Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2015.1067839

57 (2016) 14833–14848
July

Taylor & Francis
Taylor & Francis Group

Enzymatic destabilization of chemical surfactant in wastewater—a potent ultrafiltration foulant: kinetic studies

Theagaraj Sailatha[b], Paranji Saranya[a], Somasundaram Swarnalatha[a], Manickam Velan[b], Ganesan Sekaran[a,*]

[a]*Environmental Technology Division, Council of Scientific and Industrial Research (CSIR)-Central Leather Research Institute (CLRI), Adyar, Chennai, Tamil Nadu 600020, India, emails: psaranyanarasimman@gmail.com (P. Saranya), swarnavinayak2009@gmail.com (S. Swarnalatha), Tel. +91 44 24452941; Fax: +91 44 24410232; email: ganesansekaran@gmail.com (G. Sekaran)*
[b]*Department of Chemical Engineering, Anna University, Adyar, Chennai, Tamil Nadu 600025, India, emails: sailathatheagaraj@gmail.com (T. Sailatha), velan@annauniv.edu (M. Velan)*

Received 2 February 2015; Accepted 23 June 2015

ABSTRACT

Chemical surfactants find a wide range of applications in leather manufacturing practice and they are present in the wastewater at a concentration of 10–200 mg/L. The wastewater after primary, secondary biological, and tertiary treatments contains chemical surfactants at a considerable concentration (60 mg/L). In the present investigation, lipolytic micro-organisms that are capable of utilizing wetting agents (vegetable fatliquor) as the substrate were used to produce lipase. The various conditions such as time, pH, temperature, and concentration of vegetable fatliquor were optimized for the production of lipase. The lipase of an activity 345 U/mL with two different molecular weights 62 and 80 kDa was produced from *Lysinibacillus* sp. The predominant amino acid present in the lipase was found to be glutamic acid. The lipase was characterized using FT-IR, circular dichroism, and XRD spectroscopy. The purified lipase could be used for destabilization of tannery vegetable fatliquor (TVFL) present in the secondary biological-treated tannery wastewater. The optimum conditions for the destabilization of TVFL using lipase was found to be time, 3 h; pH, 7; temperature, 35°C; lipase concentration, 18 μL by response surface methodology. The destabilization of TVFL by lipase followed pseudo-second-order kinetic model with the rate constant, 9.20×10^{-5} mg L^{-1} min^{-1}. The destabilization of TVFL was confirmed using UV–visible, NMR, and FT-IR spectroscopy and surface tension measurement.

Keywords: Tannery wastewater; *Lysinibacillus* sp.; Lipase; Vegetable fatliquor; Kinetics

1. Introduction

The leather manufacturing industry holds a dominant position in the Indian economy. This sector is a major contributor of export market from India, thereby earning a high foreign exchange for the country. The process of leather manufacturing is classified under three steps: pre-tanning, tanning, and post-tanning operations. A further finishing process of surface coating of the leather was employed to soften it, which is an important aspect of leather making.

*Corresponding author.

Post-tanning operations include retanning, softening, and dyeing of tanned leather. The softening of leather is achieved using chemical surfactants known as wetting agents.

Surfactants are an exceptional class of chemicals that possess cleaning property. They are the compounds that lower the surface tension of liquids, interfacial tension between liquid–liquid and solid–liquid interfaces. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants. They generally consist of a polar head group having high interaction with water, and a non-polar hydrocarbon tail, having weak interaction with water [1]. Hence, surfactants are usually an integrated assembly of both hydrophobic and hydrophilic moieties in one molecule. They find extensive application in household cleaning detergents, leather processing, personal care products, textiles, paints, polymers, pesticide formulations, pharmaceuticals, mining, oil extraction, and pulp and paper manufacturing industries [2].

About 7.2 million tons of various surfactants are being manufactured every year, in different forms and names for various purposes [3]. They are mainly classified into three types based on their charge: anionic, non-ionic, and cationic surfactants. The most extensively used surfactants are linear alkyl-benzene sulfonates, alkyl phenol ethoxylates, and quaternary ammonium compounds. Other surfactants less commonly used are alkyl ethoxy sulfates, alkyl sulfates, and alkyl ethoxylates [4].

Wetting agents are the type of surfactants that reduce the surface tension of the liquid on a surface causing the liquid to spread across and adhere to the surface. Wetting agents act on the tanned hides/skins, penetrate more effectively into the surface of the leather fibers, adhere to them, and prevent them from drying out hard. They are sulfated (reacted with sulfuric acid and neutralized with alkali), sulfited (reacted with sodium bisulfite and neutralized with alkali) vegetable or synthetic oils. Some wetting agents are emulsified with anionic, non-ionic, or cationic emulsifiers to enhance their solubility and binding capacity to leather fibers. The wetting agents penetrate the leather conventionally without mechanical means and adhere to the collagen fibers to impart stiffness to leather [5]. The wetting agents of vegetable origin (vegetable oils reacted sulfuric acid) used in leather industry are termed as tannery vegetable fatliquors (TVFL) containing both hydrophobic (aliphatic chain) and hydrophilic moieties (sulfates, sulfites, or phosphates). The major components of vegetable fatliquors are triacylglycerols obtained from soap oil, vegetable oils (castor oil), and sodium bicarbonate.

Around 10–15% of applied TVFL remains to be unexhausted and unspent TVFL is discharged into wastewater, thereby the surfactant concentration in tannery wastewater is in the range of 10–200 mg/L. The excessive foam formation in the aerobic biological treatment in effluent treatment plants/common effluent treatment plants is majorly due to the presence of TVFL in tannery wastewater. The presence of TVFL in wastewater not only causes choking of wastewater conveyance pipelines but additionally interfere with the oxygen transfer efficiency in aerobic treatment processes [6].

The undegraded TVFL escape the post-aerobic biological treatment processes and carried to other unit operations such as sand filtration, activated carbon filtration, and membrane filtration. The residual surface active compounds foul the ultrafiltration system in association with the calcium salts in the wastewater [7]. It has also been proven that calcium ion could act as a fouling facilitator through a bridging mechanism, enhancing adsorption, and complexation with the membrane functional groups [7]. The combination of solid particulates (kaolin and cellulose) and surfactants could cause stabilization of the foulants due to sorption onto the colloid surfaces and possibly enhanced fouling [8]. Thus, the service life of the membranes is very much shortened.

The spent surfactants, surfactant residues, metabolites, and breakdown products of target chemicals need to be eliminated or recovered from wastewater to prevent them from hampering further wastewater treatment activities. Surfactants are removed by clarifying wastewater using flocculants and coagulants like alumina that bind with surfactant molecules intact agglomerating them and aiding in their removal by filtration. While destabilizing surfactants by coagulation or chemical treatment, the agglomerates or resultant products needed to be removed with care to prevent intervention with further treatment additives [9,10]. Specially fabricated membrane bioreactors to separate surfactant molecules and advanced oxidation processes to oxidize the surfactants present in wastewater were employed in wastewater treatment processes [11]. Similarly, membrane destabilization techniques focused on removing surfactants with respect to frequent replacement of membranes due to pore plugging and low-economic feasibility [11,12]. A technology to destabilize chemical surfactants by solvent extraction using butanol or pentanol and recover surfactants using a combination of coagulants was also suggested [13]. Solvent extraction is limited by the solubility of surfactant in wastewater. An enzymatic destabilization with lipase to enhance the biodegradability of surfactant byproducts besides eliminating

pore plugging possibilities in membranes was also attempted [14]. However, there are no reports on the enzymatic destabilization of TVFL in secondary biological-treated tannery wastewater (SBTTW).

In the present investigation, bacterial lipase produced from TVFL as the substrate was used to destabilize the residual surfactant present in the SBTTW.

2. Materials and methods

2.1. Isolation and identification of lipolytic bacterial strain

Soil acclimatized with commercial TVFL BL_2 (Natural oil base fatliquor) manufactured by Balmer and Lawrie Chemicals India Pvt. Ltd. (Chennai) was serially diluted and bacteria were isolated by pour plate method followed by incubation for 24–48 h at 35˚C for the growth of micro-organisms. Microbial colonies, appeared on the agar plates, were further screened by subsequent subculturing and obtained as pure cultures and subjected to qualitative screening for the identification of lipase-producing lipolytic micro-organisms on tributyrin agar. The strain, which showed the maximum lipolytic activity, was identified by 16S ribosomal DNA (16S rDNA) sequencing and phylogenetic analysis [15].

