X-100) and sweet oil on the lipolytic activity of *B. atrophaeus* FSHM2, except for hazelnut oil (6270 U L^{-1} lipase activity), the lipase production was negatively affected by other applied oil and emulsifying agents. The proportional effect of vegetable oil on the lipase production of microbial strains has been previously reported. For example, although Hasan-Beikdashti *et al*. (2012) determined the negative effect of olive oil on the lipase activity originated from *Stenotrophomonas maltophilia*, the results obtained by Dandavate *et al*. (2009), revealed 2.2-fold (14 U mL^{-1}) enhancement in the lipase productivity of *Burkholderia multivorans* V2 in the presence of olive oil. The inverse effect of Tween 80 on the lipase productivity of *B. atrophaeus* FSHM2 (obtained in the present study) was in agreement with the finding by Brozzoli *et al*. (2009), who determined 50% reduction of the lipase activity after adding Tween 80 to the culture medium of *Candida cylindracea* NRRL Y-17506.

Effects of different metal ions on the production of lipase by *B. atrophaeus* FSHM2 are illustrated in Fig. 2d. It can be noted that except for calcium ion which enhanced the lipase activity to 5505 U L^{-1}, other metal ions (Mg^{2+}, Fe^{3+}, K$^+$ and Zn^{2+}) negatively affected the lipase activity of *B. atrophaeus* FSHM2 compared to that of the basal medium (Fig. 2d). Several authors have reported the stimulatory effects of calcium, magnesium and iron ions on lipase production by different organisms. For instance, in the study by Kumar *et al*. (2005), activity of an extracellular lipase from *Geotrichum marinum* was enhanced in the presence of Mg^{2+} and K$^+$ ions. After the primary screening of ten factors (using Plackett-Burman design).
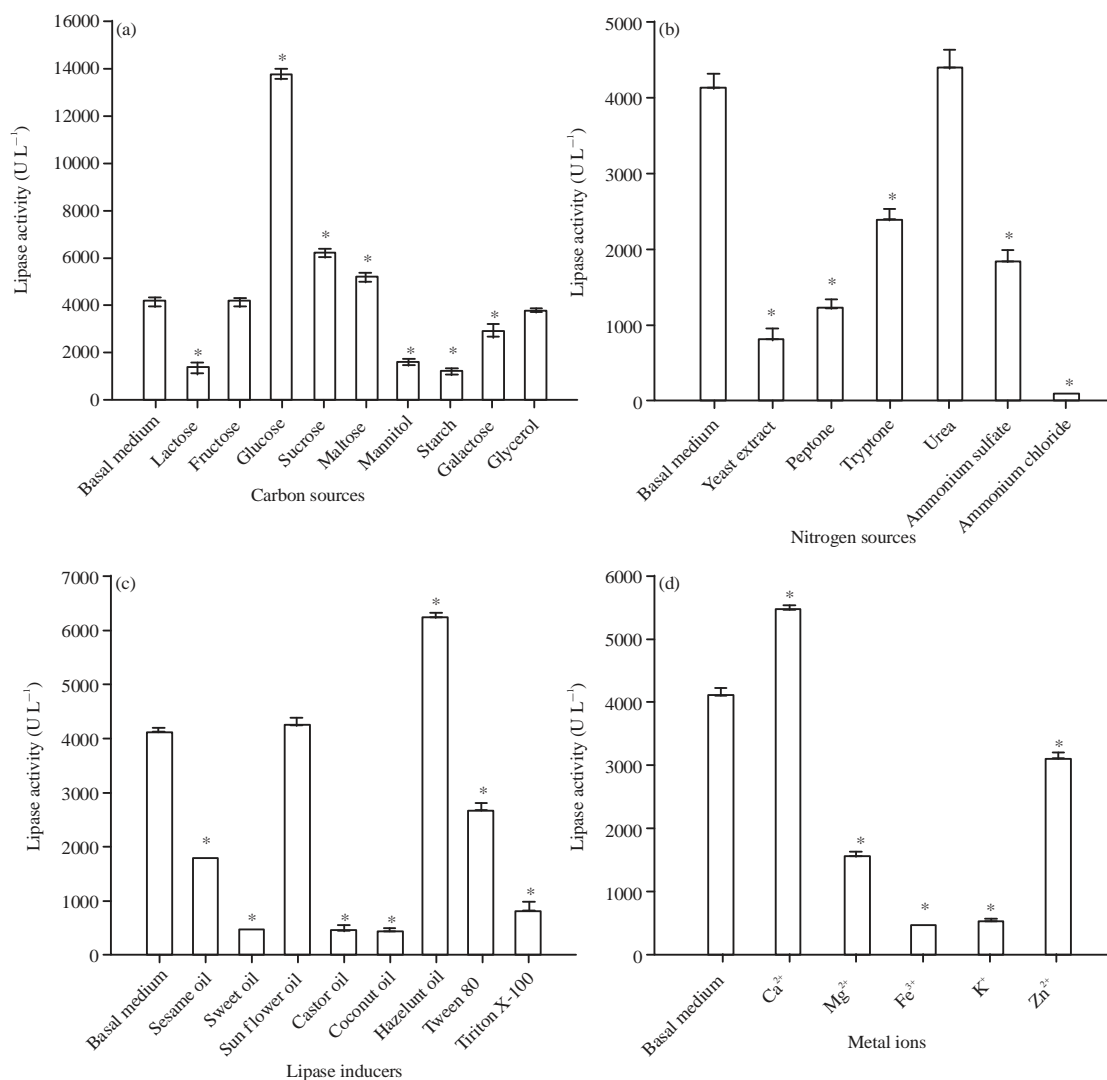
Fig. 2(a-d): Effect of (a) Carbon sources, (b) Nitrogen sources, (c) Lipase inducers and (d) Metal ions on the lipase productivity of *Bacillus atrophaeus* FSHM2 compared to the basal medium

