



# An organic solvent-tolerant lipase of *Streptomyces pratensis* MV1 with the potential application for enzymatic improvement of n6/n3 ratio in polyunsaturated fatty acids from fenugreek seed oil

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Revised: 18 August 2020 / Accepted: 9 September 2020 / Published online: 17 September 2020  
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**Abstract** Lipase-catalyzed esterification is an efficient technique in the production of polyunsaturated fatty acid (PUFA) concentrates which are applied for nutrition and health purposes. In this project, a solvent-tolerant lipase from *Streptomyces pratensis* MV1 was immobilized and purified by a hydrophobic support. The purified lipase revealed enhanced activity and stability towards chemicals, organic solvents, and a broad range of pH values. The production of lipase was enhanced to 7.0 U/mL after optimization by a central composite design. Acylglycerols (AGs) rich in  $\alpha$ -linolenic acid (45%, w/w) were produced and a favorable n-6/n-3 free fatty acid (FFA) ratio of 1.1 was achieved in fenugreek seed oil using the immobilized lipase. The ability of *S. pratensis* lipase in ester synthesis and the improvement of n6/n3 FFA ratio make it a suitable candidate in food production industries.

**Keywords** n-6/n-3 free fatty acid ratio · Polyunsaturated fatty acids · Fenugreek seed oil · Solvent tolerant lipase · *Streptomyces pratensis*

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s13197-020-04784-w>) contains supplementary material, which is available to authorized users.

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## Introduction

Polyunsaturated fatty acids (PUFAs) particularly  $\alpha$ -linolenic acid (ALA) are health-promoting fatty acids, because of their role in reducing blood LDL cholesterol, regulation of inflammation, brain functions, fluidity or integrity of membrane bilayer structures, vasodilation, preventing cardiac arrhythmias, and reducing thrombosis by conversion to eicosanoids (Nicholson et al. 2013). Recent studies have shown that PUFAs can strengthen the immune system and increase resistance and recovery from virus diseases such as the Middle East Respiratory Syndrome (MERS) and the Severe Acute Respiratory Syndrome (SARS-CoV-2) infections (Galanakis 2020). The human body does not contain enzymes to biosynthesize ALA, so it should be supplied in the diet. One of the current strategies to develop PUFA functional foods is mainly focused on the addition of one or a combination of PUFAs from natural sources to nutrients to enhance their total content (Granato et al. 2020). Utilization of conventional recovery methods such as thermal technologies are often restricted due to high energy consumption, overheating of the food matrix, loss of functionality and poor stability of the final product (Galanakis 2012). In order to produce functional foods, it is important to explore alternative processing technologies (e.g., high hydrostatic pressure (Kovačević et al. 2018), pulsed electric fields (Barba et al. 2015), ultrasounds (Deng et al. 2015; Zinoviadou et al. 2015a, b), and microwaves (Sarfarazi et al. 2020; Deng et al. 2015; Zinoviadou et al. 2015). These sustainable technologies provide more efficient preservation of native nutrients in food, and consume less energy (Galanakis et al. 2013). PUFAs are very sensitive to autoxidation causing undesirable taste and odor during food processing. To overcome these problems during

processing and storage, multiple strategies have been explored such as the use of antioxidants (Galanakis 2018; Galanakis et al. 2018), encapsulation approaches (e.g., freeze-drying, spray-drying, and spray granulation), electrospray, microgels, and enzymatic synthesis. Enzymatic synthesis of PUFA in acylglycerols (AGs) form in the presence of organic solvents provides various industrial advantages such as suppression of water-dependent side reactions, increase in solubility of reactants, and elimination of microbial contamination (Granato et al. 2020; Imanparast et al. 2018; Umesha, 2014).

Lipases (acylglycerol acyl hydrolases, EC 3.1.1.3.) can be successfully used as biocatalysts for hydrolysis, esterification, interesterification, and modification of oils (Gandhi 1997). Lipase-catalyzed esterification can be a suitable method for producing PUFA concentrates as AGs which are applied in nutrition, clinical, health, pharmaceutical, and medical purposes (Akoh and Sista 1995). Productions of lipases are widely reported in animals, plants, and microorganisms, but microbial lipases are commercially applied in biotechnological applications due to their greater stability and wider availability (Chakraborty and Raj 2009).

The concentration of PUFA from a wide range of organisms has been reported in previous studies, such as fish oil, marine alga (Guil-Guerrero et al. 2007), vegetable oil (Senanayake and Shahidi 2004), and some edible oils (López-Martínez et al. 2005). Vegetable oils could be an appropriate source for high concentrates of PUFA owing to their low cost, availability, and absence of heavy metal and hence toxicity concerns (Zahir et al. 2005).

Fenugreek belongs to the family of Fabaceae and is indigenous to southeastern Europe and Western Asia (El Nasri and El Tinay 2007). Fenugreek seeds containing 7.5% oil are a suitable source of PUFA containing large amounts of ALA and linoleic acid with 66–82% and 26–40%, respectively. The concentration of PUFAs in the oil of fenugreek seeds by means of enzyme has not been reported so far. There are several studies on the physiochemical characteristics of fenugreek seeds (El-Sebaïy and El-Mahdy 1983). The ratio of n-6/n-3 free fatty acid (FFA) in the common diet of western people is 10–30. Due to high consumption of plant oils and relatively low-available sources of marine products, a ratio of less than 5 is recommended by nutritionists (Orsavova et al. 2015). Thus, functional foods rich in n-3 PUFAs are suggested mainly for vegetarians, whose blood has characteristically high n-6/n-3 ratios (Imanparast et al. 2018).

The present study was designed to screen and identify a solvent-tolerant lipase producing microorganism followed by simultaneous purification and immobilization of the enzyme. The efficiency of an immobilized solvent-tolerant lipase of *Streptomyces pratensis* MV1 was investigated for

the enzymatic esterification of acylglycerols rich in omega-3 PUFA of fenugreek seed oil. According to Literature, there is no report on the isolation of a solvent-tolerant lipase of this microorganism; hence, this is the first attempt to report a solvent-tolerant lipase from *S. pratensis* MV1 and its application for production of acylglycerols rich in n-3 PUFA. Furthermore, the immobilized lipase showed high efficiency to n-3 for enzymatic production of functional foods with high content of n-3 PUFAs.