2.2. Production, extraction, and purification of lipase using TVFL

Lipase was produced by growing the culture in a broth medium containing 10 g/L of (1:1 v/v) TVFL. The same medium supplemented with isolated culture was used to optimize lipase production. Lipase production was optimized by varying the time (0–120 h), pH (1–10), temperature (20–50˚C), and concentration of TVFL/water emulsions (0.5:1, 1:1, 1.5:1, 2:1, and 2.5:1 v/v). The lipase from the cell-free supernatant was precipitated using ammonium sulfate at 4˚C. Solid ammonium sulfate was added to the cell-free supernatant at 20% saturation and allowed to stand for 4 h. The precipitate obtained was separated by centrifugation and the supernatant was further treated with ammonium sulfate at 40, 60, and 80% saturation. All the precipitates, separated by centrifugation at 8,000 rpm for 15 min at 4˚C, were dissolved in 0.1 M phosphate buffer of pH 7.0. This aliquot was dialyzed (Dialysis membrane-150, HiMedia) against the same buffer for overnight under refrigerated conditions. The fraction showing the maximum lipase activity at 80% saturation was used for further studies. The quantitative estimation of the lipase activity was determined by the standard method mentioned below. The dialyzed enzyme was loaded onto the DEAE-cellulose column (1.09–15 cm) antecedent equilibrated with 0.1 M phosphate buffer (pH 7.0). After washing with two bed volumes of the buffer, elution was performed with a negative linear gradient of 0–1.0 M NaCl at a flow rate of 30 ml/h. The fractions with lipase activity were pooled up and lyophilized for overnight under vacuum, 1 Pa; condenser temperature, −70˚C; and shelf temperature, 20˚C for further analysis.

2.3. Enzyme activity assay

Lipase activity was quantified by titrimetric assay, utilizing an olive oil emulsion with remote modifications as followed by Ramani and Sekaran [16]. One activity unit of lipase was defined as the amount of enzyme that relinquished 1 μM of fatty acid per minute under assay conditions.

2.4. Determination of molecular weight of lipase

The molecular mass of the lipase was determined by utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [17], on a 5% stacking gel and 12% resolving gel. The protein marker ranging from 14.3 to 94.7 kDa was utilized as a standard marker for the determination of molecular weight. Protein bands were visualized by silver staining.

2.5. Amino acid composition of lipase

Lipase was hydrolyzed with 6 N HCl at 110˚C for 20 h in a sealed and evacuated glass tube, and the amino acid composition was evaluated utilizing C18 column in Agilent 1100 HPLC amino acid analyzer and the data analysis was carried out, utilizing HP chem. Station [18].

2.6. Physicochemical characterization of lipase

2.6.1. Effect of pH

The optimum pH for the purified lipase was determined by incubating the enzyme at various pH values ranging from 1 to 10 at room temperature in the following buffers: 0.1 M HCl–KCl buffer (pH 1.0 and 2.0), acetate buffer (pH 3.0 and 4.0), 0.1 M phosphate buffer (pH 5.0, 6.0, and 7.0), and 0.1 M Tris buffer (pH 9 and 10). The residual enzyme activity was determined by following the standard assay procedure (Section 2.3). The pH stability was studied by incubating the lipase at each desired pH for 4 h and then the lipase activity was quantified.

2.6.2. Effect of temperature

In order to determine the thermal stability of enzyme, the lipase was incubated at different temperatures and at optimum pH. The thermal stability of the purified lipase was determined by incubating the reaction mixture at temperatures ranging from 20 to 80°C at optimized pH value for 4 h and lipase activity under same assay conditions was measured.

2.6.3. Effect of metal ions

One milli mole of potassium chloride (KCl), calcium chloride ($CaCl_2{\cdot}2H_2O$), zinc chloride ($ZnCl_2{\cdot}H_2O$), magnesium chloride ($MgCl_2$), ferrous sulfate ($FeSO_4{\cdot}7H_2O$), copper sulfate ($CuSO_4{\cdot}5H_2O$), manganese chloride ($MnCl_2{\cdot}2H_2O$), and ethylene diamine tetra acetic acid (EDTA) were added to 0.1 M phosphate buffered (pH 7.0) enzyme solution and incubated at 35°C for 1 h to determine the stimulatory or inhibitory effect of metal ions on lipase activity.

2.7. Kinetic parameters

The olive oil and wetting agent emulsions separately at different concentrations (1–10 (w/v)%), at a constant temperature (35°C), and pH (7.0) were employed to determine the kinetic parameters such as maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) for the purified lipase. One unit of lipase activity (U) is defined as the amount of enzyme that hydrolyzed the substrate to release 1 μmol of fatty acid per minute. The kinetic parameters were obtained from Lineweaver–Burk equation plot where [S] is the substrate concentration (mM) and v is the initial reaction rate of the enzyme (mM/min).

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{[s]}\right) + \left(\frac{1}{V_{max}}\right) \quad (1)$$

2.8. Instrumental characterization of lipase

2.8.1. FT-IR spectral analysis of lipase

The functional groups of the purified lipase, produced using the wetting agent as the substrate, were characterized using a FT-IR spectrophotometer (Perkin Elmer). The samples were lyophilized to form pellets with dimensions of thickness, 1 mm and diameter, 13 mm using spectroscopic grade KBr. The spectrum was analyzed in the spectral range of 400–4,000 cm^{-1}.

2.8.2. XRD pattern of lipase

The XRD pattern of free lipase was determined for 2θ values from 10 to 80°C using Cu-Kα radiation at a wavelength of λ = 1.514 Å (40 kV, 30 mA) with silicon as the reference using high-resolution GUINER powder X-ray diffractometer (SEIFERT -3003TT, Germany).

2.8.3. Circular dichroism of lipase

The secondary structures of lipase were studied in the wavelength of $\lambda_{150–250}$ nm with a path length of 1 mm and width of 1 nm in circular dichroism using a Peltier temperature-controlled system (JASCO J715, Japan).

2.9. Destabilization study of TVFL in SBTTW using lipase and its kinetic studies

The kinetic studies were carried out to determine the efficiency of lipase on hydrolysis of the TVFL in SBTTW. The optimum conditions for the hydrolysis of TVFL in SBTTW was carried out by varying the time (30–240 min), pH (1–10), temperature (20–50°C), and concentration of lipase (2–20 μL at 345 U/mL) for 25 ml of SBTTW in the batch experiment under shaking conditions (150 rpm). The initial concentration of TVFL in the SBTTW was found to be 60 mg/L [19]. The experiment was carried out by adding lipase (14 μL) to SBTTW of volume, 25 ml at pH 7.0, and incubated at 30°C. The sample was withdrawn from the incubator and analyzed for the residual TVFL content [19]. The % hydrolysis of TVFL was calculated as follows.

$$\%\text{Hydrolysis of TVFL} = \left(1 - \left(\frac{\text{Final lipid content}}{\text{Initial lipid content}}\right)\right) \times 100 \quad (2)$$

The same experiment was repeated for different time, pH, and temperature values. The kinetic rate constants for the degradation of TVFL using the purified lipase were determined by applying the non-linear kinetic models. The pseudo-first-order [20] and pseudo-second-order [21] models were employed, respectively.

$$r_t = r_e\left(1 - \exp^{-K_1 t}\right) \quad (3)$$

$$r_t = \frac{K_2 r_e^2 t}{1 + K_2 r_e t} \quad (4)$$

where r_e and r_t are the amounts of TVFL destabilized (mg of TVFL/μL of lipase) at equilibrium and at time (t); k_1 and k_2 are the first- and second-order rate constants.

2.10. Statistical tool for optimization of destabilization model

The most significant range of parameters such as time, (2.5–3.5 h); pH, (6–8); temperature, (30–40˚C), and lipase concentration, (14–20 μL) at constant volume of SBTTW of 25 ml and agitation speed (150 rpm) were optimized using response surface methodology (RSM). The four independent parameters were studied at three different levels and a set of 30 experiments were carried out.

The analysis was done as per the following model Eq. (5)

$$Y = \alpha_0 + \sum_{i=1}^{k} \alpha_i x_i + \sum_{i=1}^{k} \alpha_i x_i^2 + \sum_{i<j} \alpha_{ij} x_i x_j \quad (5)$$

where Y is the predicted response, k is the number of parameters, α_0 is the design factor of interest, and α_i and α_{ij} are coefficients. The second-degree term (X_i^2) gives the optimal values of the response and the interactive term ($x_i x_j$) represents the influence of one parameter over the other. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Each experimental design was carried out in triplicate, and the mean values were derived.

Design expert, version 9 (Statease Inc., Minneapolis, USA) was used for the experimental designs and regression analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2 and its statistical significance was determined by an F-test.

2.11. Instrumental evidences for destabilization of TVFL

2.11.1. UV–visible spectroscopy

The UV–visible scans of TVFL and the degraded TVFL by lipase were evaluated in the range $\lambda_{200–800}$ nm using a UV–visible spectrophotometer (Cary varion; Agilent Technologies). The enzymatic degradation of TVFL was studied by measuring the absorbance at a wavelength of λ_{291} nm.

2.11.2. FT-IR analysis

The functional groups of the degraded TVFL using purified lipase were identified using a FT-IR spectrophotometer (Perkin Elmer). The samples were lyophilized and made in the form of pellet with dimensions of thickness, 1 mm and diameter, 13 mm using spectroscopic grade KBr. The spectrum was analyzed in the spectral range of 400–4,000 $cm^{-1.}$

2.11.3. Surface tension analysis

Surface tension and interfacial tension can be defined as the boundary between two immiscible phases; they may be a gas, a liquid, or a solid. Interfaces are extremely important in colloid systems such as emulsions (liquid in a liquid) or foams (gas in a liquid). The surface tension of the liquids was measured using surface tensiometer (NIMA). The surface tension of water was measured using the Wilhelmy plate method.