for optimizing lipase production (Hasan-Beikdashti *et al.*, 2012), Fe^{2+} introduced as one of the most effective factors significantly increased the lipolytic activity of *S. maltophilia*.

Partial purification of lipase: The obtained results of the partial purification of produced lipase of *B. atrophaeus* FSHM2 using two consequent steps of ethanol precipitation and dialysis are summarized in Table 2. The specific activity of the enzyme increased from 1.73-12.48 U mg^{-1} after the precipitation of culture broth with ethanol (80%), which was related to 7.21-fold improvement in purification steps. After the first step of partial purification (0-70% acetone precipitation) of the organic-solvent tolerant lipase from *Burkholderia multivorans* V2, the specific activity of 24.3 U mg^{-1} related to 13.8 purification fold was achieved in

Table 2: Partial purification of the extracellular lipase from the culture broth of *Bacillus atrophaeus* FSHM2

Parameters	Centrifuged culture broth (crude)	Ethanol precipitation (0-80%)
Total activity (U)	168	67.4
Total protein (mg)	96.84	5.4
Specific activity (U mg^{-1})	1.73	12.48
Recovery (%)	100	40.12
Purification (fold)	1	7.21

the study performed by Dandavate *et al.* (2009). Ugur *et al.* (2014) reported the purification fold of 2.47 and specific activity of 149.78 U mg^{-1} after the precipitation of crude lipase from *Streptomyces bambergiensis* OC 25-4 using ammonium sulfate (90%) and dialysis by Tris-HCl buffer (50 mM, pH 8).