## Materials and methods

### Chemicals and reagents

Rhodamine B, Pluronic P123, tetraethoxysilane (TEOS), (3-aminopropyl)triethoxysilane (APTES), dimethylformamide (DMF), and *p*-nitrophenyl palmitate (*p*-NPP) were supplied from Sigma (St. Louis, MO, USA). Surfactants, bovine serum albumin, sodium dodecyl sulfate (SDS), urea, and media components were purchased from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade.

### Isolation and identification of the solvent-tolerant lipase producing strain

Lipase-producing bacteria were simultaneously isolated and screened by Rhodamine B agar plate from the oil-contaminated regions, petroleum refineries, and coal mines in Iran. The Rhodamine B agar plates were incubated at 28 °C for 48–168 h and the isolates with orange fluorescent halos under UV irradiation (350 nm) were applied for secondary screening by a colorimetric assay (Hasan-Beikdashti et al. 2012). The stability of the enzyme was examined in the presence of organic solvents (25%, v/v) including methanol, *tert*-butyl alcohol, toluene, hexane, and cyclohexane. The lipolytic isolate with maximal stability in multiple solvents was identified based on molecular characterization and preserved at –196 °C for further investigation. Further details are mentioned in the Supplementary Material.

### The enzyme assay

The enzyme substrate *p*-NPP was used to assay the lipase activity according to the method of Hasan-Beikdashti et al. (2012). The substrate solution (1 mM) was prepared and added to the reaction with the incubation of mixture performed at 45 °C for 10 min. The lipase activity was stopped by acetone/ethanol (1:1, v/v) and the release of *p*-NP was monitored at 420 nm. One lipase unit is defined as the

amount of the enzyme required to release 1  $\mu\text{mol}$  *p*-NP/ min mL.

### Production optimization of lipase

The optimization of the lipase production was performed in the fermentation medium containing (g/L) yeast extract, (0.3–6.2); peptone, (1.6–18.4); olive oil, (0.5–3.0% v/v),  $\text{NH}_4\text{Cl}$ , 5;  $\text{NaCl}$ , 9;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 by means of response surface method (RSM). A central composite design (CCD) was employed including peptone ( $X_1$ ), yeast extract ( $X_2$ ), and olive oil ( $X_3$ ). The variables were determined at five levels ( $-\alpha$ ,  $-1$ ,  $0$ ,  $+1$ , and  $+\alpha$ ), and the central point with six replications. A total of 20 experiments with different settings were obtained by Design Expert software (version 10.0.7, Stat-Easy Inc. Minneapolis, MN, USA). The significance of experimental data was evaluated by Analysis of Variance (ANOVA) statistical test.

### Immobilization and purification of the bacterial lipase

The mesoporous SBA-15@oleic acid was synthesized according to the method reported previously (Imanparast and Faramarzi 2018). Briefly, Pluronic P123 and TEOS were utilized as a structure-directing agent and silica source, respectively. The crystallization of the resulting gel was performed at 130 °C for 24 h followed by calcination to remove surfactant at 550 °C for 6 h. APTES was used for amino-functionalization of SBA-15. SBA-15@oleic acid was prepared by mixing the amine-functionalized support with DMF, alkalized oleic acid, and trimethylamine for 24 h. The enzyme immobilization was carried out by mixing 1 g of SBA-15@oleic acid with 50 mL of concentrated protein solution in phosphate buffer (50 mM, pH 7.4) at 25 °C for 6 h. The obtained immobilized enzyme was washed three times with phosphate buffer to discard unbound proteins. Thereafter, a linear gradient of 0–1% SDS in Tris buffer (50 mM, pH 8) was applied to elution of the support to separate unwanted bound proteins. The eluted fractions with lipase activity were pooled and lyophilized. Specific activity of the purified lipase was calculated with measuring total activity of the purified enzyme/ mass of the enzyme in mg. The total amount of the enzyme was estimated according to the method of Bradford (1976) by bovine serum albumin (BSA) as a standard. The yield (IY) and efficiency (IE) of lipase immobilization was calculated by the following equations (Tarasi et al. 2018):  $\text{IY}(\%) = [(Y_0 - Y_1)/Y_0] \times 100$ ,  $\text{IE}(\%) = [E_1/E_0] \times 100$ ; where  $E_0$  and  $E_1$  represent the activity of total and immobilized enzyme, respectively.

Also,  $Y_0$  and  $Y_1$  indicate the amounts of total protein and protein that did not bind to SBA-15@oleic, respectively.

### SDS-PAGE and zymography

The crude and purified lipases were subjected to SDS-PAGE gel electrophoresis on 12% polyacrylamide and stained with silver nitrate (Laemmli 1970). The molecular weight of proteins was determined with the migration rates of standard proteins. Zymography was also performed to visualize the lipase activity. Triton X-100 (2.5%) was utilized to wash and remove SDS. The washed gel along with an agar plate containing phenol red (0.01%) and 1% olive oil (pH 7.3–7.4) were incubated at 45 °C for 20 min. The emergence of distinct yellow bands against a pink background represented lipase activity on the gel (Singh et al. 2006).

### Biochemical characterizations of the purified lipase

#### *Thermal and pH stability and the activity of lipase*

Glycine–NaOH (pH 10.0–11.0), Tris–HCl (pH 9.0), phosphate (pH 7.0–8), and citrate (pH 3.0–6.0) buffers were used as general solutions to evaluate the simultaneous effect of pH (3.0–11.0) and temperature (15–65 °C) on the relative enzyme activity. The pH and temperature stability were investigated after 60 min of incubation at 25–65 °C in the mentioned buffers. The percentage of residual lipase activity was determined under standard assay conditions.

#### *Organic solvent stability of the enzyme*

The effects of different organic solvents on the lipase activity under the standard assay conditions were determined by mixing the enzyme aliquots in 25, 50, and 80% (v/v) of solvents at 25 °C for 60 min. The residual activity was calculated by measuring the enzyme activity in the presence of organic solvent/ the enzyme activity in the absence of organic solvent  $\times 100$ .