2.11.4. Nuclear Magnetic Resonance (NMR) spectroscopy

The sample preparation for nuclear magnetic resonance (NMR) analysis was carried out by dissolving 5–30 mg of lyophilized SBTTW in 650 μl of deuterated water (Brucker NMR FT 500 mHz). The degraded products of TVFL were identified using ^{13}C NMR analysis.

3. Results and discussion

3.1. Isolation and identification of micro-organisms

Among the five strains isolated from the soil that utilized TVFL as the sole carbon source, the strain which exhibited the maximum lipolytic activity (345 U/ml) and a clear zone formation on tributyrin agar plates was culled for lipase production. The 16S rDNA sequencing data indicated that the isolate was *Lysinibacillus* sp. The Basic Local Alignment Search Tool results showed 98% similarities to other *Lysinibacillus* sp. and it is an aerobic, Gram-positive soil bacterium survives at 35˚C and pH 7, and produces endospores.

3.2. Production of lipase using TVFL and its purification

The optimum conditions such as time, pH, temperature, and concentration of TVFL for the production of lipase were optimized. The optimum incubation time for maximum lipase activity of 197 U/mL was found to be 72 h at room temperature (Fig. 1(a)). The optimum conditions for the maximum production of lipase with activity 197 U/mL were pH 7; time, 72 h; and

temperature, 30˚C (Fig. 1(b)). The bacterial species was pH sensitive, unable to withstand extreme acidic or alkaline pH. It was observed that good biomass yield at the neutral pH 7 with a considerable biomass yield in weakly acidic and basic pH. The optimum temperature for the maximum lipase activity (235 U/mL) was found to be at 35˚C at the optimum time 72 h and pH 7 (Fig. 1(c)). The effect of different concentrations of TVFL emulsions containing TVFL/Water (0.5:1, 1:1, 1.5:1, 2:1, and 2.5:1 v/v) on lipase production in the nutrient broth medium was studied and the optimum ratio was found to be 1:1 (TVFL/water emulsion) with the highest activity of 345 U/mL (Fig. 1(d)), also the lipase activity decreased at higher concentrations and the reason could be attributed to substrate inhibition.

At the optimized conditions, the culture supernatant was utilized for the extraction of lipase. The lipase was obtained by ammonium sulfate precipitation and purified with DEAE-cellulose column chromatography and the protein content was estimated by Lowry's method [22]. The lipase obtained was observed to have incremented in purification fold by 1.57 times, 77.9% recovery of lipase, and specific activity of 52.26 U/mg.

3.3. Molecular weight determination of lipase

The SDS-PAGE of lyophilized lipase showed two protein bands of molecular weights 80 and 62 kDa (Fig. 2(i)). They were observed to be similar to lipases reported from other substrates such as slaughter house waste, blood tissue lipid, beef tallow, and cooked sunflower oil with a molecular weight of 94 [23], 92 [24], 56 [16], and 39 kDa [15], respectively. The two lipases with different molecular weights may be due to the presence of soap oil and castor oil in TVFL. The high molecular weights of lipases corresponding

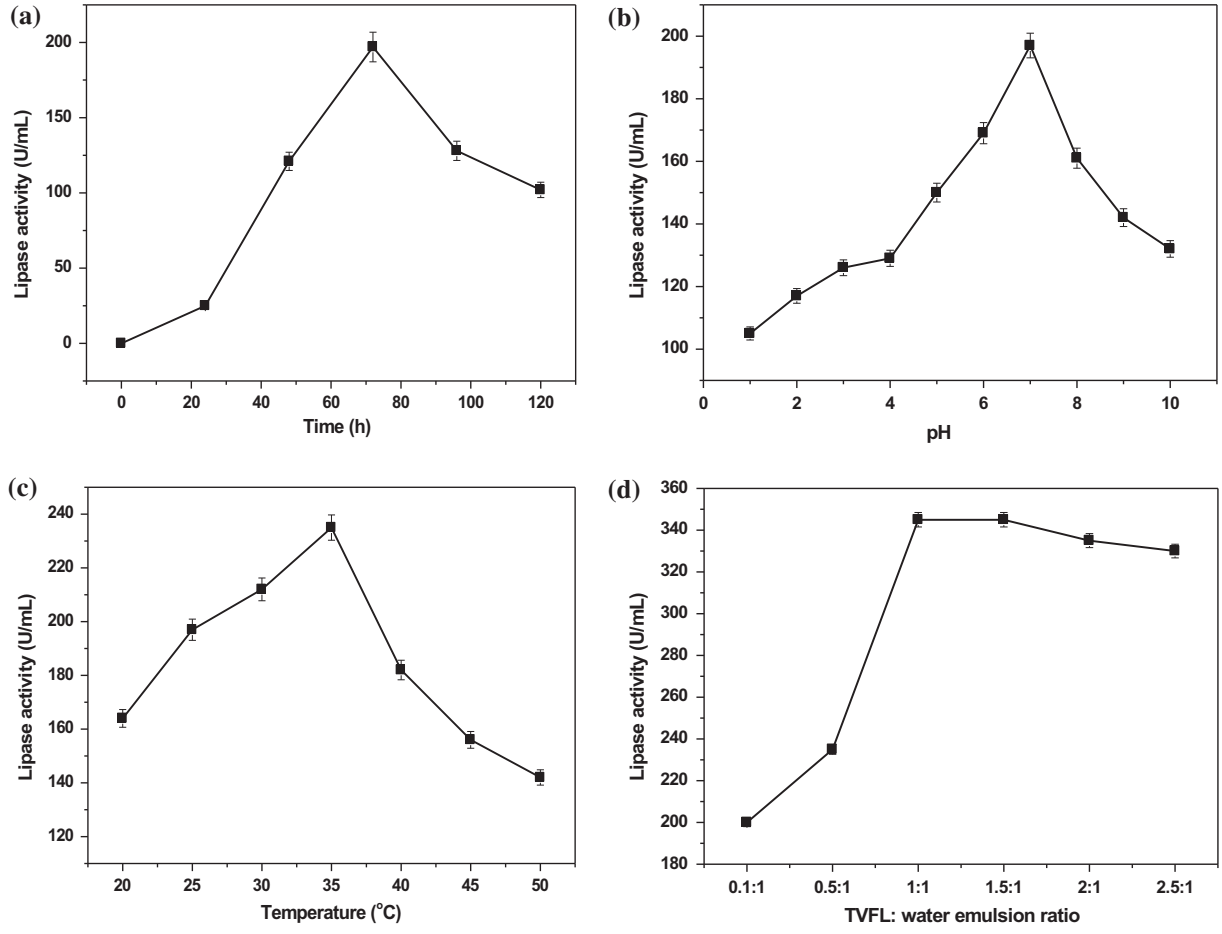

Fig. 1. Optimization of production of lipase activity (U/mL) by *Lysinibacillus sp.* as the function of (a) time (h) (reaction conditions: pH 7, 25˚C, and 0.5: 1 TVFL), (b) pH (reaction conditions: time 72 h, 25˚C, and 0.5: 1 TVFL), (c) temperature (˚C) (reaction conditions: time 72 h, pH 7, and 0.5: 1 TVFL), and (d) substrate concentration (reaction conditions: time 72 h, pH 7, and 35˚C).

to 80 and 62 kDa could be due to the high formula weight of the TVFL. The molecular formula of the TVFL is $C_{18}H_{32}Na_2O_6S$ and formula weight of the TVFL is 422.49. This high formula weight of the TVFL and the complexity in its composition could produce specific lipase. The major novel attribute of the lipase from *Lysinibacillus* sp. may be due to the usage of complex substrate with high molecular weight TVFL as the carbon source for the growth of the bacterial strain. The molecular weight of the lipase obtained in the present investigation was different from conventional lipases which could be attributed to the characteristic constituents of TVFL. It is well known that the substrate employed to induce the enzyme production controls the characteristics of the enzyme produced [25]. There are many reports that slight variations in the activity and characteristics of the enzymes could be obtained by altering the carbon source and varying their concentration [25,26]. Thus, it is evident that the novel property i.e. high molecular weight of the enzyme is due to the complex composition of the substrate containing both hydrophobic and hydrophilic moieties and a very high formula weight when compared to other reported lipid substrates employed to produce lipases. The high molecular weight is expected to make the enzymes more stable and tolerant to a wide range of experimental conditions.

