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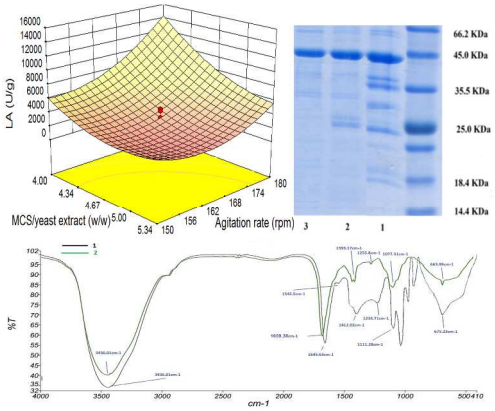
Graphical abstract



Optimization and partial purification of a high-activity lipase synthesized by a newly isolated *Acinetobacter* from offshore waters of the Caspian Sea under solid-state fermentation

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Abstract

A new aerobic mesophilic bacterium was isolated from the southern coastal waters of the Caspian Sea which substantially produced an extracellular lipase in solid-state fermentation using milled coriander seeds (MCS) as support substrate. This bacterium was identified as a strain of genus *Acinetobacter* based on morphological and biochemical characterization and 16S rRNA gene sequence. The various medium components and culture parameters to achieve a more cost effective and economically viable bioprocess were screened and optimized using the Plackett-Burman and central composite designs. The highest lipase activity (20480.2 U/g) was achieved at optimum levels of predominant factors of MCS/yeast extract (4.0 w/w), olive oil concentration (30 g/L), moisture content (65.0%), and agitation rate (180.0 rpm). The enzyme with molecular weight of 46 kDa was purified 26.9-fold to homogeneity by ammonium sulfate precipitation and phenyl-Sepharose hydrophobic interaction chromatography. The functional groups of the lipase were also assigned using Fourier transform-infrared spectroscopy.

Keywords: Microbial lipase, *Acintobacter* sp., Solid state fermentation, Validation methods, Response surface methodology (RSM)

1 Introduction

Lipase (E.C. 3.1.1.3) enzymes are carboxyl esterases that catalyze the hydrolysis of acylglycerols composed of long-chain fatty acids with more than 10 carbon atoms.[1] These enzymes have attracted scientific and commercial attentions over the past few years, because they are industrially considered to be the most important enzyme groups after proteases and amylases.[2] Although lipases are produced by animals, plants and microorganisms, mainly synthesized by biotechnologically methods using bacteria and fungi.[3] The microbial lipases are extracellular

metabolites with high potential to catalyze broad range of reactions in the different aqueous and non-aqueous phases.[4] These biocatalysts have interesting characteristics such as high activity under mild conditions, suitable stability in organic solvents, high substrate specificity, and regio- and enantio-selectivity.[5] It was demonstrated that the genera *Acinetobacter*, *Pseudomonas* and *Burkholderia* among other microbial strains had the unique activities at a wide range of pH and temperature, and have shown the excellent properties of chemo-, regio-, and enantio-selectivity that made them the interest biocatalysts to use by most organic chemists and pharmacologists.[3]

Although submerged liquid fermentations (SLFs) nowadays are the most important of commercial bioprocesses, the lipase production using solid state fermentations (SSFs) due to the significant reduction of final product cost by decreasing downstream processes is recently considered.[6] Microbes in the SSF process are grown on a porous solid substrate in the absence of free water. Large number of agricultural/food residues have been used as suitable substrates for SSF. The growth of cells is provided by the water and nutrient absorbed on the surface of the solid support and within the support matrix.[7]

In the present study, the novel extracellular lipases from various natural sources such as Caspian Sea water (CSW), extract olive (EO), linseed cake (LC), soy bean cake (SBC), cotton seed cake (CSC), fat milk (FM), olive oil pressing waste (OPW) and dairy industrial effluent (DIE) were isolated and partially purified. The cheap carbon substrates including milled seeds of grape, pomegranate and coriander were also analyzed to achieve the best conditions for batch fermentation process. Response surface methodology (RSM) was then applied to determine the optimal culture media for lipase SSF-production with the highest activity using the best bacteria isolate. RSM development for this bioprocess can result in improved product yields, reduced

process variability, and closer conformance of the output responses to nominal and target requirements, thus reducing the development time and the overall costs.[8,9]

2 Materials and methods

Chemicals and raw materials

P-nitro phenol (p-NP) and p-nitro phenyl palmitate (p-NPP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Rhodamine B, nutrient broth (NB), peptone, yeast extract, *iso*-propanol, Triton X-100, Tris HCl, gum arabic and agar were provided by Merck Chemical Co. (Darmstadt, Germany). Coriander, grape and pomegranate seeds and olive oil were purchased from a local market in Tehran (Iran). The contents of protein, lipid and dry matter for the milled seeds of coriander, grape and pomegranate were 15.99, 12.90, and 12.35%, 15.50, 24.85 and 20.05%, and 96.1, 93.2 and 93.4%, respectively. All other chemicals were of analytical grade.

Isolation and screening of lipolytic bacteria

Various natural sources including CSW, EO, LC, SBC, CSC, FM, OPW and DIE were used to isolate a bacterial strain with the highest lipolytic activity. 1.0 g each sample to enrich was cultured into 250 mL-Erlenmeyer flasks containing 50 mL of broth (NB, 8 g/L (w/v); NaCl, 3 g/L (w/v); and emulsified olive oil (EOO), 25 g/L (w/v)) at pH 6.5. The flasks were incubated at 37°C with a constant shaking rate of 160 rpm for 24 h. Initial qualitative screening for lipase producing isolate was conducted by serially spreading the diluted samples on a specific medium containing 10 g/L extract yeast, 3 g/L NaCl, 25 g/L EOO, 0.01 g/L rhodamine B and 20 g/L agar at pH 7.0. Incubation temperature for the plates was chosen in range of 20-45°C. The formation

of orange fluorescent halos around colonies under ultraviolet light (350 nm) indicates lipase positive isolates. Bacterial colony with the largest halo were isolated by repeated pure culture technique and stored for the further studies according to the described method by Sengun *et al.*[10] with some modifications. Briefly, after the purification and isolation steps, the number of isolated colonies using sampler tip were collected and inoculated into the test tubes containing 10 g/L yeast extract, 3 g/L sodium chloride and 25 g/L EOO (pH = 6.5). The test tubes were then incubated at 37ºC for 24 h in order to achieve the favorable growth and suitable turbidity. In the next step, 1000 µL of microbial suspension was transferred to a 1.5 mL pre-sterilized microtube and centrifuged at 4000 rpm for 15 min. After the discarding supernatant, 500 µL of the suspension of the isolated microbe and 500 µL of pre-sterilized liquid culture containing 30% glycerol added to the microbial sediment of microtube bottom. Glycerol concentration in the final solution should be 15%.