#### *Effect of metal ions, detergents, and inhibitors*

The enzyme stability was evaluated in the presence of metal ions including  $\text{Mg}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cu}^{2+}$  at the concentrations of 1, 5, and 10 mM. Additionally, the influence of some inhibitors and surfactants on the enzyme stability was estimated after 60 min of incubation at 25 °C. The enzyme inhibitors and surfactants were 2-mercaptoethanol (2-ME) (0.1, 0.25, and 0.5%), ethylenediaminetetraacetic acid (EDTA; 1, 5, and 10 mM), urea (1, 5, and 10 mM), sodium dodecyl sulfate (SDS; 0.1, 0.25, and 0.5%), tween 80% (0.1, 0.25, and 0.5%), triton

X-100 (0.1, 0.25, and 0.5%), and docusate sodium salt (AOT; 1, 5, and 10 mM). The mentioned materials were prepared in potassium phosphate buffer (pH 7.0).

### Enzymatic improvement of n6/n3 ratio in fenugreek seed oil

#### Extraction of the crude oil

Fenugreek (*Trigonella foenum-graceum*) seeds were purchased from a local grocery store in Kerman, Iran. They were cleaned and crushed using an electric grinder. The Soxhlet apparatus was used to extract 100 g of the seed flour. The obtained oil was maintained under nitrogen inside dark bottles at  $-20\text{ }^{\circ}\text{C}$  prior to further analysis. The synthesis process was divided into four main stages: (i) the extraction of FFAs from fenugreek seed oil, (ii) PUFA concentration, (iii) enzymatic esterification, and (iv) GC analysis. In all cases, the yield of each step was calculated as follows:

$$\text{Yield} = \left( \frac{\text{Recovered mass of the desired compound; g}}{\text{Initial mass of substrate or raw material; g}} \right) \times 100.$$

#### Preparation of FFAs from fenugreek seed oil

Free fatty acids from fenugreek seed oil were obtained by saponification (Wanasundara and Shahidi 1999). The oil sample (50 g) was saponified in 1.2 N KOH/EtOH (172 mL) and water (22 mL) at  $62\text{ }^{\circ}\text{C}$  for 1 h under a blanket of nitrogen. The unsaponifiable part was discarded by adding 100 mL distilled water and extracted using hexane. The saponifiable layer was acidified by 3 N HCl and the released free fatty acids were extracted using hexane. FFAs with mass yield of 77.7% (w/w) were recovered by a rotary evaporator and stored at  $-60\text{ }^{\circ}\text{C}$ .

#### Concentration of PUFAs

The PUFAs were concentrated using urea-fatty acid complexation method according a recognized process (Wanasundara and Shahidi 1999). FFAs (10 g) were added to a solution of urea (30 g) in 150 mL of 95% aqueous ethanol, with the mixture heated ( $60\text{--}65\text{ }^{\circ}\text{C}$ ) and stirred until a homogeneous solution was obtained. The urea complex was incubated at room temperature for 4 h followed by storage overnight at  $4\text{ }^{\circ}\text{C}$ . The formed crystals were separated from the liquid by vacuum filtration. The ethanolic solution consisting of PUFAs was diluted with water, where pH decreased to 4–5. Additionally, hexane was added in equal volume while stirring for 1 h. Then, the

hexane layer was eluted with distilled water to discard any residual urea. The mass yield of 45.2% (w/w) was calculated for PUFA concentrations.

#### Esterification reaction

The acylglycerols were synthesized in a screw-capped crystal flask composed of PUFA concentrates (0.25 g), glycerol (1 g), *tert*-butanol/n-hexane (3 mL; 40:60, v/v), and 200 mg of the immobilized lipase. The reaction solution was incubated for 4 h in an orbital shaker (150 rpm,  $45\text{ }^{\circ}\text{C}$ ), with molecular sieves added to remove the water formed during the reaction after 1 h. Finally, the immobilized lipase and the molecular sieves were separated by centrifugation. The obtained FFAs were neutralized by titration using 0.025 mol/L NaOH and subjected to thin-layer chromatography (TLC) and gas chromatography (GC) analyses. The acid value was calculated according to the following equation:

$$\text{FFA content (w/w \%)} = \left[ \frac{(40 \times N \times V \times MW_{\text{FFA}})}{m} \right] \times 100;$$

where V is the used volume in mL of NaOH solution, N represents normality of NaOH, m indicates the mass in g of the product taken for the titration, and  $MW_{\text{FFA}}$  is the molecular weight of FFA. The molecular weight of 279 g/mol was considered as an average molecular weight of FFAs.

#### Analysis of fatty acid compositions

The samples were applied for TLC on silica 60 F254 and developed in an acetic acid/chloroform/acetone (1:95:4, v/v) mobile phase. The reaction products were visualized by iodine vapor as the reagent. The obtained acylglycerols were converted into fatty acid methyl ester (FAME) according to the Metcalfe method (1966) and analyzed via gas chromatography (GC) equipped with a fused silica capillary column (BPX70, 0.25 mm  $\times$  30 m i.d.) and a flame ionization detector (FID). The column temperature was initially set at  $180\text{ }^{\circ}\text{C}$  for 9 min and increased at the rate of  $20\text{ }^{\circ}\text{C}/\text{min}$  to  $200\text{ }^{\circ}\text{C}$ , and then maintained for 25 min. The identification of fatty acids from Fenugreek oil was performed based on the comparison of their retention times with the pure FAME standards. The GC analysis of the Fenugreek oil revealed that it was not contained pentadecanoic acid (C15:0), so for quantification of fatty acids pentadecanoic acid methyl ester (10 mg/mL) was applied as internal standard (IS) without interfering with the results and the injection volume was 1  $\mu\text{L}$ . Based on the area of IS peak, the concentration of fatty acids were reported as weight percentages according to the following equation:

FAME content = [The peak area of each methyl ester  
 × (The concentration of IS/ The peak area of SI)]/  
 Mass of the sample.