3.4. Amino acid composition of lipase

The amino acid composition of lipase was determined using HPLC as shown in Table 1. It was found that the lipase contained polar amino acids by 40% and non-polar amino acids by 60%. The amino acid composition of the lipase (Table 2), unlike the lipases reported by other researchers [15,16,23,24], suggests that it contained a higher percentage (57.14%) of aliphatic amino acids. The molecular weight of lipase and its amino acid composition depend on the substrate used for its production. Hence, it could be concluded that the molecular weight of lipase produced was a function of substrate characteristics. The non-polar amino acids

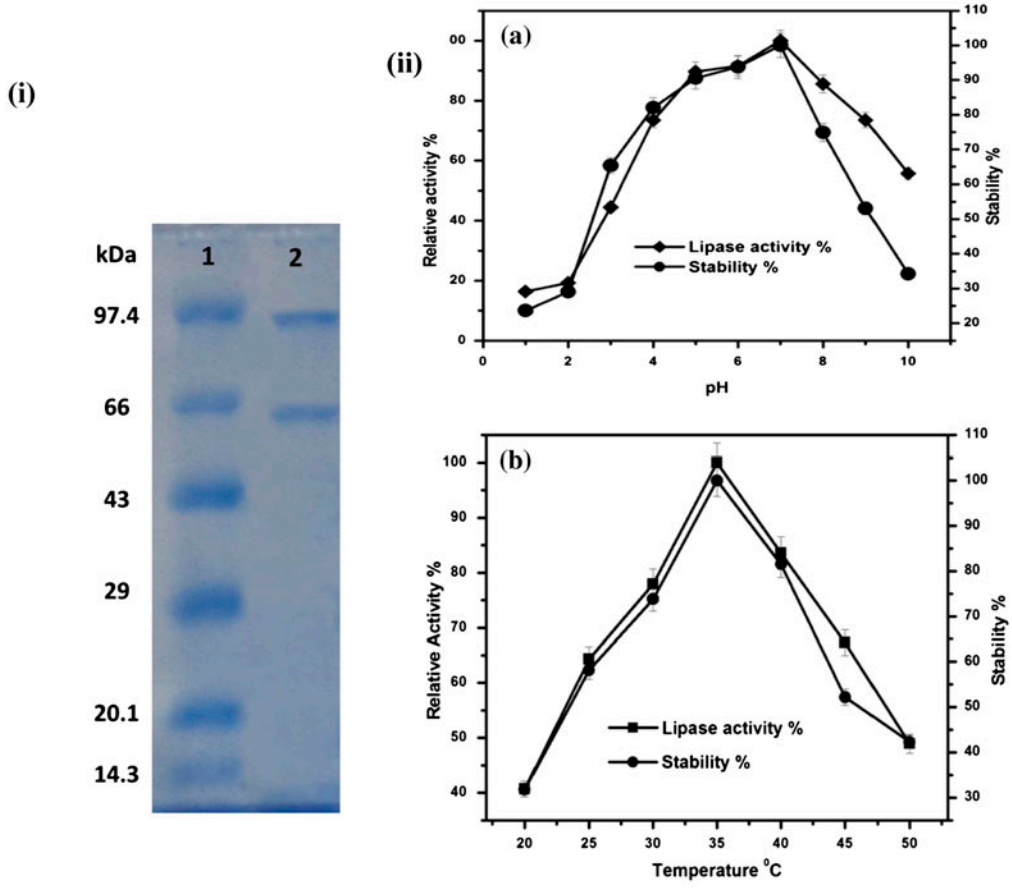

Fig. 2. (i) SDS-PAGE showing the molecular weight of purified lipase. Lane 1 molecular weight marker, lane 2 purified lipase, (ii) relative lipase activity and stability of purified lipase at different (a) pH values and (b) temperatures.

Table 1
Amino acid composition of purified lipase

S.no	Amino acid	nmoles/mg
1	Glutamic acid	962
2	Serine	56
3	Arginine	66
4	Valine	140
5	Threonine	192
6	Alanine	78
7	Tyrosine	130
8	Phenylalanine	24
9	Isoleucine	32
10	Leucine	54
11	Lysine	40

(22.9%) of the lipase was found to be higher than the other lipases, which could be due to the unique composition of TVFL.

3.5. Physicochemical characteristics of lipase

3.5.1. Effect of pH

The purified lipase was found to be active in the broader pH range between 4 and 8. The maximum relative lipase activity was observed at pH 7.5 (Fig. 2(ii)a). The enzyme retained the relative lipase activity by more than 50% in the pH range between 4 and 8. The purified lipase was incubated for 1 h at selected pHs to determine the stability of the purified lipase over the acidic, neutral, and alkaline pH. The study showed that the lipase possessed good stability over the wide range of pH. About 65–100% of enzyme stability was observed in strongly acidic to weakly alkaline pH (4–8). More than 90% of the stability was observed in the pH range between 5 and 7. This proves that the lipase is functional and stable in the pH range of 4–8 and is a neutral lipase in nature with an ability to retain activity in strongly acidic and weakly alkaline conditions. The broad working pH range of the lipase proves to be an integrated advantage in utilizing alkaline or acidic lipases in many environmental processes.

3.5.2. Effect of temperature

The purified lipase was active in the temperature range from 20 to 60℃ with enhanced activity at 35℃. A steep decline in relative activity was observed at relatively higher (50℃) and lower temperatures (20℃). The thermal stability profile of lipase suggests the purified lipase was highly stable (100%) at 35℃ (Fig. 2(ii)b). The purified enzyme was thermostable in the temperature range of 30–45℃. This proves that the lipase could be employed at ambient temperature with wide temperature fluctuations (within ± 10℃) in enzymatic processes with lipase activity of 100%. Thermal resistance of the lipase may be attributed to high molecular weight that imparts a stable intermolecular bonding leading to compactness in the tertiary structure of the molecule. The conformational entropy of enzyme molecule is generally attributed to the presence of low molecular weight amino acids like valine and serine. These residues in lipase require high thermal energy to cause unfolding of enzyme molecule and the thermal stability of lipase may be due to the presence of these two amino acids. This key potential property may be harnessed to make the process more cost efficient by eliminating external temperature maintenance systems for utilization of the enzyme.

3.5.3. Effect of metal ions on lipase activity

It is well known that Ca^{2+} ions bind to the enzyme molecule and change the conformation of the protein to impart greater stability to the enzyme [27]. Metal enzymes are the group of enzymes that contain tightly

Table 2
Comparison of pseudo-first- and second-order kinetic parameters of hydrolysis of TVFL by lipase (pH 7; concentration of lipases, 14 μL; and volume of SBTTW, 25 ml)

		Pseudo-first-order				Pseudo-second-order			
Temperature (℃)	r_e (exp)(mg/L)	k_1 (min^{-1})	r_e (Cal) (mg/L)	R^2	χ^2	k_2 (mg L^{-1} min^{-1})	r_e (Cal) (mg/L)	R^2	χ^2
20	47.4	0.0113	59.0	0.931	2.28	8.49 *10^{-5}	48.74	0.999	0.04
25	55.2	0.0148	74.47	0.925	4.98	0.18*10^{-5}	55.55	0.999	0.002
30	57	0.0367	78.5	0.946	5.88	9.22*10^{-5}	62.04	0.998	0.41
35	57.9	0.0265	94.4	0.894	14.11	9.20*10^{-5}	63.09	0.998	0.42
40	49.2	0.0206	84.03	0.937	14.4	6.30*10^{-5}	65.26	0.976	3.95
45	45	0.0171	72.28	0.823	10.2	7.07*10^{-5}	58.49	0.978	3.11
50	40.8	0.011	90.0	0.932	26.89	9.79*10^{-5}	58.02	0.999	5.11

bound metal ions as cofactors, conventional enzymes contain transition metal ions viz. Fe^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. Metal enzymes loosely bound with metal ions and the most commonly bound metal ions in metal activated enzymes are alkali and alkaline earth metal ions K^+, Mg^{2+}, and Ca^{2+}. Among the metal ions tested, Ca^{2+} significantly enhanced lipase activity by 120%. The other metal ions acted as potent inhibitors of the lipase activity by 4–50%. The stimulatory effect of Ca^{2+} on the lipase activity suggests that the enzyme requires calcium ion as a catalytic activator. Moreover, the results suggest that the lipase activity was enhanced by the presence of alkali and alkaline earth metal ions than the transition metal ions [15]. Hence, the presence of calcium ions in the tannery wastewater may be considered as an advantage to enhance the activity of lipase and thus, it was considered as the promoter of TVFL destabilizer.

3.6. Kinetic parameters of lipase

The Michaelis–Menten enzyme kinetic parameters, K_m and V_{max}, for the purified acidic lipase were calculated from the Lineweaver–Burk plot (Fig. 3(a)). The crude extract of lipase showed a lower K_m value of 2.04 mM and higher V_{max} value of 7.20 mM/min. The relatively lower value of K_m represents the higher affinity between enzymes and substrates, whereas V_{max} represents the higher catalytic efficiency of lipase from *Lysinibacillus* sp.

3.7. Instrumental characterization of lipase

3.7.1. FT-IR spectral analysis of lipase

The FT-IR spectrum of the purified lipase is shown in Fig. 3(b). The spectrum shows the major stretching bands owing to the peptide bonds occurred in the spectral range of 1,100–1,700 cm^{-1}. The band at 1,654.77 cm^{-1} is due to the C=O stretching vibrations of amide I bond which may be contributed by peptide linkage present in lipase. The band at 1,401.15 cm^{-1} may be attributed to C–N stretching vibrations of amide II bond [28]. The peak at 3,168.52 cm^{-1} may be attributed to N–H stretching vibrations present in amino acids like valine [29]. The in-plane CH_3 stretch of glutamic acid is observed at 1,354.12 cm^{-1}.