Characterization of the partially purified lipase
Effects of pH and temperature on activity and stability:
The effect of pH on the activity of partially purified lipase originated from *B. atrophaeus* FSHM2 was investigated by performing enzyme assay in the pH range of 3-10 using ρ-NPP as substrate. As illustrated in Fig. 3a, the lipase activity was gradually increased by increasing pH from 3-9 and the lipolytic activity maximally occurred at pH 9. The same profile was obtained in the case of pH stability experiments (Fig. 3a), where the partially purified lipase retained 100% relative activity after 24 h of incubation at 4°C. However, the relative activity dropped into 66 and 13% after 24 h of incubation of the partially purified lipase at alkaline pH 10 and acidic pH 3, respectively (Fig. 3a). The obtained results of the present study was in accordance with the findings by Patel *et al*. (2014) who reported that the alkaline lipase originated from *Pseudomonas* sp. DMVR46 retained 100% of its maximum activity at pH 8.5 after 30 min of incubation. They found 50% decrease in the relative activity following pH alteration to 10 (Patel *et al*., 2014).

As presented in Fig. 3b, the catalytic activity of the partially purified lipase from *B. atrophaeus* FSHM2 were increased by elevating temperature from 4-80°C and decreased by 92% at 100°C. The stability curve (Fig. 3b) exhibited that the partially purified lipase retained 88.2% of its initial activity after incubation at 100°C for 1 h. Based on the above mentioned results, the lipolytic enzyme of *B. atrophaeus* FSHM2 could be categorized as thermoalkalophilic lipases (Salameh and Wiegel, 2007). Salameh (2006) launched *Thermosyntropha lipolytica* as the alkalithermophilic bacteria and produced two lipases, LipA and LipB, which represented their maximal activity at 96°C and retained 50% relative activity after 24 h of incubation at 75°C. In the study by Emtenani *et al*. (2013), in which the thermoalkalophilic lipase from *B. subtilis* DR8806 was functionally expressed in *E. coli* BL21, the purified enzyme represented its maximum activity at 70°C and retained 87.5% of its initial activity after incubation at 80°C for 1 h.

Influence of metal ions, EDTA, detergents and salt concentration on lipase activity: Effects of some metal ions, EDTA and detergents on the lipase activity are summarized in Table 3. As presented in Table 3, except for Zn^{2+} (relative activity of 91.25%) and K^+ (relative activity of 86.05%), all the tested metal ions significantly decreased the relative activity of the partially purified lipase. In general, metal ions are able to affect the catalytic function of lipases by changing the solubility and behavior of ionized fatty acids via the complex formation with metal ions and direct enzyme inhibition (Emtenani *et al*., 2013; Ugur *et al*., 2014). The obtained results of the present study were in accordance with the findings of Emtenani *et al*. (2013), who achieved negative influence of metal ions on the lipase activity of *B. subtilis* DR8806. On the other hand, Patel *et al*. (2014) determined that Mg^{2+}, Ca^{2+} and Ba^{2+} significantly stimulated the lipase activity of *Pseudomonas* sp. DMVR46. They attributed such a positive effect of the mentioned metal ions to the formation of fatty

Table 3: Effect of metal ions, EDTA and detergents on the activity of the partially purified lipase originated from *Bacillus atrophaeus* FSHM2

Metal ions	Final concentration	Relative activity (%)
Control	-	100.00
Mg^{2+}	10 mM	72.05*
K^+	10 mM	86.05
Cu^{2+}	10 mM	48.8*
Ca^{2+}	10 mM	62.8*
Ag^+	5 mM	58.15*
Co^{2+}	5 mM	53.45*
Cd^{2+}	5 mM	34.85*
Mn^{2+}	5 mM	76.7*
Zn^{2+}	5 mM	91.25
Fe^{3+}	5 mM	57.2*
EDTA	2.5 mM	75.6*
Detergents		
Triton X-100	1%	41.4*
Tween-80	1%	36.5*
Tween-40	1%	7.25*
SDS	1 mM	1.8*