Selection of the best lipase-producing bacteria isolate

One loopful from orange halo colonies of fresh cultures was inoculated into a medium containing of 10 g/L extract yeast, 3 g/L NaCl and 25 g/L EOO at pH 7.0, and then incubated for 24 h at 37°C under a shaking rate of 160 rpm. Then, the cultures were centrifuged twice at 6000 rpm for 30 min (Hettich Centrifuge, D-78532 model, Tuttlingen, Germany), filtered through 0.2 µm filters to remove the cell mass and other solids. The obtained supernatant was used in lipase activity (LA) assay.

Morphological, biochemical and molecular identification of selected isolate

The selected bacterium was identified by morphological and biochemical properties according to Bergey's Manual of Determinative Bacteriology. [11] The identification was further confirmed by the 16S rRNA gene sequencing method. The genomic DNA of isolate was extracted as previously described by Ausubel *et al*.[12]. The DNA was then amplified by PCR using the following universal 16S ribosomal RNA (rRNA) gene primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').The amplification of 16S rRNA gene was conducted in BioRad PCR cycler (USA). PCR was carried out by subjecting a reaction mixture to initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 1.5 min and a final extension step at 72°C for 15 min. The 16S rRNA gene sequence was compared with sequences available in the nucleotide database using the BLAST algorithm at the NCBI server. Phylogenetic tree was constructed by the neighbor joining method using molecular evolutionary genetics analysis (MEGA) software version 4. [11]

Solid-state fermentation process

The batch experiments for lipase production were carried out in 500 mL-Erlenmeyer flasks based on the milled seeds of coriander, grape and pomegranate. These solid substrates were prepared by washing with distilled water and next air-drying. The powders were sieved and divided to two major fractions based on the particle diameter of 1-2 mm (Tyler Standard Sieve Series of 9-16) and 0.5-1 mm (Tyler Standard Sieve Series of 16-32). Owing to the low accessibility of bacterial cells to nutrients and inappropriate aeration in the cultures with substrate particles size more than 1 mm, the fraction with particle diameter of 0.5-1 mm was selected for use in the fermentation process because of the better cell growth and higher enzyme production.

A stable oil-in-water emulsion with 20% w/w gum arabic was prepared to use olive oil (15% w/w) in the culture medium. The coarse emulsion was produced by Ultra-Turrax (IKA T25 Digital, Germany) at 24,000 rpm for 2.5 min and, further emulsification was performed using a 20 kHz-ultrasounic homogenizer (UP200S, Hielscher Ultrasonics GmbH, Teltow, Germany) equipped to a water bath (45°C) at a total nominal output power of 45 W for 3 min.[8] Each sterilized flask was inoculated with the constant amount of the active isolate (10%) and then incubated at 37°C for 72 h. Activation of the stored bacterium by placing at ambient laboratory conditions (in a sterile environment under the hood) was started. When it was a little freezing outside, amount of the liquid into microtube using sampler tip was transferred to the culture medium containing 10 g/L yeast extract, 3 g/L sodium chloride and 25 g/L EOO (pH = 6.5) and incubated at 37ºC for 18 h (Fig. 1). According to the RSM design, the used ranges for ratio of solid substrate to yeast extract, olive oil, moisture content (MC) and agitation rate were 3.33 to 6.01 (w/w), 7.5 to 37.5 g/L, 57.5 to 87.5% (w.b.), and 135 to 195 rpm, respectively.

Lipolytic activity determination

The LA was assayed by measuring the absorbance increase at 410 nm using an UV-visible spectrophotometer (CE2502, BioQuest & BioAquarius Series, Cecil Instr. Inc., Cambridge, UK). This increase was due to the p-NPP hydrolysis and p-NP release at pH 8.0 (37°C) after 30 min of reaction time. To initialize the reaction, 0.1 mL of the resulted supernatant was added to 0.9 mL of substrate solution containing 3 mg p-NPP dissolved in 1 mL isopropanol diluted in 9 mL of 50 mM Tris–HCl (pH 8.0) containing 40 mg of Triton X-100 and 10 mg of gum arabic. [13] One unit (U) of lipase was defined as the amount of enzyme that releases 1 µmol p-NP per min under the assay conditions. The calibration curve with p-NP as standard was prepared.

152

153 **Statistical analysis**

154 **Plackett–Burman experimental design**

155 The Plackett–Burman (PB) experimental design was chosen to screen eight medium components
156 (yeast extract, peptone, milled seeds of coriander, grape and pomegranate, molasses, olive oil
157 and $MgCl_2$) and three culture parameters (particle size, agitation rate and MC) affecting lipase
158 production with the highest activity by the selected bacterial isolate using the statistical software
159 package Design-Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA) (Table 1). These
160 variables were selected based on a preliminary literature review and experiment and designed in
161 term of a matrix with 14 experiments. Table 1 listed the minimum and maximum range of
162 variables investigated and their values in actual and coded form. Two replicates of the center
163 point were performed in runs 13 and 14 in order to detect the curvature that may exist in the first-
164 order model.

165

166 **Central composite experimental design**

167 Based on the results of the PB design, five variables of olive oil, yeast extract, milled coriander
168 seed (MCS), MC and agitation rate were found to have greater influences on the lipase
169 production with high activity by the selected isolate. SSF process was then conducted with a
170 central composite rotatable design (CCRD) using the same software, as a function of MCS/yeast
171 extract (3.33-6.01 w/w, X_1), olive oil (7.5-37.5 g/L, X_2), MC (57.5-87.5% w.b., X_3), and agitation
172 rate (135-195 rpm, X_4). With CCRD, three levels for each factor are used which enables to fit
173 second-order polynomials to the experimental data points. Therefore, curved surfaces can be
174 fitted to the experimental data. A total of 31 experiments were carried out in CCRD (**Table 2**):

sixteen factorial points (coded levels as (+1) and (-1)); eight axial points (coded as (-1) and (+1)) and seven center points (coded as 0).