3.7.2. XRD pattern of lipase

The XRD pattern of lipase was determined using high-resolution GUINER powder X-ray diffractometer (SEIFERT, Germany). The peaks of 2θ at 17˚, 19˚, 20˚, 22˚, 29˚, 32˚, and 35˚ (Fig. 3(c)) in the XRD spectrum of lipase confirm the crystalline phases of lipase. This is in accordance with the values reported by Gupta et al. [30].

3.7.3. Circular dichroism of lipase

The absorption of the polarized light in the region between $\lambda_{190–220}$ nm indicates absorption due to the peptide bonding in the enzyme. The peaks at $\lambda_{199.5}$, λ_{213}, and $\lambda_{210.5}$ nm (Fig. 3(d)) indicate the absorption due to the presence of prominent beta sheets in the lipase enzyme. The peaks in the region between $\lambda_{170–200}$ nm are generally due to the presence of amide bonds [31].

3.8. Destabilization of TVFL in SBTTW using lipase

The percentage hydrolysis of TVFL, at an initial concentration of 60 mg/L, in SBTTW was observed over the period of 4 h in a batch reactor. The concentration of TVFL in SBTTW decreased with time of enzymatic reaction. It was found to be 3 mg/L at 3 h with the maximum percentage of hydrolysis, 95% (Fig. 4(a)). The hydrolysis of TVFL in buffers of different pH values was studied. The maximum hydrolysis of TVFL by 95% was performed by lipase at pH 7 (Fig. 4(b)) and the residual TVFL left in the lipase-treated SBTTW was observed to be 3 mg/L. More than 60% hydrolysis of the TVFL was observed in the pH range between 5 and 8. This confirms that the lipase is functional over the weakly acidic, weakly alkaline, and neutral range with maximum hydrolysis at the neutral pH.

The hydrolysis of TVFL by lipase was evaluated at different temperatures (20–50˚C). It was found that hydrolysis of TVFL by 96.5% was obtained at an optimum temperature of 35˚C (Fig. 4(c)). The residual TVFL was 2.1 mg/L at time, 3 h; pH, 7; and lipase concentration, 14 µL. The destabilization of TVFL at different lipase concentrations with activity 345 U/mL (2–20 µL) was studied. It was found that TVFL was hydrolyzed by 98% at an optimum enzyme concentration of 18 µL. The residual TVFL concentration was 1.92 mg/L at optimum conditions such as time, 3 h; pH 7; temperature, 35˚C; and volume of SBTTW, 25 ml (Fig. 4(d)).

Enzymatic destabilization of TVFL by 98% in 3 h was achieved using lipase which was comparatively higher than the reported values in primary degradation of 96% in 30 d in a closed bottle test [32], 87% in ozonation [33], and about 80% in other conventional treatment methods [14]. This is the first report on the enzymatic destabilization of TVFL by lipase produced using TVFL as the substrate.

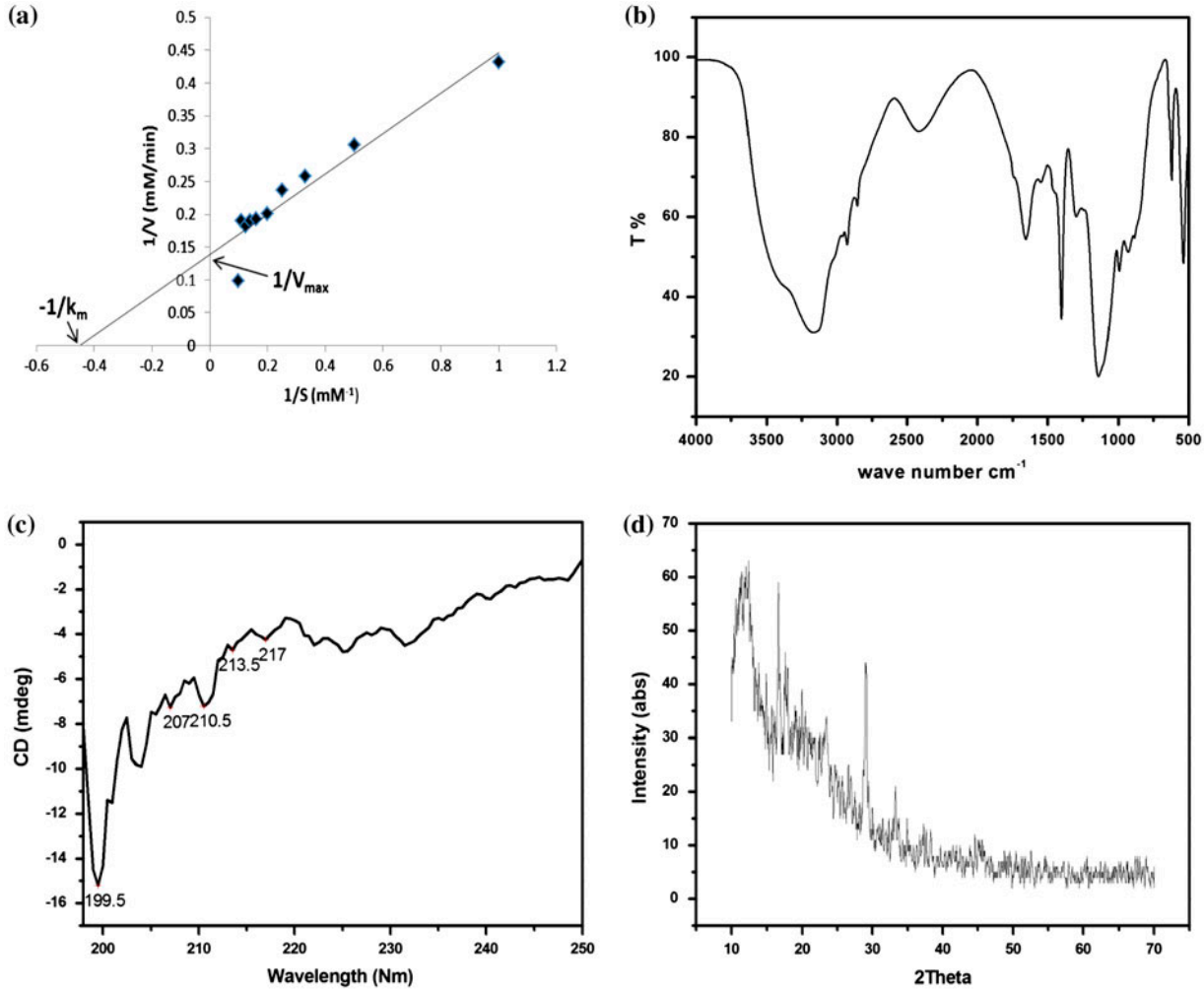

Fig. 3. (a) Lineweaver–Burk plot using TVFL as substrate, (b) FT-IR spectrum, (c) XRD plot, and (d) circular dichroism spectrum of purified lipase from *Lysinibacillus sp.*

3.9. Non-linear kinetic model for enzymatic destabilization of TVFL

The validity of kinetic order for the enzymatic destabilization of TVFL using the purified lipase process was based on the regression coefficients and χ^2 values. The experimental data were plotted under each model (Figures not shown). The values of k_1, k_2, and r_e of each hydrolysis process were determined from the slopes and intercepts of the plots and they are presented in Table 2 along with their corresponding regression coefficients (R^2) and χ^2 values. Relatively higher R^2 values, least χ^2 values, and more or less closer experimental r_e, and calculated r_e values showed a better agreement of enzymatic destabilization of TVFL using the purified lipase from *Lysinibacillus* sp. with the pseudo-second-order kinetic model. The pseudo-second-order kinetic model suggests that the rate of destabilization of TVFL by enzymatic process depends both on the concentration of lipase and TVFL.

3.10. Statistical optimization of destabilization of TVFL using lipase

RSM using central composite design was employed to determine the optimal levels of the four significant parameters that affected destabilization of TVFL. Three levels with the coded levels for the parameters are shown in Table 3. Based on the regression analysis of the data from the Table 3, the effects of four parameters on the destabilization of TVFL were predicted by the second-order polynomial function as

$$\text{Destabilization of TVFL}(\%) = +98.00 + 4.08 \times A - 2.17 \times B - 0.33 \times C - 0.42 \times D - 0.50 \times AB + 2.00 \times AC - 1.62 \times AD + 1.75 \times BC + 1.88 \times BD + 0.63 \times CD - 3.52 \times A^2 - 15.15 \times B^2 - 3.40 \times C^2 - 2.15 \times D^2$$

where A, B, C, and D are time, pH, temperature, and lipase concentration, respectively.

3.10.1. Analysis of variance for response surface quadratic model

3.10.1.1. ANOVA (partial sum of squares—Type III). The statistical significance of equation was checked by F test and ANOVA for the second-order polynomial model as shown in Table 1. The analysis of factor (F test) showed that the second-order polynomial model was well adjusted to the experimental data and the coefficient of variation (CV) indicated the degree of precision of the experiment.

In general, higher the value of CV, lower the reliability of the experiment. In the present investigation, the lower value of CV (5.56) with a regression coefficient of 0.9612 indicated a better precision and reliability of experiments [34]. Linear and quadratic terms were both significant at the 1% level.