## Statistical analyses

The experiments of this study were performed in triplicate with the obtained results mentioned as mean. Statistical assessments were conducted by ANOVA and values < 0.05 were considered significant. The statistical analyses were done by SigmaPlot version 14.0.

## Results

### Solvent-tolerant lipase producing strains

Thirty-five lipase-producing isolates were obtained during a screening program using Rhodamine B-agar plate from soil samples. Among them, 5 isolates with a high activity in the liquid medium were then selected for stability screening in various organic solvents (Tables S1 and S2). An isolate was chosen with the maximal lipase activity and organic solvent stability. The isolate was identified as *Streptomyces pratensis* MV1 (Gene-Bank accession No. MK447746) based on 16S rRNA gene-sequence analysis following comparison to reference strains by EzTaxon database (Fig. S1). The extracellular lipase production of the strain reached to 1950 and 900 U/L after approximately 5 days in LB and basal salt medium, respectively (Fig. S2). More details have been mentioned in the Supplementary Material.

### Optimization of lipase production by statistical model

According to the obtained results of RSM (Tables S3 and S4), a quadric polynomial model was proposed. The coefficients of independent variables were determined as follows:

$$R_1 = 6.55 - 0.9 X_1 + 0.42 X_2 + 0.42 X_3 \\ + 0.52 X_1 X_2 - 1.32 (X_1)^2 - 0.98 (X_2)^2 - 0.51 (X_3)^2.$$

The analysis of variance (ANOVA) and F-test were applied for checking the statistical significance of the equation with  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_1 X_2$ ,  $X_1^2$ ,  $X_2^2$ , and  $X_3^2$  considered as the most significant factors on the response. Figure 1 presents the interaction effect of variables through 3D surface and counter plots. The optimized medium composition

contained (g/L) yeast extract, 1.5; peptone, 7.0; NaCl, 9;  $MgSO_4 \cdot 7H_2O$ , 0.5;  $NH_4Cl$ , 5; and olive oil 2.0% v/v. Under the optimized conditions, the production of lipase was three times higher than that of the basal fermentation medium.

### Simultaneous purification and immobilization of *S. pratensis* lipase

The solvent tolerant lipase from *S. pratensis* was immobilized and purified by SBA-15@oleic acid nanoparticles. The obtained results of SDS-PAGE and silver staining analysis showed no band at 0–0.5% and 1% of SDS, while one protein band with the molecular weight of about 63 kDa was recognized at 0.75% of SDS (Fig. S3). More results and details have been provided in the Supplementary Material. The obtained results of the purification process of lipase are summarized in Table 1. At the end of the procedure, the purity of the enzyme was estimated to be about 14.7-fold greater than that of the crude extract. The purified lipase with a specific activity of  $445.45 \pm 8.4$  U/mg accounted for about  $58.0 \pm 0.5$  of the total activity. The maximum immobilization efficiency and yield were obtained as  $59 \pm 2.3\%$  and  $72 \pm 3.2\%$ , respectively, at pH 7 and 6 h in this research.