Both linear and quadratic effects of the four variables under study, as well as their interactions, on the dependent variable namely LA (U/g, *Y*) were calculated. Their significance was evaluated by analysis of variance (ANOVA). The experimental design results were fitted by a second-order polynomial equation in order to correlate the response to the independent variables. The general equation to predict the optimal point was explained as follows (Eq. 1):

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} x_i + \sum_{i=1}^{4} \beta_{kii} x_i^2 + \sum_{i<j=2}^{4} \beta_{kij} x_i x_j \qquad (1)$$

where Y is the predicted response (LA); β_{k0}, β_{ki}, β_{kii} and β_{kij} represent regression coefficients; and x_i x_j are the coded independent factors. The goodness-of-fit of the polynomial model was checked accounting for R^2 and adjusted-R^2, and adequate precision.[8]

Optimal conditions for the constructed model solution depended on combination of the independent process variables were obtained through the predictive equation of RSM and the LA. Three additional confirmation experiments were carried out to verify the accuracy of statistical experimental design. Finally, the experimental and predicted values were compared in order to determine the model validity.

The obtained data for LAs produced by the screened bacterial isolates and LA of the *Acinetobacter* isolated from CSW as a function of different metal ions were subjected to analysis of variance (ANOVA) using SPSS 13 software (SPSS Inc., Chicago, Illinois, USA). The means were compared using the Duncan's multiple ranges test at a significant level of P <0.05.

Partial purification of the produced lipase

The calculated amount of solid ammonium sulphate was added to cell free supernatant with constant stirring at 4°C to give a concentration of 80% (w/v) saturation. The precipitate was allowed to settle overnight, harvested by centrifugation at 13,000×*g* for 30 min and resuspended in minimum volume of 25 mM sodium phosphate buffer (pH 7.2). This enzyme solution was subjected to dialysis for 24 h at 4°C against the same buffer, with three intermittent changes of the buffer. The filtrate was further concentrated using centricon centrifugal filters (30-kDa cutoff; Millipore, Billerica, MA) at 800×*g* for 1 h at 4°C. LA and protein content (by the method of Lowry) were determined for both the dialyzed and the concentrated filtrate sample. [14]

A hydrophobic interaction chromatography (HIC) column of phenyl Sepharose^{TM} (1.5×6 cm) pre-equilibrated with the solution containing 0.6 M ammonium sulphate dissolved in 25 mM phosphate buffer (pH 7.2) was used to assay purity of the concentrated lipase solution. This solution was injected to elute the bound enzyme with a flow rate of 1 mL/min through negative linear gradient.

Molecular weight determination

The procedure of Laemmli *et al*.[15] was applied to perform the electrophoresis of sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE on a 5% polyacrylamide stacking gel and a 12% polyacrylamide-resolving gel. A protein marker in range of 14.3 to 94.7 kDa as a standard marker was used to evaluate molecular weight. Protein bands were visualized by silver staining.

Fourier transform-infrared spectroscopy (FT-IR)

An infrared absorption spectrum between 500-2000 cm^{-1} was recorded using a Fourier transform infrared spectroscopy (FT-IR, Model 1725X, Perkin Elmer, Norwalk, CT, USA) with the

specimen prepared as a potassium bromide (KBr) pellet in order to determine the functional groups present in the purified lipase. The samples made in pellet form had a thickness of 1 mm and a diameter of 13 mm.

3 Results

Preliminary study

The objectives of preliminary experiments were to (I) select the most ideal natural source among CSW, EO, LC, SBC, CSC, FM, OPW and DIE to isolate the lipase-producing bacterium, (II) identify and characterize the best bacterial isolate, (III) determine the most suitable trace element and solid substrate to increase lipase production with the highest activity and then (IV) found the best culture formulation and operation conditions among the various variables.

Twelve bacteria were isolated from eight sources by culturing in NB enriched with olive oil. The isolates were screened based on their lipolytic potential using rhodamine B-agar plate. The formation of orange fluorescent halos around colonies ultraviolet light (350 nm) indicates lipase positive isolate. Among the screened isolates, the CSW isolate due to the highest LA (3124 U/mL) was selected for the further studies (Fig. 2a). The cells of this bacterial isolate were Gram-negative, aerobic, short rod- and coccoid-like, non-motile, non-endospore-forming, non-acid fast, oxidase-negative, and catalase-positive. The selected isolate was positive for H_2S and indole production and urea hydrolysis, but they could not reduce nitrates to nitrogen. The biochemical tests of Voges–Proskauer and citrate utilization were negative and positive, respectively. This bacterial isolate was able to utilize the sole carbon sources of D-glucose, D-mannose, D-tagatose, L-rhamnose, lactose, maltose, sucrose, trehalose, gentiobiose, melezitose and melibiose. However, it was unable to utilize inulin, inositol, D-adonitol, D-arabitol, L-

arabinose, arbutin, cellobiose, raffinose and cellobiose. Moreover, optimum range for growth temperature, salinity (tolerance level to NaCl) and pH for the selected isolate were 15-45°C, 0-5% and 6.5-8, respectively. Sequencing and PCR amplification of 16S rRNA gene sequences as a phylogenetic tree revealed that this isolate is closely associated with the genus *Acintobacter* and represents a distinct sub-line within its members (Fig. 3).

Fig. 2b showed that the best metal ion (25 mM) to maximize the enzyme activity by the newly isolated *Acinetobacter* in a SSF was magnesium (as $MgCl_2 \cdot 6H_2O$, 4181.25 U/g), followed by calcium (as $CaCl_2$, 3903.5 U/g), sodium (as NaCl, 3482.5 U/g), manganese (as $MnCl_2 \cdot 4H_2O$, 3070.0 U/g), potassium (as KCl, 3070.0 U/g), copper (as $CuCl_2$, 1431.25 U/g), and iron (as $FeCl_3 \cdot 6H_2O$, 566.25 U/g). The PB design revealed that MCS was the solid substrate better than grape and pomegranate seeds (Table 1). As earlier mentioned, the process parameters of agitation rate and MC were more effective than the particle size according to the PB design.

Optimization study

The predicted model for LA was highly significant ($p< 0.0001$, Table 3). There was a non-significant lack of fit that further validates the model ($p>0.05$). The values of the R^2 (0.975) and R^2_{adj} (0.953) also confirmed that the model was highly significant. Adequate precision compares the range of the predicted values at the design points to the average prediction error. This factor measures the signal-to-noise ratio so that a ratio greater than 4 is desirable.[8] For the proposed model, this value was 19.74, a very good signal-to-noise ratio. For the LA, effect of MCS/yeast extract (X_1), MC (X_3) and agitation rate (X_4) was significant ($p< 0.05$) in first-order linear effect, second-order quadratic effect (X_1^2, X_2^2, X_3^2 and X_4^2) and interactive effect (X_1X_2, X_1X_3, X_1X_4, X_2X_3 and X_3X_4) (Table 3). The variables with the largest effect were the linear terms of MCS/yeast

extract and agitation rate followed by the quadratic effect of agitation rate. The three-dimensional (3D) surface plot was drawn to visualize the significant ($p< 0.05$) interaction effect of independent variables on the LA (Fig. 4). As considered in Fig. 4, the LA considerably increased as the MC was decreased, but increased with increasing rotation rate. Moreover, an decrease in concentration of olive oil and MCS/yeast extract provided high activity value for the produced lipase. The optimum location led to the highest LA (20480.2 U/g) for the model was estimated to be achieved by a set level of 4.0 w/w, 30 g/L, 65.0% and 180.0 rpm for MCS/yeast extract, olive oil concentration, MC and agitation rate, respectively. The suitability of the presented model for predicting the optimum value of LA was delaminated using the recommended optimum conditions. The maximum activity by lipase synthesized experimentally was found to be 20892.7 U/g, which was clearly very close to the predicted value ($p >0.05$).