3.10.2. Localization of optimum conditions

The contour plots described by the regression model were drawn to illustrate the effects of the independent parameters and interactive effects of each independent parameters on the response factors. It also showed that the optimum conditions required for the maximum destabilization of TVFL (98%) using

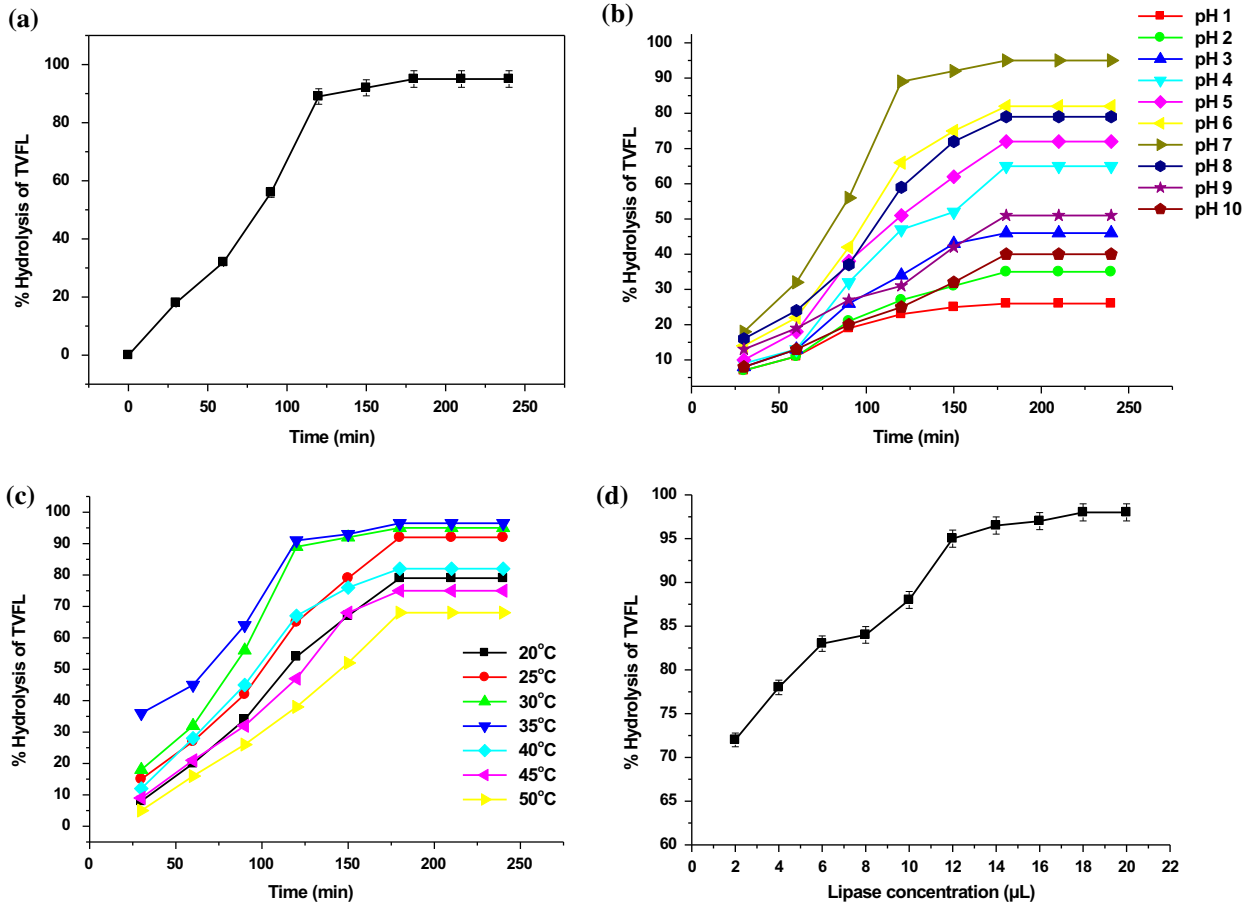

Fig. 4. Degradation of TVFL using free enzyme (a) effect of time (experimental conditions: pH 7; temperature, 30˚C; lipases concentration, 14 µL; and volume of SBTTW, 25 ml), (b) effect of pH (experimental conditions: temperature, 30˚C; lipases concentration, 14 µL; and volume of SBTTW, 25 ml), (c) effect of temperature (experimental conditions: pH 7; lipases concentration, 14 µL; and volume of SBTTW, 25 ml), and (d) effect of lipase concentration (experimental conditions: time, 180 min; pH 7; temperature, 30˚C; and volume of SBTTW, 25 ml).

Table 3
Coded and real values of the factors tested in the RSM experimental design and ANOVA for the second-order polynomial model for destabilization of TVFL

Factor		Levels of factors		
		−1	0	+1
A	Time (h)	2.5	3	3.5
B	pH	6	7	8
C	Temperature (°C)	30	35	40
D	Concentration of lipase (μL)	14	18	20

Source	Degree of freedom	Mean square	*F*- value	P value prob> *F*
Model	14	508.56	26.56	<0.0001 (significant)
Residual	15	19.14		
Lack of fit	10	28.72		
Pure error	5	0.000		
Total	29			
R^2				0.9612

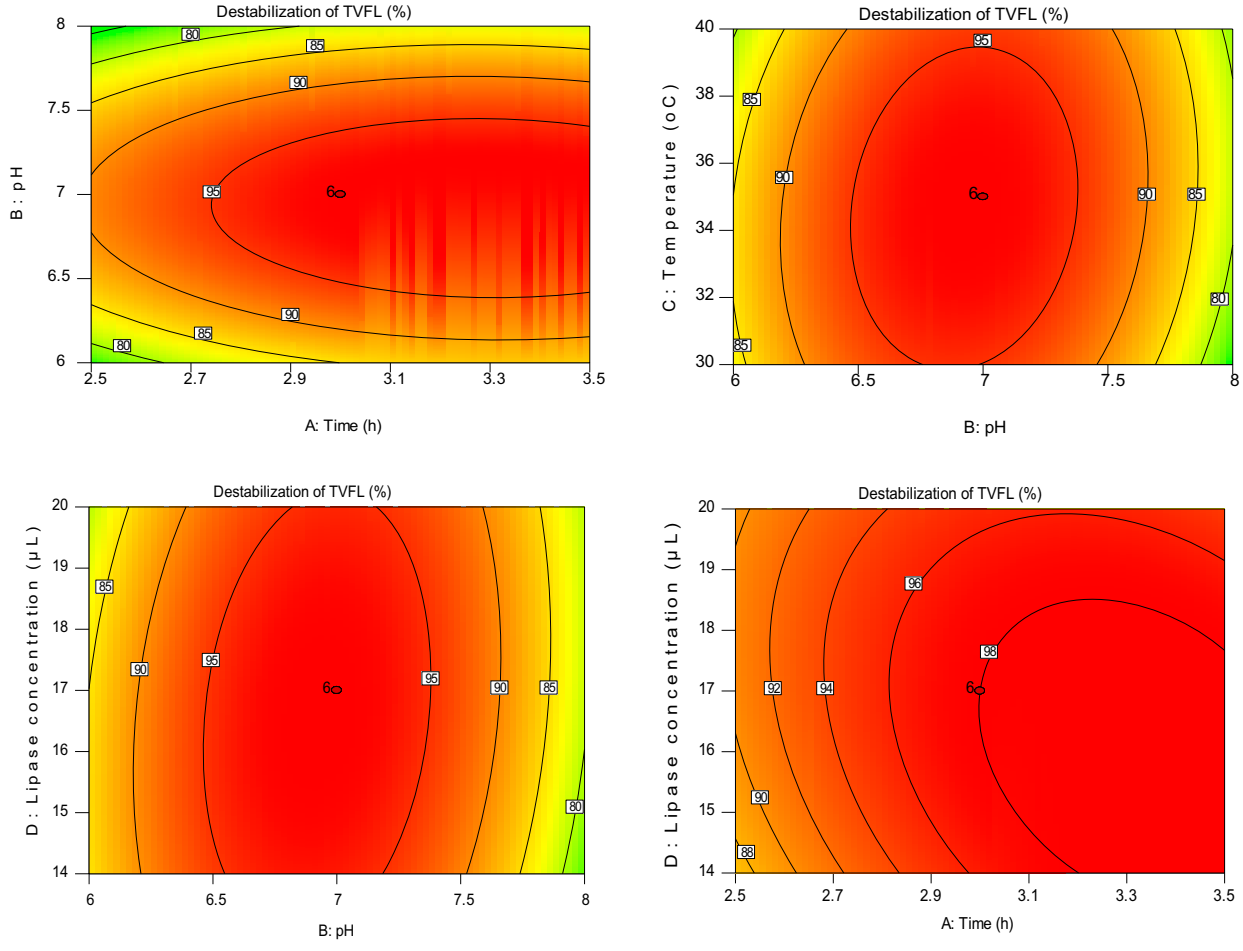

Fig. 5. Contour plots for destabilization of TVFL using lipase (%) as the function of (a) time (h) and pH, (b) pH and temperature (°C), (c) pH and concentration of lipase (μL), and (d) time (h) and concentration of lipase (μL).