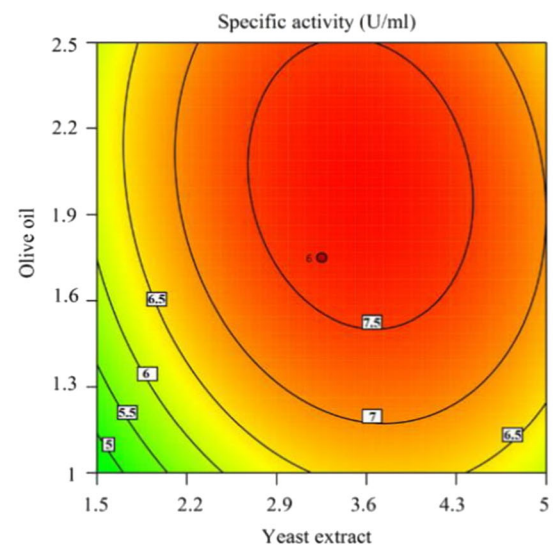
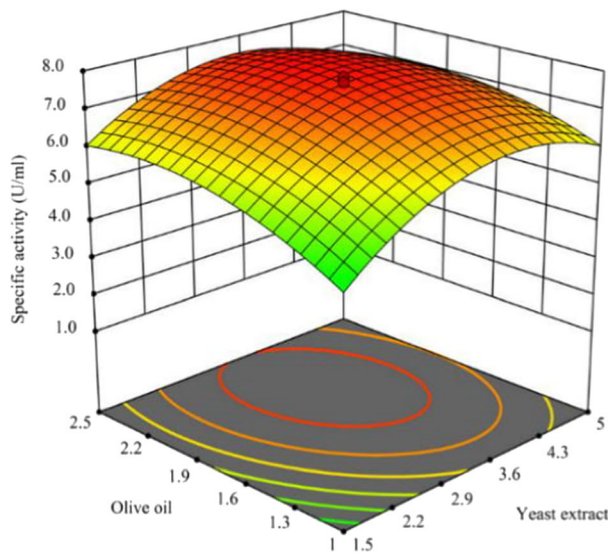
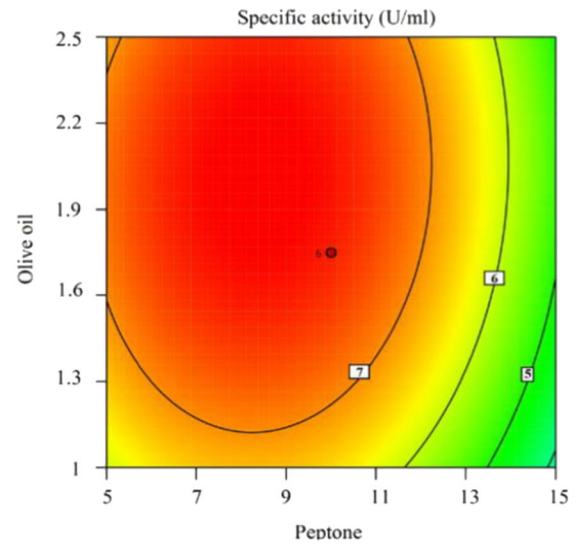
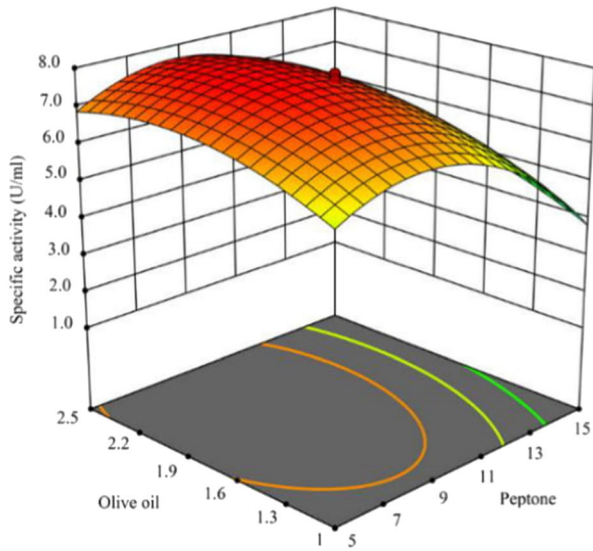
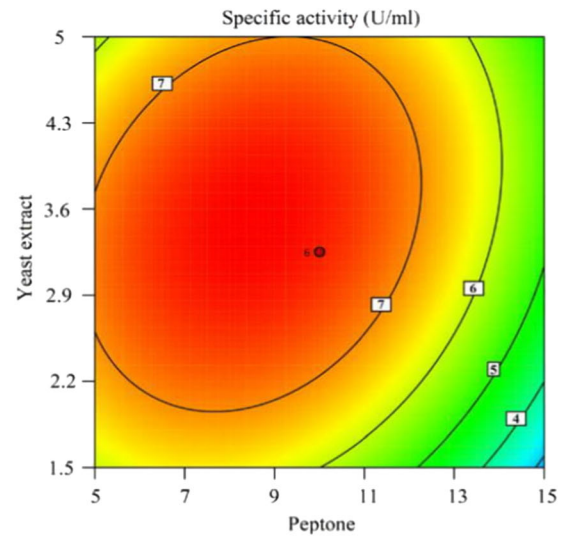
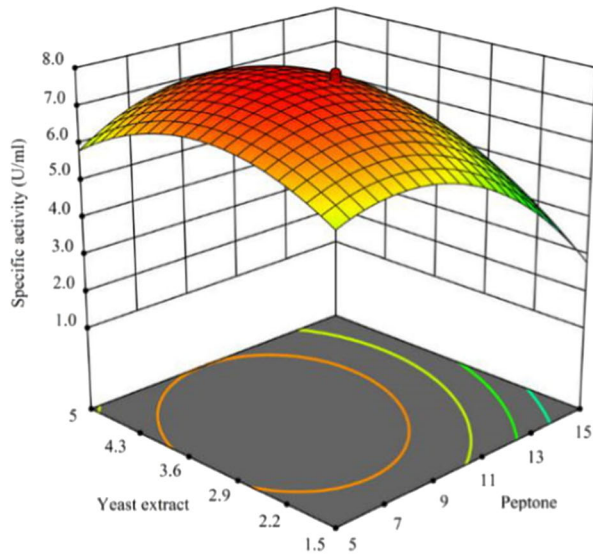
### Biochemical characterization of the purified enzyme

#### Optimal pH and temperature of the enzyme stability and activity

The lipase revealed maximum stability at pH 8, and maintained 80% of the activity at a pH of 5.0–9.0 at 25 °C. The lipase kept about 50% of the activity after 1 h incubation at 55 °C and pH 7. The results from our study indicated that the maximum activity occurred at 45 °C and pH 8 with the enzyme maintaining 65% of its maximum activity at pH 9.0. The lipase exhibited pH-sensitivity, with a considerable decline in the enzyme activity detected below pH 8.0 and above 9.0. The data of Fig. 2 also displayed that the enzyme had maximal activity at slightly alkaline pH. The enzyme was active within 15–65 °C and retained about 40% of its total activity at 55 °C and pH 8.0.

#### Stability of lipase in the presence of organic solvents

The enzyme showed approximately 25, 30, 32, and 45% increases in the activity compared to control in the presence of 25% cyclohexane, hexane, n-hexadecane, and isooctane, respectively (Table 2). Although the stability and activity of lipases in organic solvents such as ethanol, methanol, DMFA, and acetone are rarely detected (Ogino and Ishikawa 2001), in the present study, the results



**Fig. 1** Production optimization of lipase by a response surface methodology. The main impacts of peptone content ( $x_1$ ), yeast extract ( $x_2$ ), and olive oil ( $x_3$ ), and their relations are revealed for the enzyme production (%)

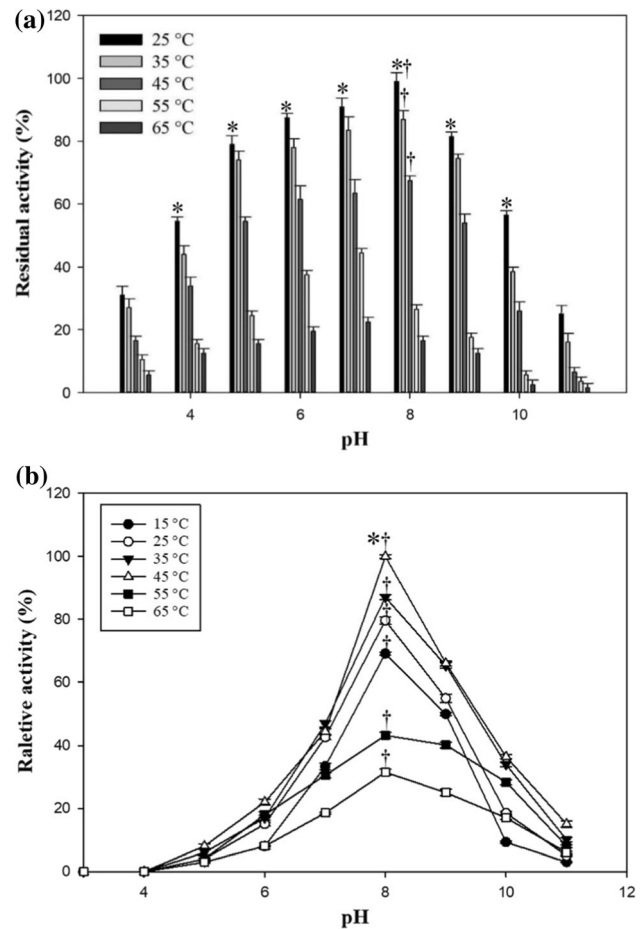
revealed that the enzyme retained 98 and 84% of activity in 25% (v/v) of DMSO and methanol, respectively.