Purification study

The produced lipase by *Acintobacter* isolated from CSW was purified using ammonium sulphate precipitation (ASP) followed by HIC. In the chromatography, the enzyme was eluted with the buffer which was highly hydrophobic. Purity and activity recovery were increased 26.9-fold and 27.8%, respectively ($p<0.01$). Fig. 5a showed the purified lipase is as a single protein band in the SDS-PAGE representing the protein content with high purity level. The molecular mass of the biosynthesized lipase was determined to be about 46 kD a proposing that the enzyme is a monomer. Fig. 5b depicted the FT-IR spectra from 500-2000 cm^{-1} for the reference lipase and the enzyme produced by the identified isolate before and after the purification process. The closeness of C=O and C–N stretching vibrations in these spectra demonstrated that the ASP and HIC can considerably improve purity of the biosynthesized lipase.

4 Discussion

Rhodamine B-agar plate (RBAP) method was used to determine lipase producing bacteria. It is suggested that the formation of fluorescent halos under the UV rays in RBAP can be due to rhodamine B dimers complexed with mono or diglycerides and fatty acid liberated by the enzyme into the medium.[16] High activity of produced lipase by the *Acinetobacter* isolated from CSW can probably be attributed to the ideal growth conditions and substrate type present in Caspian Sea water. [17] *Acinetobacter* sp. have a high distribution and survival on moist and dry surfaces. A number of lipase producing bacteria especially *Acinetobacter* sp. inhabit the outer body of many fishes and can feed upon different minerals and protein sources. It has been reported that *Acinetobacter* sp. are one of the most important lipase-producing microorganisms such as *Ac. calcoaceticus* [18], *Ac. radioresistens* [19], and *Ac. baylyi* [20].

Minerals play a significant role in production of extracellular lipase. They, as cofactors of several enzymes involved in the biosynthetic pathway of enzymes, can improve valuable metabolites at certain concentrations.[21,22] Among the different ions, magnesium and calcium led to a considerable increase in LA by the newly isolated *Acinetobacter*. Kok *et al.*[23] found that production of extracellular lipase by *A. calcoaceticus* BD 413 was significantly enhanced when the medium was supplemented with divalent cations of Mg^{2+}, Ca^{2+}, Cu^{2+} and Co^{2+}. An increase in lipase biosynthesis by *A. niger* and *Pseudomonas pseudoalcaligenes* F-111 in the presence of Mg^{2+} ions was observed.[24,25] Hasan *et al*.[3] had reported that Mg^{2+}ions would generally increase the lipase production by forming complexes with ionized fatty acids, changing their solubility and behavior at interfaces.

The higher effect of MCS than the milled seeds of grape and pomegranate on the LA can be attributed to the high nitrogen content of this seed. It was demonstrated that microorganisms provide high yields of lipase when organic nitrogen sources together with olive oil as carbon source are used at optimal concentrations.[23] Moreover, response surface optimization showed that the MCS/yeast extract and agitation rate had more effect than MC and olive oil concentration on the lipase productivity of aerobic microorganism of *Acinetobacter* in shake flask cultures. These factors at their optimum points can improve substrate's particle structure with the increasing MCS porosity and solubility to transfer oxygen to the isolated *Acinetobacter* and can increase the LA. [26] A higher MC to achieve the highest LA by *A. niger* (71.0%) was reported than the isolated *Acinetobacter* (65.0%) in this study.[27] However, Mahanta *et al.*[28] found an optimum MC of 50% for the lipase production by *P. aeruginosa* in SSF using *Jatropha curcas* seed cake as substrate. The LA reduction by increasing MC can be attributed to the aggregation of substrate particles, poor aeration, and possible anaerobic conditions.[6] Similarly, MC lower than optimum level can considerably decrease the solubility and/or degree of swelling of the solid substrate, and thus by providing a higher water tension can produce lipase with a lower activity. [26] The LA enhancement by increasing agitation rate can be due to the improved oxygen transfer rate as well as the mixing efficiency of the culture, thus enhancing the cell growth and lipase production. However, higher agitation rate more than 180 rpm by increasing shear force led to a negative impact on cell growth and enzyme activity.[29] Alonso *et al.* [30] have also reported 200 rpm, stirring rates as optimum while Freire *et al.*[31] have reported maximum production of lipase at agitation rate of 300 rpm with *Pencillium* sp. These researchers showed that an increase in agitation rate more than the optimal level (200 or 300 rpm) resulted in decreased enzyme production because of mechanical and/or oxidative stress, excessive foaming,

disruption and physiological disturbance of the cells. [30,31] Activity reduction of the synthesized lipase at low agitation rates can be attributed to the limited oxygen levels along with the lacking of homogeneous suspension of the fermentation medium and breaking of the clumps of the cells.[32] Low increase of the enzyme activity in presence of olive oil inducer in the culture medium could be due to its high content of long, unsaturated fatty acyl chains, such as oleic acid. This fact was earlier demonstrated by other researchers on the lipase production from *Amycolatopsis mediterranei* and *Microbacterium luteolum*. [17,33] The LA (20480.2 U/g) obtained at the optimal conditions was considerably more than activity of lipase produced by *Yarrowia lipolytica* (69.0 U/g)[34], *Penicillum sp*. (140.7 U/g)[35], *Rhizopus oryzae* (96.52 U/g)[36], *Ac. radioresistens* (54 U/L)[37], *Bacillus sp.* (168 U/mL)[38], *A. niger* (33.03 U/mL)[39], *Acinetobacter* sp. EH28 (57.1 U/mL)[40], *Geobacillus thermoleovorans* CCR11 (2283 U/mL)[41], *A. terreus* (1566 U/mL)[42] and *P. aeruginosa* AAU2 (0.432 U/mL)[43] in SSF cultures.