*: Significant means (p-value<0.05) were obtained after ANOVA analysis with dunnett's T3 post hoc test, SDS: Sodium dodecyl sulphate
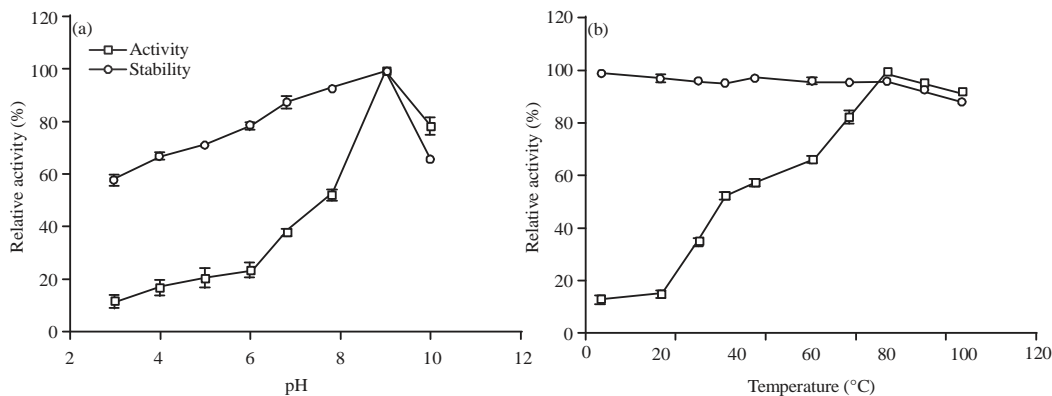

Fig. 3(a-b): Effects of (a) pH and (b) Temperature on the activity and stability of the partially purified lipase of *Bacillus atrophaeus* FSHM2

acids complexes produced during the catalysis and probable elimination of product inhibition on lipase activity (Patel *et al*., 2014).

The inhibitory effect of the chelating agent (EDTA) on the lipolytic activity of the partially purified enzyme of *B. atrophaeus* FSHM2 (Table 3) was in agreement with the results by Dandavate *et al*. (2009). However, EDTA had no effect on the lipase activity of *Burkholderia ambifaria* YCJ01 (Yao *et al*., 2013). The lipolytic activity of the partially purified lipase from *B. atrophaeus* FSHM2 was significantly inhibited in the presence of non-ionic surfactants including Triton X-100, Tween 80 and Tween 40, which was in contrast to the reports by Yao *et al*. (2013), who observed 123, 125 and 107%, relative activity of *Burkholderia ambifaria* YCJ01 lipase treated with Triton X-100, Tween 80 and Tween 20, respectively. Examination of the influence of salt concentration (1-4 M) on the lipase activity of *B. atrophaeus* FSHM2 exhibited that the relative activity increased by 2.3-fold after increasing NaCl concentration up to 4 M and the relative activity dropped to 45% at 5 M NaCl concentration (Fig. 4).

Organic solvent stability of lipase: Examination of lipases for the maintenance of their activity in the presence of various organic solvents is a key feature in the potential industrial applications (Yu *et al*., 2010). So, stability of the partially purified lipase from *B. atrophaeus* FSHM2 treated with fourteen common hydrophilic and hydrophobic organic solvents with different log p values (-1.35-4.0) (Table 4) was evaluated in the present study. As summarized in Table 4, except for xylene, n-hexane, isoamyl alcohol, n-butanol and isopropyl alcohol which increased the relative activity of the partially purified lipase to 319, 144, 116.3, 111.5 and 104%, respectively, other applied organic solvents significantly decreased the residual lipolytic activity (Table 4). All six applied solvents in the study by Yu *et al*. (2010), demonstrated a positive effect on the catalytic activity of the recombinant lipase from *Fervidobacterium nodosum* Rt17- B1. On the other hand, in the study performed by Masomian *et al*. (2013), it was found that among twelve tested organic solvents, 75 and 23% of the initial activity of lipase obtained from *Aneurinibacillus thermoaerophilus* were lost in the presence of dodecanol and xylene, respectively.