*Effectors and inhibitors on lipase stability*

The lipase stability was moderately inhibited by  $Al^{+2}$ ,  $Cu^{+2}$ ,  $Fe^{+2}$ , and  $Mn^{+2}$  at 10 mM and strongly inhibited by  $Hg^{+2}$  at 5–10 mM concentrations. No stabilizing effect was observed in the presence of  $Mg^{+2}$  and  $Zn^{+2}$ , but > 100% activity was achieved with some ions such as  $Fe^{+2}$ ,  $Ca^{+2}$ ,  $Mn^{+2}$ , and  $Al^{+3}$  at 1 mM. Note that after incubation with reducing agents and detergents, the enzyme not only exhibited high stability but also presented > 50% increase in the activity against 2-ME and AOT (Table S5). In this study, it was found that tween 80%, Tris–HCl, and urea stimulated the lipase activity.

**Enzymatic improvement of n6/n3 FFAs ratio in fenugreek oil**

The esterification reaction was carried out in a substrate molar ratio [1 g glycerol (about 10 mmol): 0.25 g PUFA (about 1 mmol)] = 10:1 and 200 mg of the immobilized lipase with an enzyme/substrate ratio of 2.2%. In general, an increase on the glycerol content will affect the theoretical equilibration values that raise the yield of AGs accordingly. Furthermore, glycerol can act as an effective stabilizer against thermal and solvent deactivation. Nevertheless, the increase of glycerol content will also influence the system polarity, which subsequently affects the system stability and homogeneity. In addition, especially for the enzymes with hydrophilic carriers, glycerol may form a coating surrounding the enzyme, which subsequently leads to enzyme clumping and inactivation (Zhong et al. 2009). In the present study, SBA-15@oleic acid as a hydrophobic support was applied for the enzyme immobilization that can be less affected by high glycerol concentration. This suggests that while glycerol concentration



**Fig. 2** The combined effects of pH and temperature on the lipase **a** stability and **b** activity by determining the residual activities after 60 min of incubation within a wide range of pH values and temperatures. (a) \* $p < 0.05$  versus 25 °C at pH 11 and pH 3; †  $p < 0.05$  versus 65 °C at pH 8. (b) \* $p < 0.05$  versus 65 °C and 55 °C at pH 8; †  $p < 0.05$  versus pH < 8.0 and > 9.0

has a significant effect, the type of carrier used to enzyme immobilization may be more important for the glyceride synthesis than glycerol concentration. In accordance to the present study, glyceride synthesis increased with increasing glycerol content and substrate molar ratio of 10:1 and 25:1 were optimal for *Candida rugosa* lipase and porcine pancreas lipase (Yesiloglu and Kilic 2004).

The obtained results of titration using 0.025 mol/L NaOH that indicated 40% (w/w) of PUFAs were remained

**Table 1** Purification of lipase by the hydrophobic support

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	1000	6900 ± 32.4	305.5 ± 7.5	22.58 ± 0.5	100 ± 1.1	1.0 ± 0.04
Dialyzed precipitate	10	4250 ± 26.0	140.6 ± 2.5	30.23 ± 0.8	61.6 ± 1.0	1.3 ± 0.1
Affinity chromatography	0.5	2450 ± 15.4	5.5 ± 0.2	445.45 ± 8.4*	58.0 ± 0.5	14.71 ± 0.5

\* Indicates significant difference ( $p < 0.05$ ,  $n = 3$ ) at confidence interval of 95% compared to the crude enzyme

**Table 2** Stability of lipase in the presence of organic solvents at 25 °C for 60 min

Organic solvent	Log <i>P</i>	Residual activity (%)		
		25%	50%	80%
Control	–	100	100	100
DMSO	– 1.4	97.8 ± 1.7	59.7 ± 1.1	8.2 ± 0.5
Methanol	– 0.7	84 ± 1.1	69.8 ± 1.0	0
Acetonitrile	– 0.2	35.3 ± 1.3	0	0
<i>tert</i> -Butyl alcohol	0.6	110 ± 1.5*	94.0 ± 1.5	84.0 ± 1.7
Ethyl acetate	0.7	32.4 ± 0.7	27.9 ± 0.4	15.2 ± 0.5
Benzene	2.0	94.1 ± 1.5	57.2 ± 1.0	22.2 ± 1.0
Toluene	2.7	111.5 ± 1.5*	85.2 ± 1.5	79.8 ± 0.5
1-Octanol	2.9	90.4 ± 2.1	27.5 ± 1.1	17.9 ± 0.6
Hexane	3.5	129.6 ± 1.7*	121.0 ± 1.5*	94.5 ± 2.1
Cyclohexane	3.6	124.8 ± 3.0*	95.5 ± 1.0	88.9 ± 1.7
Isooctane	4.5	145.4 ± 3.3*	84.7 ± 1.0	82.6 ± 1.1
<i>n</i> -Decane	5.6	103.6 ± 1.2	97.9 ± 1.7	38.5 ± 1.2
<i>n</i> -Dodecane	6.6	96.6 ± 2.1	88.1 ± 1.6	29.6 ± 0.4
<i>n</i> -Tetradecane	7.0	97.1 ± 2.5	76.2 ± 1.3	1.2 ± 0.2
<i>n</i> -Hexadecan	8.8	131.8 ± 1.7*	90.0 ± 1.5	43.5 ± 1.2