A suitable lipase purity is mostly required for use in the different areas such as food processing, detergent, paper and pulp industry. Therefore, it is necessary to remove some unwanted impurities like proteins because of their antagonistic effects on the desired enzyme's activity.[3] Partial purification of the extracted lipase from free fraction of liquid culture by ASP and HIC led to a considerable increase in the purity (26.9-fold) in comparison to other reports. Ahmed *et al*.[40] and Lee *et al*.[44] using the same methods respectively achieved 24.2-fold and 9-fold purification for lipases produced by *Acinetobacter* sp. EH28 and *Acinetobacter* ES-1. Uttatree *et al.*[18] reported a successful purification for lipase synthesized by *A. baylyi* (21.89-fold) to homogeneity by ASP and gel-permeable column chromatography with a relative molecular mass of 30 kDa. The enzyme recovery (27.8%) by the studied *Acinetobacter* was higher than the recovery of lipase biosynthesized by *Geotrichum* sp. SYBC WU-3 (20.4%) and *Ralstonia* sp.

CS274 (20.8%).[45,46] However, the low recovery may be attributed to strong affinity of the produced lipase with the matrix which may hold some activity even using highly hydrophobic elution condition.[47] The molecular mass of lipase produced by newly isolated *Acinetobacter* (~ 46 kDa) was found in the range of other lipases synthesized from the same genus such as *A. colcoaceticus* BD413 (32 kDa), *A. radioresistens* CMC-1 (45 kDa), *A. calcoaceticus* LP009 (23 kDa), and *Acinetobacter* sp. RAG-1 (43 kDa).[4,5,18,20] IR radiation is adsorbed by the polypeptide chain backbone in IR spectroscopy and excited the vibrational modes of basic amide (I and II) bonds to determine protein secondary structure. Main protein bonds because of the peptide group vibration occurred in the spectral region of 1000-1700 cm^{-1}.[47] The signals at 1645 cm^{-1}in the partial purified sample is due to the C-O stretching vibrations of amide I. The bonds at 675, 1111, 1412 and 3390 cm^{-1} are respectively assigned to C-N stretching vibrations of amide I, partly wide peak of amide I, bending peak of amide I and O-H functional group. Comparison of between infrared absorption spectrum of the purified and crude enzymes revealed that the bands at 1659, 1393 ,663, 1077 and 3436 cm^{-1} in the crude enzyme can be due to the C-O stretching vibrations of amide I, C-N stretching vibrations of amide I, partly wide peak of amide I, bending peak of amide I and O-H group, respectively. In the purified enzyme sample, the band at 1545.5 cm^{-1} is also assigned to the N–H bending (amide II region) with a contribution of the C–N stretching vibrations.[47,48] Moreover, the band at 1234.7 cm^{-1} in this sample can be ascribed to the N–H bending and, C–C and C–N stretching vibrations that this peak shows random coil conformation of proteins. The amide I band at 1645.63 cm^{-1} is due to the stretching vibrations of the C=O bonds in the backbone of the protein; therefore, the frequency of this peak is sensitive to protein secondary structure.[48]

5 Conclusion

The microbial lipase production by a new strain of *Acinetobacter* sp. using milled seeds of coriander and olive oil in batch SSF flasks was studied. The obtained results are promising because this strain produces lipase with the highest activity (20480.2 U/g) in an inexpensive medium which facilitates its recovery and purification. Partial purification led to an increase in the purity (26.9-fold) and activity recovery (27.8%). The biosynthesized lipase from *Acinetobacter* isolated from CSW will be exposed for its potential esterification and transesterification reaction in organic solvents, synthesis of some industrial esters and biotreatment of lipid-rich industrial wastewaters. However, further studies are needed to purify and characterize the produced lipase to utilize in industrial applications.

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Table 1 PB matrix for the evaluation of the lipase activity produced by *Acinetobacter* isolated from CSW

A	Independent variables											
Run	Yeast extract (g/L)	Peptone (g/L)	Particle size (mm)	MCS[a] (g/L)	MPC[b] (g/L)	MGC[c] (g/L)	$MgCl_2$ (mM)	Agitation rate (rpm)	MC (%)	Molasses (g/L)	Olive oil (g/L)	**LA(U/g)[d]**
1	15	15	0.5	40	40	40	20	130	50	15	10	5452.5
2	5	15	2	20	40	40	40	130	50	5	30	5234.0
3	15	5	2	40	20	40	40	180	50	5	10	6543.0
4	5	15	0.5	40	40	20	40	180	80	5	10	6787.0
5	5	5	2	20	40	40	20	180	80	15	10	5456.0
6	5	5	0.5	40	20	40	40	130	80	15	30	6634.0
7	15	5	0.5	20	40	20	40	180	50	15	30	7431.0
8	15	15	0.5	20	20	40	20	180	80	5	30	7518.0
9	15	15	2	20	20	20	40	130	80	15	10	6300.0
10	5	15	2	40	20	20	20	180	50	15	30	8478.7
11	15	5	2	40	40	20	20	130	80	5	30	6845.0
12	5	5	1.25	20	20	20	20	130	50	5	10	5294.0
13	10	10	1.25	30	30	30	30	155	65	10	20	7638.0
14	10	10	1.25	30	30	30	30	155	65	10	20	7605.5

[a] MCS: Milled coriander seeds; [b] MPC: Milled pomegranate seeds; [c] MGC: Milled grape seeds; [d] The activity was measured after 24 h incubation

Table 2 CCRD matrix for the evaluation of the lipase activity produced by *Acinetobacter* isolated from CSW

Run	Independent Variables				Response variable (LA, U/g)	
	MCS/yeast extract (w/w)	Olive oil (g/L)	MC (%)	Agitation rate (rpm)	Experimental value[a]	Predicted value
1	4.00	15	65	150	7887.5±126	9239
2	5.34	15	65	150	6510±45	6221
3	4.00	30	65	150	10577±79	9996
4	5.34	30	65	150	11412±122	12323
5	4.00	15	80	150	14792±412	14148
6	5.34	15	80	150	5738±321	6546
7	4.00	30	80	150	5955±152	6556
8	5.34	30	80	150	3135±27	4298
9	4.00	15	65	180	20832±119	20162
10	5.34	15	65	180	11725±349	11102
11	4.00	30	65	180	21310±487	20480
12	5.34	30	65	180	15627±29	16764
13	4.00	15	80	180	21027±245	20094
14	5.34	15	80	180	5376±216	6450
15	4.00	30	80	180	11280±450	12062
16	5.34	30	80	180	5135±32	3762
17	3.33	22.5	72.5	165	15187±161	15883
18	6.01	22.5	72.5	165	5736±49	4566
19	4.67	7.5	72.5	165	11497±146	11693
20	4.67	37.5	72.5	165	10433±87	9763
21	4.67	22.5	57.5	165	15632±246	15663
22	4.67	22.5	87.5	165	8075±87	7570
23	4.67	22.5	72.5	135	8713±341	7287
24	4.67	22.5	72.5	195	16723±246	17675
25	4.67	22.5	72.5	165	3142±21	3387
26	4.67	22.5	72.5	165	4651±444	3387
27	4.67	22.5	72.5	165	3362±12	3387
28	4.67	22.5	72.5	165	4360±32	3387
29	4.67	22.5	72.5	165	3271±145	3387
30	4.67	22.5	72.5	165	3387±64	3387
31	4.67	22.5	72.5	165	1536±316	3387