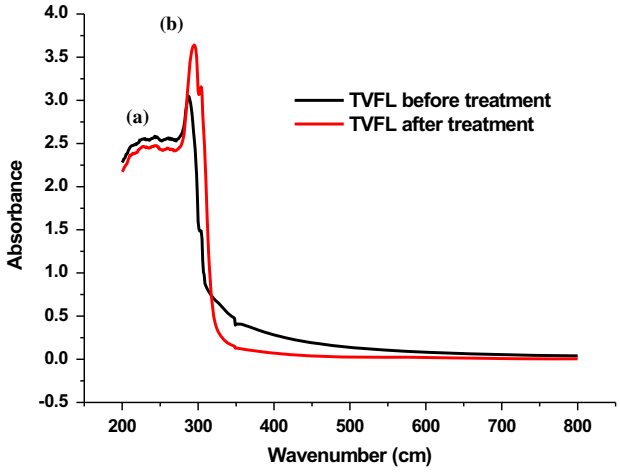

Fig. 6. UV–visible spectra of (a) TVFL in SBTTW before and (b) TVFL in SBTTW after lipase treatment.

lipase were reaction time, 3 h; temperature, 35˚C; pH 7.0; agitation speed of 150 rpm; and lipase concentration, 18 µL (Fig. 5). Each figure presented the effect of two parameters, while the other parameter was held at the zero level.

3.11. Instrumental evidences for destabilization of TVFL

3.11.1. UV–visible spectroscopy

UV–visible spectrophotometric scans of destabilization of TVFL were measured in the wavelength range of 200–800 nm. In Fig. 6(a), the peak at λ_{291} nm corresponds to TVFL. After destabilization of TVFL using lipase, the peak at a wavelength of λ_{291} nm disappeared and new peaks at wavelengths λ_{295} nm and λ_{304} nm were appeared. The observed changes in UV–visible spectroscopy confirm that TVFL was destabilized by lipase from *Lysinibacillus* sp.

3.11.2. FT-IR analysis of TVFL before and after destabilization

The FT-IR spectrum of TVFL is shown in Fig. 7(a). Well defined and characteristic absorption peaks in the region of 3,200–2,500 cm^{-1} are due to C–H stretching of carbon and hydrogen containing compounds. The presence of long chain aliphatic compound is identified by the presence of absorption bands at 2,534.89 and 2,925.10 cm^{-1} and the absorption peak at 1,465 cm^{-1} also corresponds to long chain aliphatic compounds. Absorption band at 1,746 cm^{-1} indicates the presence of carboxylates in TVFL [15]. The FT-IR spectrum of TVFL after destabilization (Fig. 7(b)) depicts several changes compared to the spectrum of untreated TVFL. The widening of the C=O ester peak at 1,784.17 cm^{-1} is due to the formation of secondary oxidation products containing carbonyl groups which resulted in shifting the absorption peak to lower wave number region [35]. Generally, weak to moderate absorption in the region around 1,650 cm^{-1} indicates the presence of unsaturated carbon chain (C=C), but lowering of this

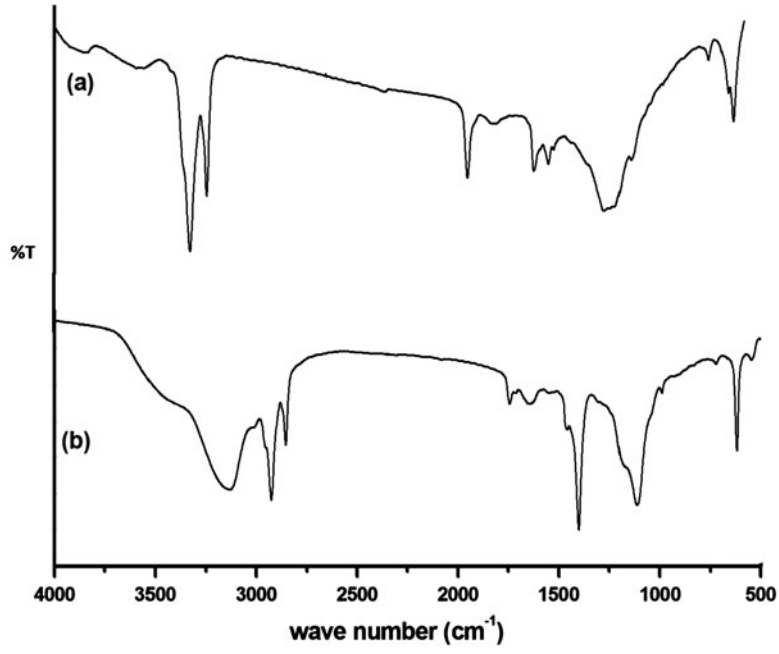

Fig. 7. FT-IR spectra of (a) TVFL before and (b) TVFL after lipase treatment.

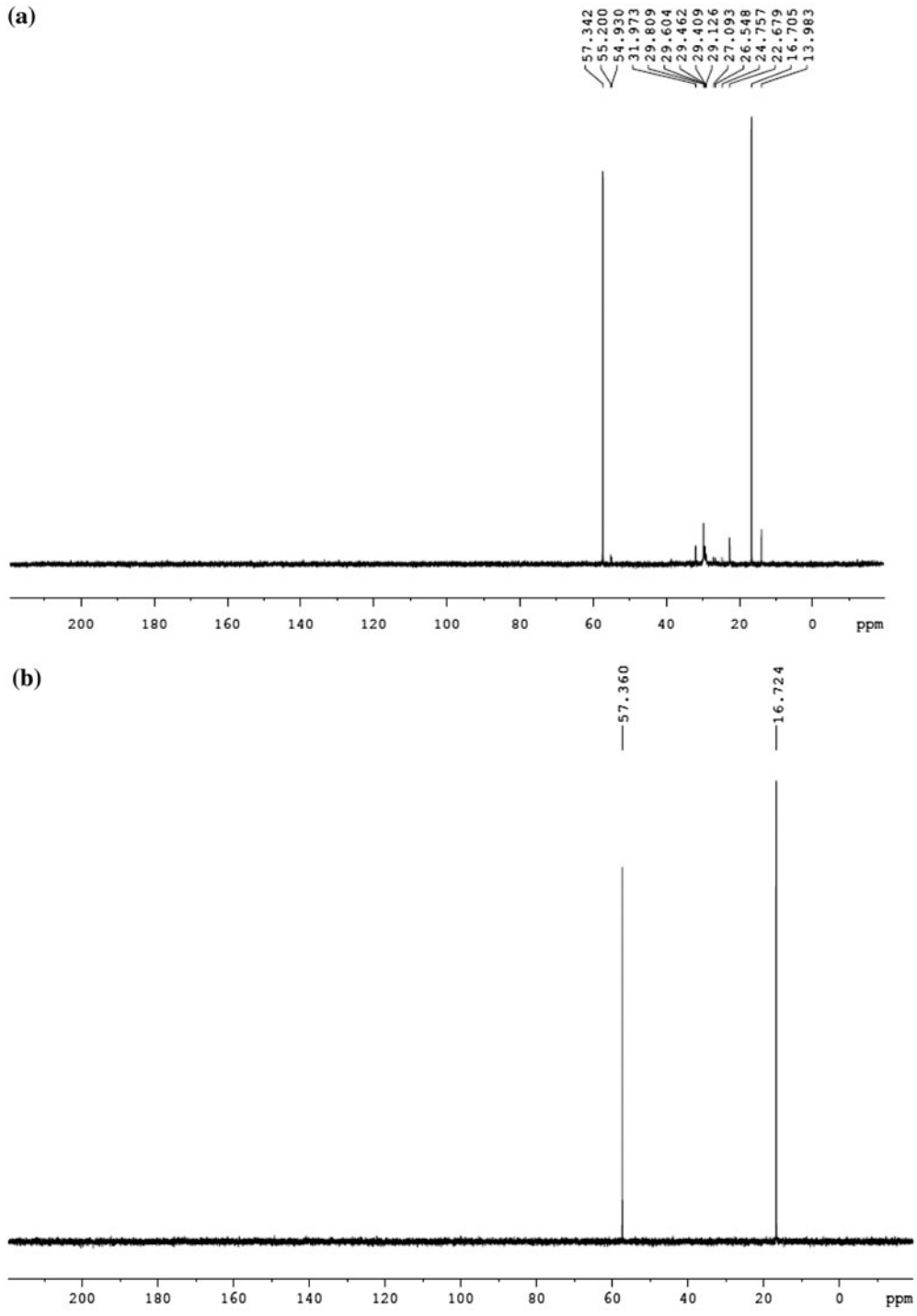

Fig. 8. C^{13} NMR spectra of (a) TVFL before and (b) TVFL after lipase treatment. .

frequency is due to formation of a compound containing C=O probably in carbonic acid [15].

3.11.3. Surface tension analysis

The surface tension of initial SBTTW sample at pH 7.2 was observed to be 13.16 mN/min. After treatment with lipase for about 3 h at pH 7.2, the surface tension was increased to 24.17 mN/min. The increase in surface tension depicts the change in emulsification property of the agent, which is due to the destabilization of TVFL catalyzed by lipase [36].