\* Indicates significant difference ( $p < 0.05$ ,  $n = 3$ ) at confidence interval of 95% compared to Control

as FFAs, so the yield of enzymatic esterification was 60% (w/w). The GC results in Fig. 3a and Table 3 indicate that linoleic acid (C18:2; n-6),  $\alpha$ -linolenic acid (C18:3; n-3), oleic acid (C18:1; n-9), palmitic acid (C16:0), and stearic acid (C18:0) were the most fatty acid composition of fenugreek oil. While the n-3 PUFA concentrates obtained by urea complexation method (Fig. 3b) showed a total reduction in saturated (C16:0 and C18:0) and mono-unsaturated (18:1) FFAs contents and remarkable increase in n-3 PUFA (C18:2; n-6 and C18:3; n-3). The percentage of  $\alpha$ -linolenic acid (n-3) was enhanced from 30% (76.3 mg) in 250 mg of PUFA concentrate to 45% (67.6 mg) in 150 mg of the final product, while linoleic acid (n-6) decreased from 68% (169 mg) to 52% (78.2 mg) by lipase esterification. These results show that the solvent-tolerant lipase has a high efficiency to n-3 compared to n-6 PUFAs and a reduction in n-6/n-3 ratio is observed. The value of n-6/n-3 ratio as 1.1 in the produced AGs is remarkably distinct from other plant seeds showing a high value of n-6/n-3 ratio (Orsavova et al. 2015).

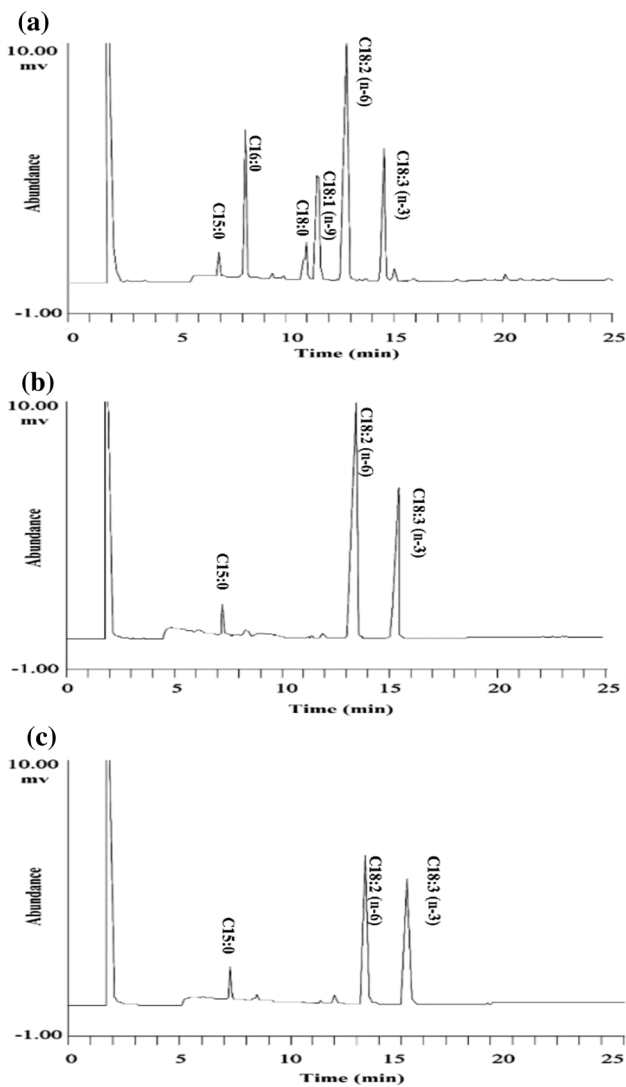
## Discussion

Polyunsaturated fatty acids/ saturated fatty acids (PUFA/SFA) index in vegetable oils is considered as an important parameter for determination of oil nutrition value. The ratio of PUFA/SFA in seed oils with high nutritional value is higher than 1 (Ahmad et al. 2018).

The n-3 and n-6 FFAs are essential PUFAs that cannot be synthesized in the human body and must be obtained through the diet. However, nutritional imbalance in the consumption of n-6/n-3 FFAs ratio can hurt human health and a ratio of 1–5 is recommended (Orsavova et al. 2015). PUFAs are easily dissociated in chemical synthesis, yielding unwanted oxidation polymers and products. Hence, lipase-catalyzed esterification as a feasible alternative could be used for PUFA concentration from vegetable oils for reducing the n-6/n-3 FFA ratio as a valuable alternative in diet. The analysis of FFAs composition of vegetable oils shows that linoleic acid (n-6) within the range of 1.6–79.0% is the most common PUFA in plant oils. On the other hand, ALA (n-3) was detected within the range of 0.1–1.6%, except for flaxseed oil (20–60%) (Orsavova et al. 2015) and fenugreek seed oil (26–40%).

In this study a solvent-tolerant lipase producing strain was isolated from the oil-contaminated regions during primary and secondary screening and identified as *Streptomyces pratensis*. Under the optimized conditions, lipase production tripled. Ebrahimpour et al. (2008) reported a 4.7-enhancement in lipase production in a similar study. Considering the fact that interfacial activation mechanism is an oriented adsorption of lipases on hydrophobic surfaces and a useful reversible method for simultaneous immobilization and purification of lipases (Bastida et al. 1998), SBA-15@oleic acid nanoparticles were applied as an appropriate functionalized support. The results are comparable with those obtained from few studies on lipase purification by affinity column strategies (Solanki and





**Fig. 3** The GC results of the FFAs composition of the original fenugreek oil (a), concentrated PUFA (b), the produced AGs (c) by lipase of *S. pratensis* MV1

Gupta 2011; Volpato et al. 2010). The pH stability of the purified enzyme was approximately similar to that of lipases, such as *Streptomyces fradiae* var. k11 (Zhang et al. 2008) and *Streptomyces coelicolor* A3 (Côté and Shareck 2008) with at a broad pH range of 4.0–10.0 and 6.0–9.0, respectively.