[a] Mean ± standard deviation (n = 3)

501 **Table 3** ANOVA of the experimental results of the RSM-CCRD

Source	Coefficient	Sum of squares (SS)	df [a]	Mean of squares	F-value	Probability>F [b,c,d]
Model	3387.00	9.807E+008	14	7.005E+007	45.24	< 0.0001**
X_1 (MCS/yeast extract)	-2829.35	1.921E+008	1	1.921E+008	124.07	< 0.0001**
X_2 (Olive oil concentration)	-	5.592E+006	1	5.592E+006	3.61	ns
X_3 (MC)	-2023.19	9.824E+007	1	9.824E+007	63.44	< 0.0001**
X_4 (Agitation rate)	2596.90	1.619E+008	1	1.619E+008	104.52	< 0.0001**
X_{11}	1709.53	8.357E+007	1	8.357E+007	53.97	< 0.0001**
X_{22}	1835.40	9.633E+007	1	9.633E+007	62.21	< 0.0001**
X_{33}	2057.53	1.211E+008	1	1.211E+008	78.17	< 0.0001**
X_{44}	2273.65	1.478E+008	1	1.478E+008	95.46	< 0.0001**
X_{12}	1336.03	2.856E+007	1	2.856E+007	18.44	0.0006**
X_{13}	-1146.09	2.102E+007	1	2.102E+007	13.57	0.0020**
X_{14}	-1510.59	3.651E+007	1	3.651E+007	23.58	0.0002**
X_{23}	-2087.47	6.972E+007	1	6.972E+007	45.02	< 0.0001**
X_{24}	-	1.935E+005	1	1.935E+005	0.12	ns
X_{34}	-1244.34	2.477E+007	1	2.477E+007	16.00	0.0010**
Residual	-	2.478E+007	16	1.549E+006	-	-
Lack of fit	-	1.873E+007	10	1.873E+006	1.86	0.2312[ns]
Pure error	-	6.045E+006	6	1.007E+006	-	-
Core total	-	1.005E+009	30	-	-	-
$R^2 = 0.975$; Adjusted-$R^2 = 0.953$; $CV = 13.12$ Adequate precision = 19.74						

502 [a] df: Degree of freedom; [b] Significant (* $p< 0.05$); [c] Highly significant (** $p< 0.01$); [d] ns: Not significant

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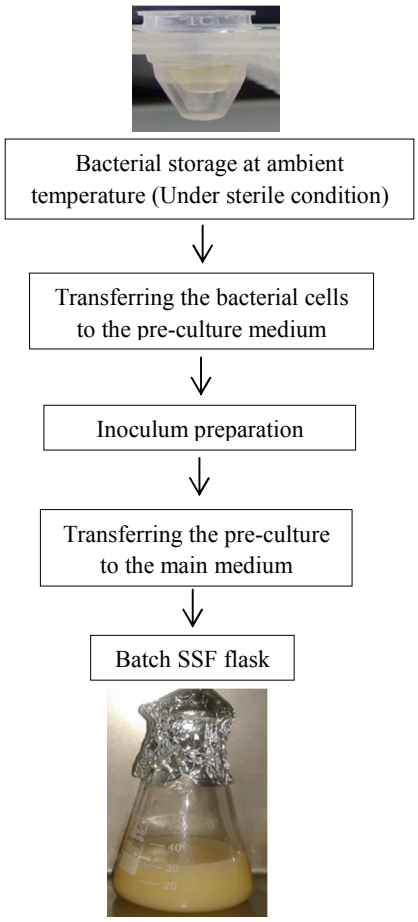

Fig. 1 The bacterial activation, inoculation steps and preparation of batch SSF flask for the lipase production

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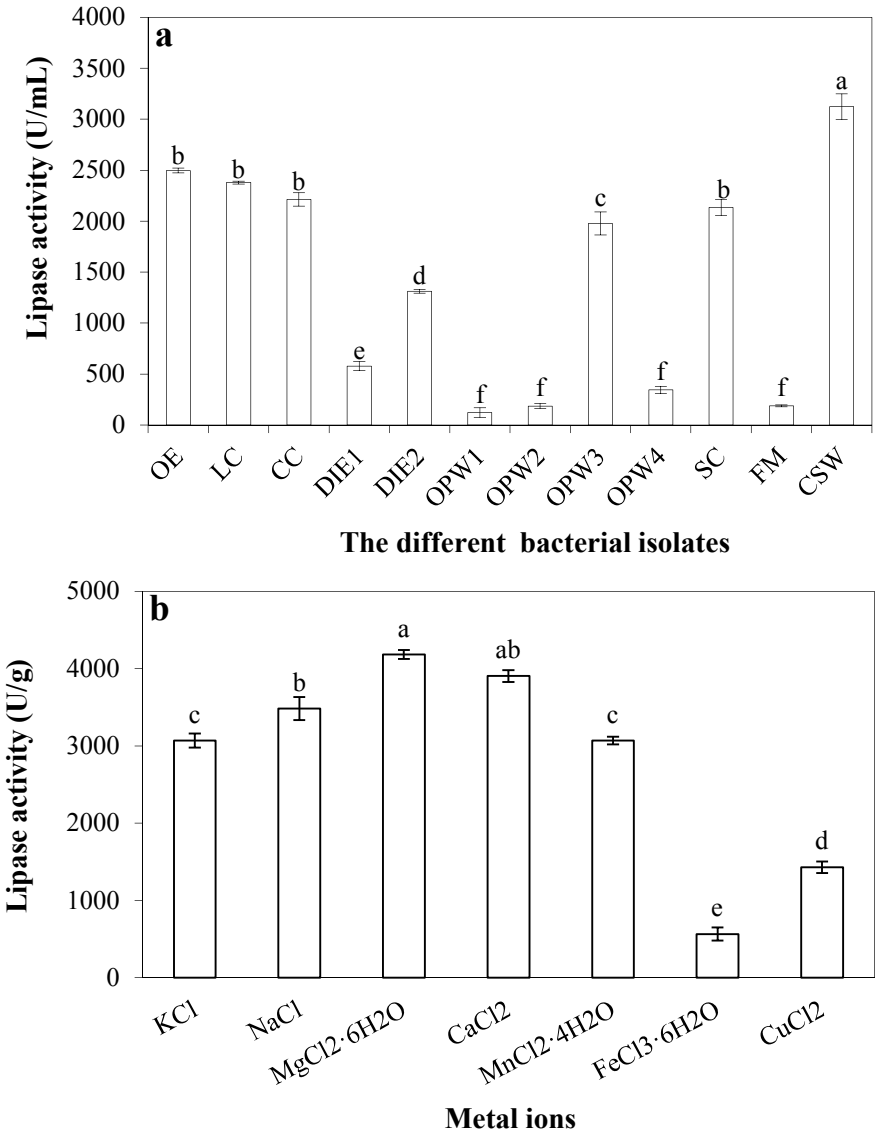