Esterification studies: Application of lipases (either free or immobilized forms) originated from bacterial or fungal strains for the biosynthesis of industrially important aroma esters as flavour and fragrance compounds has received great attention during the last decades (Li *et al*., 2013; Kumar *et al*., 2013). It has been shown that lipase-mediated esterification reaction is influenced by several parameters such as molar ratio of acid to alcohol, type of applied solvent and temperature of reaction mixture (Dhake *et al*., 2013). In order to evaluate the effect of molar ratio of acid to alcohol on the esterification assisted by

Table 4: Organic solvents (25% v/v) stability of the partially purified lipase from *Bacillus atrophaeus* FSHM2

Organic solvent	Log p	Relative activity (%)
Control	-	100
Ethanol	-0.24	81.9*
Methanol	-0.76	36.5*
n-Butanol	0.89	111.4*
n-Octanol	2.9	65.5*
Xylene	3.1	319*
n-Hexane	3.5	144*
n-Heptane	4.0	98.4
Isopropyl alcohol	0.28	104
Isoamylalcohol	1.3	116.3*
1,4-Dioxane	-0.27	55.7*
Chloroform	2.0	8.2*
Acetone	-0.23	20.5*
DMF	-1	49.2*
DMSO	-1.35	55.7*
Dichloromethane	1.25	100

The partially purified lipase and organic solvents were mixed in a 3:1 ratio and the mixture was incubated at 60°C for 1 h and assayed for residual lipolytic activity. Data is mean of three replicated experiments, *: Significant values (p-value<0.05) were achieved after ANOVA analysis with Dunnett's T3 post hoc test, DMSD: Dimethyl sulfoxide, DMF: Dimethyl form amide
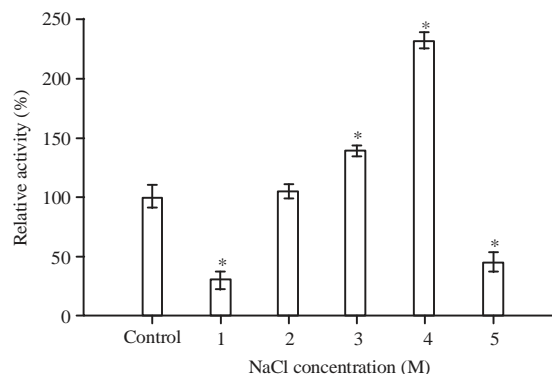

Fig. 4: Effect of NaCl concentrations on the lipolytic activity of the partially purified lipase from *Bacillus atrophaeus* FSHM2. *Significancy (p-value<0.05) was estimated after the ANOVA analysis of the obtained results (n = 3)
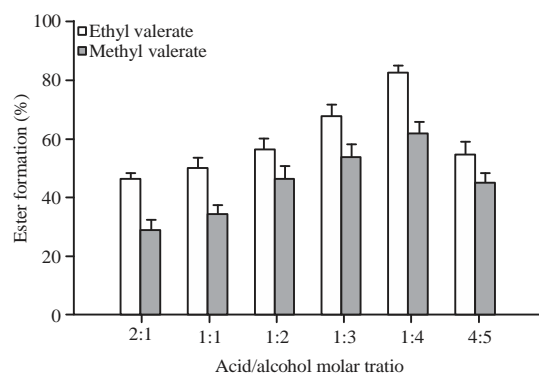