Lipase-catalyzed reactions are heavily influenced by organic solvent hydrophobicity in the reaction media. Log *P* as the logarithm of the octanol/water partition coefficient is used as an operative index of the solvent polarity (Lyman et al. 1990). The enzyme stability at 10% polar solvent concentrations have also been reported in few cases such as *Serratia marcescens* (Zhao et al. 2008), *Pseudomonas aeruginosa*, *Bacillus megaterium*, and *P. mendocina* (Gaur et al. 2008). Water-miscible hydrophilic solvents with log *P* < 2 such as methanol, acetonitrile, and ethyl acetate are

usually incompatible with the activity of a protein; whereas, water-immiscible lipophilic solvents, such as hexane, cyclohexane, isooctane, and n-hexadecan retain the enzyme's high catalytic activity due to this fact that they do not remove the crucial bound water from the enzyme's surface leading to its unfolding and reducing stability of the biocatalyst. Also, the activation of lipase by hydrophobic solvents could explain that the molecules of organic solvents interact with the hydrophobic amino acid residues present in the lid that cover the catalytic site of the enzyme, thereby maintaining the enzyme in its open conformation and conducive to catalysis. The other reason for higher stability of the enzyme in the presence of hydrophobic solvents may be resulted from the surface-solvent interaction leading to interfacial activation. However, the solvent-tolerant enzyme showed the activity > 100% in the presence of 25% (v/v) *tert*-butyl alcohol as a water-miscible hydrophilic solvent. A possible explanation for the lipase stability in the presence of metal inhibitors is that the metals bind to the active site of lipase and change the conformation of the protein for its more proper function (Kumar et al. 2016). This phenomenon can be similar to mechanism of the enzyme stability in water-immiscible lipophilic solvents. The enzyme stability in the presence of 2-ME indicated the absence of disulfide linkages in the purified lipase in contrast to other studies on an isolated lipase. Lipases-catalyzed reactions occur at the lipid-water interfacial area and the positive effect of tween 80% as a surfactant could be due to the surfactant's role in decreasing the surface tension of the liquid and preventing the aggregation of the enzyme and hence increase the enzyme stability (Samaei-Nouroozi et al. 2015). Non-ionic and anionic surfactants improve the stability of surface-active enzymes (Gupta et al. 2004). Urea is a well-known protein-denaturing agent that can affect the enzyme structure by direct interaction with the macromolecule or by an indirect action through effects on the structure and property of the surrounding solvent. However, the enzyme exhibited 109% residual activity in the presence of 10 mM urea. The possible explanation of the urea tolerance of lipase could be due to a conformational change caused by this inhibitor. In accordance to the present study, *Pseudomonas aeruginosa* mutant, *Bacillus licheniformis*, and *Burkholderia cepacia* lipases were reported to be stable with a significant residual activity in the presence of urea (Sonkar and Singh 2020; Chakraborty and Paulraj 2008; Yu et al. 2009). Tris-HCl also forms the complexes with metal ions in solution and can inhibit the enzyme activity in metalloenzymes via chelation. The maintenance > 100% of the activity after incubation with EDTA and Tris-HCl suggests that lipase is not a metalloenzymes (Bose and Keharia 2013). On the other hand, Tris-HCl can protect the enzyme from a variety of denaturing stresses with decreasing the mobility

**Table 3** Fatty acids composition of fenugreek seed oil, concentrated PUFA, AGs produced by lipase of *S. pratensis* MV1, and also the n-6/n-3 FFAs ratio

Fatty acids	Fenugreek seed oil (%)	Concentrated PUFA (%)	AGs produced (%)
C8:0	0.2	0.0	0.0
C14:0	0.9	0.2	0.0
C16:0	11.3	0.8	0.9
C16:1	0.0	0.0	0.0
C17:0	0.3	0.0	0.0
C17:1	0.1	0.0	0.0
C18:0	4.0	0.2	0.2
C18:1 (n-9)	13.6	0.6	1.7
C18:2 (n-6)	45.7	67.8	52.1
Linoleic acid			
C18:3 n6	0.0	0.0	0.0
$\gamma$ -linolenic acid			
C18:3 (n-3)	22.5	30.5	45.1
$\alpha$ -linolenic acid			
C20:0	1.2	0.0	0.0
C20:1	0.2	0.0	0.0
n6/n3	2.0	2.2	1.1

of water molecules and inhibiting autoxidation of the biocatalyst (Ugwu and Apte 2004).

Characterizations of the FA composition of more vegetable oils show that the most abundant PUFA in vegetable oils is linoleic acid (n-6). By contrast, ALA (18:3, n-3) is found to range from 0.1% to 1.6%, so a ratio of n-6/n-3 fatty acids exist too high in most vegetable oils, while a ratio of n-6/n-3 fatty acids of less than 5 is recommended by nutritionists in a healthy diet. This creates a need for functional foods with high content of n-3 PUFAs, especially for vegetarians whose blood contains a high n-6/n-3 ratio. The value of 1.1 for the n-6/n-3 ratio of the produced AGs with *S. pratensis* MV1 lipase was markedly distinct from seed oils, which normally show a high n-6/n-3 ratio (Orsavova et al. 2015).

## Conclusion

The AGs high in n-3 were successfully one-step synthesized via the immobilized lipase with a decline in n-6/n-3 ratio in fenugreek seed oil. The results show that the solvent-tolerant lipase has a high efficiency to n-3 compared to n-6 PUFAs, and it could be considered as a promising candidate in the production of PUFA concentration by a direct esterification procedure. In addition, the purified lipase with stability in organic solvents can be used for the production of fatty acid esters of sugars or sugar alcohols that exhibit high emulsifying, stabilizing, and detergency effects, thus finding applications in the food, cosmetic, detergent, and pharmaceutical industries. Also, it can be applied in inter-esterification and trans-esterification

reactions in enantioselective synthesis of drug substances that are a key process in modern chemistry and are particularly important in the field of pharmaceuticals (Kumar et al. 2016).

**Acknowledgements** Research reported in this publication was supported by Elite Researcher Grant Committee under award number 976958 from the National Institutes for Medical Research Development (NIMAD), Tehran, Iran to M.A.F.

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