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525 **Fig. 2** (a) The lipase activities of the screened bacterial isolates in SLF and (b) lipase activity of

526 the *Acinetobacter* isolated from CSW in SSF as a function of different metal ions at

527 concentration of 25 mM.

528 .

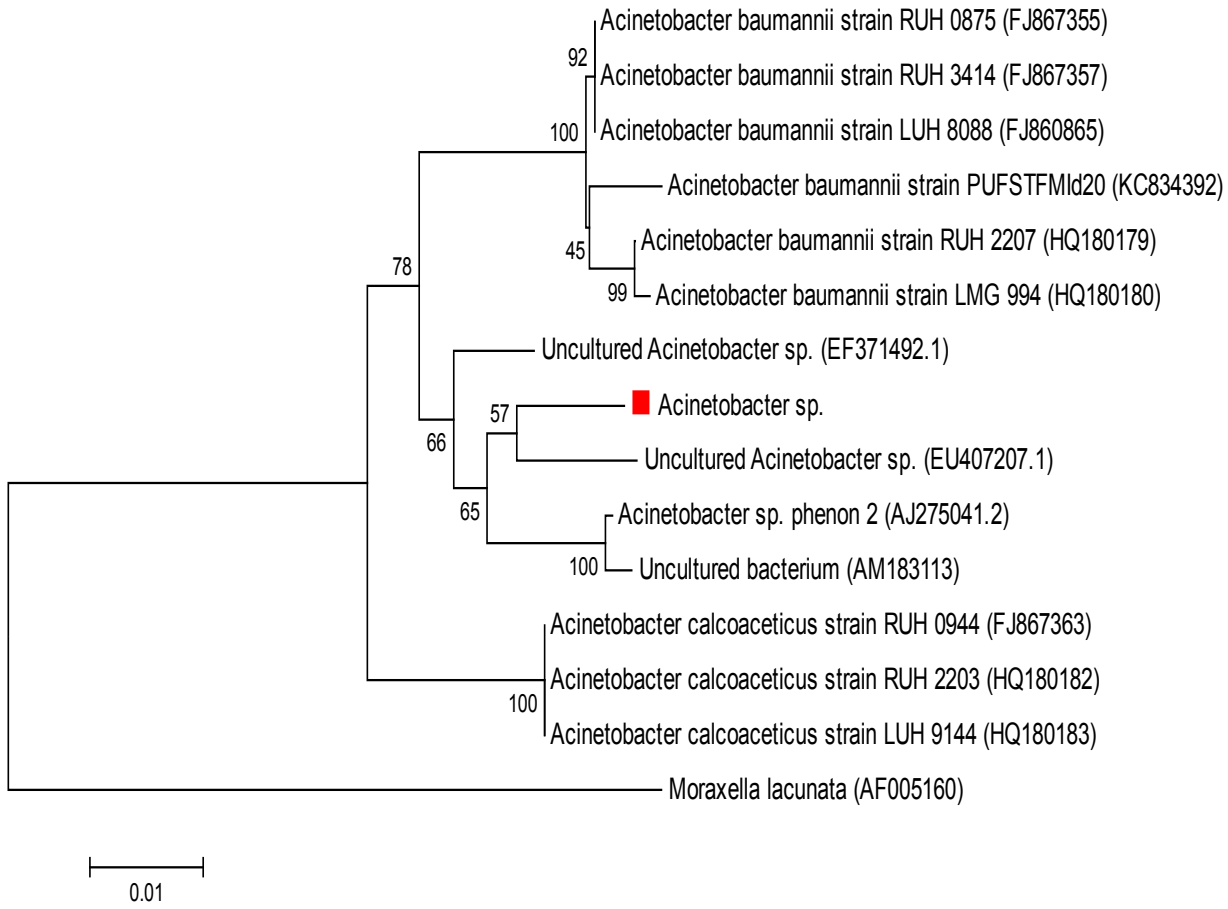

Fig. 3 Phylogenetic tree analysis displaying the relationship between strain *Acinetobacter* sp. (■) and other *Acinetobacter* species based on 16S rRNA gene sequence data using the neighbor-joining method. Scale bar represents 0.01 substitutions per nucleotide position. The sequence of *Moraxella lacunata* AF005160 was used as an outgroup (Tamura et al. 2013).