3.11.4. ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of TVFL was scanned between δ 0.0 and 200 ppm. The chemical shift at δ 13.9 ppm indicates the presence of terminal methyl group of TVFL. The chemical shift at δ 16.7 ppm indicates the long chain methylene (CH_2) groups present in the carbon chain of the TVFL. The chemical shift at δ 31.9 ppm indicates the evidence for methylene (CH_2) group bonded to sulfur group present in TVFL. The chemical shift in the region δ 22–29 ppm indicates the evidence for methylene (CH_2) group present in different environments other than the free methylene groups. The chemical shift around δ 54–57 ppm indicates the terminal methyl and methylene groups attached to the carboxyl group (Fig. 8(a)).

The ^{13}C-NMR spectrum of destabilized TVFL sample shows that the peaks in the region δ 22–31 ppm were disappeared. The peaks at δ 13, 54, and 55 ppm were also disappeared during treatment of SBTTW by lipase (Fig. 8(b)). This confirms the hydrolysis of TVFL into smaller fragments using lipase produced from *Lysinibacillus* sp.

4. Conclusions

The results confirmed that the lipase produced from *Lysinibacillus* sp. using TVFL as the substrate could hydrolyze TVFL. The lipase produced has the activity of 345 U/mL with higher molecular weights of 80 and 62 kDa. The optimum conditions for the destabilization of TVFL using lipase were enzymatic reaction time, 3 h; pH, 7; temperature, 35˚C; and lipase concentration, 18 μL by RSM. The hydrolysis of TVFL followed pseudo-second-order kinetic model. The destabilization of vegetable fatliquor was confirmed using UV–visible, NMR, and FT-IR spectroscopy and surface tension measurement. The lipase from *Lysinibacillus* sp. has tolerance to wide-range temperature fluctuations, thus holds within a wide range of biocatalytic application in a environmental management. This is the first report on the enzymatic hydrolysis of TVFL (chemical surfactant) in SBTTW.

Acknowledgments

The financial assistance from Network project STRAIT (CSC0201)—Council of Scientific and Industrial Research (CSIR), New Delhi, is highly acknowledged. The authors have declared no conflict of interest.

References

[1] K. Kosswig, Surfactants' in Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH, Weinheim, 2005, p. 1.
[2] M.J. Rosen, J.T. Kunjappu (Ed.), Surfactants and Interfacial Phenomena, fourth ed., New Jersey, NJ, John Wiley and Sons, 2010.
[3] A. Di Corcia, R. Samperi, Monitoring aromatic surfactants and their biodegradation intermediates in raw and treated sewages by solid-phase extraction and liquid chromatography, Environ. Sci. Technol. 28 (1998) 850–858.
[4] G.G. Ying, Fate, behavior and effects of surfactants and their degradation products in the environment, Environ. Int. 32 (2006) 417–431.
[5] K.T. Sarkar, Neelachal Abasan Bishaka, Theory and practice of leather manufacture, The Author Publication, Kolkata, 2005, pp. 199–219.
[6] K. Chitra, K. Sri Bala Kameswari, V. Sudharsan Varma, J. Sahil Tagra, Raghava Rao, Studies on biodegradation of vegetable-based fat liquor-containing wastewater from tanneries, , Clean Technol. Environ. Policy 15 (2013) 633–642.
[7] Q. Li, M. Elimelech, Organic fouling and chemical cleaning of nanofiltration membranes: Measurements and mechanisms, Environ. Sci. and Technol. 38 (2004) 4683–4693.
[8] N. Oschmann, L.D. Nghiem, A.I. Schäfer, Fouling mechanisms of submerged ultrafiltration membranes in greywater recycling, Desalination 179 (2005) 225–233.
[9] M.L. Braden and R.A. Marble, Method for clarifying wastewater containing surfactants, US 5433863 A, 1993.
[10] A. Adak, M. Bandyopadhyay, A. Pal, Removal of anionic surfactant from wastewater by alumina: A case study, Colloids Surf., A: Physicochem. Eng. Asp. 254 (2005) 165–171.
[11] S. González, M. Petrovic, D. Barceló, Removal of a broad range of surfactants from municipal wastewater —Comparison between membrane bioreactor and conventional activated sludge treatment, Chemosphere 67 (2007) 335–343.
[12] M. Petrovic, S. Gonzalez, D. Barceló, Analysis and removal of emerging contaminants in wastewater and drinking water, TRAC-Trend Anal. Chem. 22 (2003) 685–696.
[13] S.S. Ashrawiand G. Prukop, Method of extracting and reutilizing surfactants from emulsions, US 4559148 A, 1984.
[14] M.J. Scott, M.N. Jones, The biodegradation of surfactants in the environment, Biochim. Biophys. Acta. 1508 (2000) 235–251.
[15] K. Ramani, P. Saranya, S. Chandan Jain, G. Sekaran, Lipase from marine strain using cooked sunflower oil waste: Production optimization and application for hydrolysis and thermodynamic studies, Bioprocess Biosyst. Eng. 36 (2013) 305–315.
[16] K. Ramani, G. Sekaran, Production of lipase from *Pseudomonas gessardii* using blood tissue lipid and thereof for the hydrolysis of blood cholesterol and triglycerides and lysis of red blood cells, Bioprocess Biosyst. Eng. 35 (2012) 885–896.

[17] S. Ramakrishnan, K.N. Sulochana, R. Punitham, K. Arunagiri, Free alanine, aspartic acid, or glutamic acid reduce the glycation of human lens proteins, Glycoconj. J. 13 (1996) 519–523.
[18] Ramakrishnan, Liquid chromatography-mass spectrometry, J. Chromatogr., A 794 (1990) 165–185.
[19] A.K. Joseph, A. Shauna, M. James, Chemical basis of the sulfo–phospho vanillin reaction for estimating total serum lipids, Clin. Chem. 18 (1972) 199–202.
[20] S. Lagergren, B.K. Svenska, The theory of adsorption on geloester substances, Veternskapsakad Handlingar 24 (1898) 1–39.
[21] Y.S. Ho, G. Mckay, Kinetic models for the sorption of dye from aqueous solution by wood, Process Saf. Environ. Prot. 76B (1998) 183–191.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, J. Randal, Protein measurement with the folin-phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[23] K. Ramani, E. Chockalingam, G. Sekaran, Production of a novel extracellular acidic lipase from *Pseudomonas gessardii* using slaughterhouse waste as a substrate, J. Ind. Microbiol. Biotechnol. 37 (2010) 531–535.
[24] K. Ramani, L. John Kennedy, M. Ramakrishnan and G. Sekaran, Purification, characterization and application of acidic lipase from *Pseudomonas gessardii* using beef tallow as a substrate for fats and oil hydrolysis, Process Biochem. 45 (2010) 1683–1691.
[25] T. Tan, M. Zhang, B. Wang, C. Ying, L. Deng, Screening of high lipase producing *Candida* sp. and production of lipase by fermentation, 39 (2003) 459–465.
[26] E. Dalmau, J.L. Montesinos, M Lotti, C CasasEffect of different carbon sources on lipase production by *Candida rugosa*, Enzyme Microb. Technol. 26 (2000) 657–663.
[27] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of biochemistry, Wiley, New York, NY, 1999, p. 511.
[28] K. Ramani, L. John Kennedy, C. Vidya, R. Boopathy, G. Sekaran, Immobilization of acidic lipase derived from *Pseudomonas gessardii* onto mesoporous activated carbon for the hydrolysis of olive oil, J. Mol. Cat. B: Enzym. 62 (2009) 59–66.
[29] B. Andreas, The infrared absorption of amino acid side chains, Prog. Biophys. Mol. Biol. 74 (2000) 141–173.
[30] M. Gupta, R. Batra, R. Tyagi, A. Sharma, Polarity Index: The guiding solvent parameter for enzyme stability in aqueous-organic cosolvent MIXTURES, Biotechnol. Prog. 13 (1997) 284–288.
[31] S. Yogesha, M. Javiya, C.S. Bhambi, K. Pundir, A. Singh Bhattacharya, Comparative study of performances of lipase immobilized asymmetric polysulfone and polyether sulfone membranes in olive oil hydrolysis, Int. J. Biol. Macromol. 42 (2008) 145–151.
[32] W.K. Fischer, The important aspects of ecological evaluation of fatty alcohols and their derivatives, in: Fatty Alcohols—Raw Materials Methods and Uses, Henkel, Dusseldorf, (1982) 187–122.
[33] S. Karthikeyan, P. Ranjith, Degradation studies on anionic and nonionic surfactants by ozonation, J. Ind. Pollut. Control 23 (2007) 37–42.
[34] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for experimenters, Wiley, New York, NY, 1978.
[35] J. Coates, Interpretation of infrared spectra, a practical approach. in: R.A. Meyers (Ed.), Encyclopedia of analytical chemistry, Wiley, Chichester, 2000, pp. 10815–10837.
[36] J.A. Flipsen, H.T. van der Hijden, M.R. Egmond, H.M. Verheij, Action of cutinase at the triolein-water interface. Characterisation of interfacial effects during lipid hydrolysis using the oil-drop tensiometer as a tool to study lipase kinetics, Chem. Phys. Lipids 84 (1996) 105–115.