Fig. 5: Effect of valeric acid/ethyl or methyl alcohol molar ratio on esterification percent using the partially purified lipase of *Bacillus atrophaeus* FSHM2 in xylene medium at 50°C
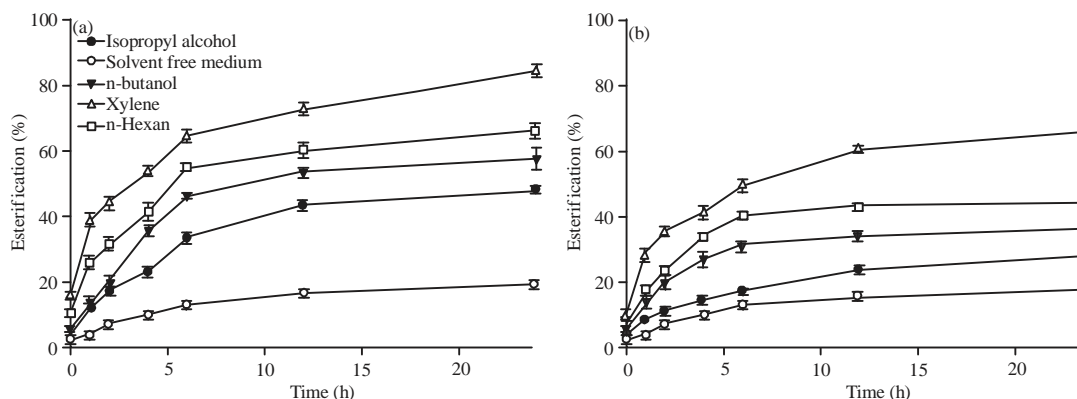
Fig. 6(a-b): Time course of (a) ethyl and (b) methyl valerate synthesis using the partially purified lipase of *Bacillus atrophaeus* FSHM2 in aqueous and non-aqueous media. Data is shown as Mean±SD (n = 3)

the partially purified lipase of *B. atrophaeus* FSHM2, various molar ratios were applied in the reaction mixtures. As illustrated in Fig. 5, in both cases of methyl and ethyl valerate the molar ratio of 1:4 (v/v) acid to alcohol was the best one to obtain the highest esterification percentages (81.6 and 62.4% for ethyl valerate and methyl valerate, respectively). In the study by Kumar *et al.* (2013), the molar ratio of 1:1 (v/v) oleic acid and ethanol was introduced as the best to get the maximum ethyl oleate biosynthesis (63%) using the immobilized lipase of *Bacillus* sp. DVL2. Khoobi *et al.* (2015) achieved the highest esterification yield (using the immobilized lipase of *Thermomyces lanuginosa* on polyethyleneimine-modified superparamagnetic Fe_3O_4) at the molar ratio of 1:4 valeric acid/ethyl alcohol. The obtained results of esterification experiments (Fig. 6) revealed that replacement of aqueous reaction medium with organic solvents increased the esterification yield. As exhibited in Fig. 6a, formation of ethyl valerate maximally occurred in the reaction medium containing xylene by 85.3% conversion after 24 h. In the case of methyl valerate, replacement of aquatic medium with xylene increased esterification percentage from 17.5-66.2% after 24 h of incubation (Fig. 6b). The same results were reported by Khoobi *et al.* (2015), who determined 72.9% conversion of valeric acid using the immobilized lipase of *Thermomyces lanuginosa* on polyethyleneimine grafted magnetic nanoparticles compared to that of the solvent-free medium (28.9%). The positive effect of organic solvents on esterification reactions could be attributed to the shifting of the reaction equilibrium toward ester synthesis by total transfer of ester to the organic phase (Khoobi *et al.*, 2015).

CONCLUSION

To sum up, an extracellular thermoalkalophilic lipase which maximally worked at 80°C and pH 9 was partially purified from the halotolerant bacterial strain *B. atrophaeus* FSHM2. Stability of the partially purified lipase was significantly increased in the presence of xylene (3.19-fold)

and n-hexane (1.44-fold) but, SDS, EDTA and all the tested metal ions had a negative influence on its activity. The enzyme activity was significantly increased (2-fold) in the presence of 4 M NaCl. Application of the obtained lipase for esterification experiments in the organic solvent (xylene) containing medium revealed 85.3 and 66.2% production of ethyl and methyl valerate, respectively, after 24 h of incubation.

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