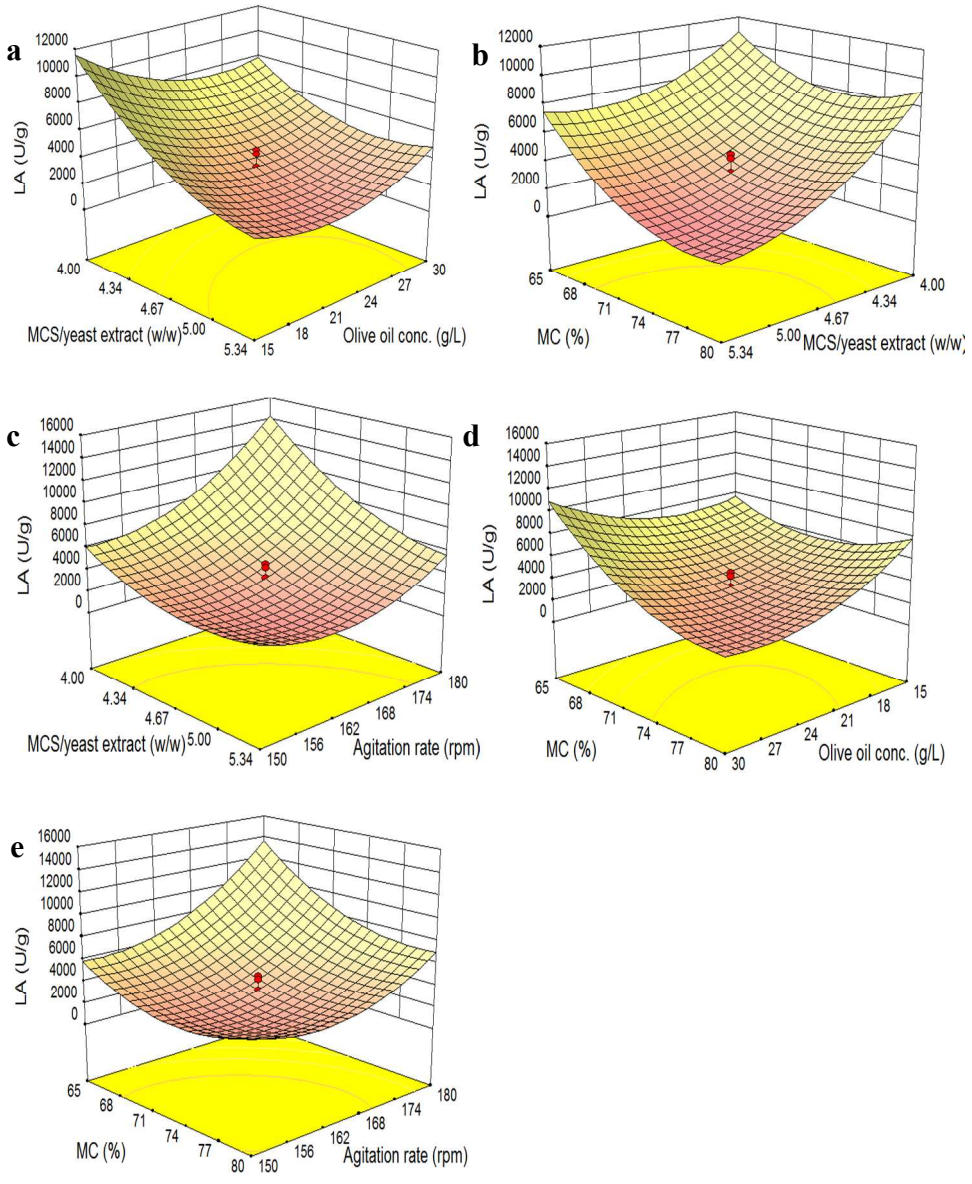

Fig. 4 3D surface plots showing the significant ($p < 0.05$) interaction effects on the activity variation of lipase (a-e) produced by *Acinetobacter* sp. isolated from CSW.

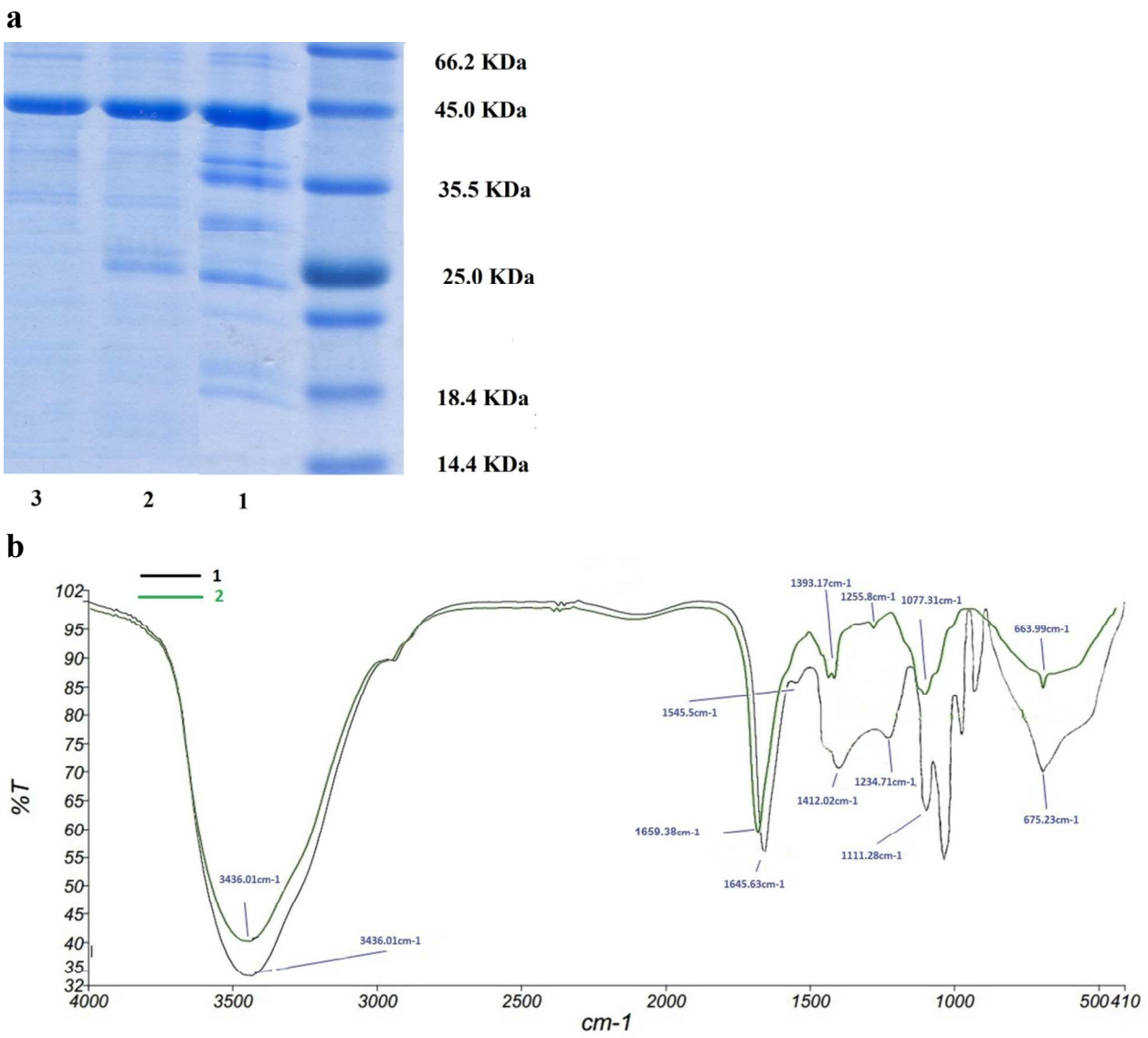

Fig. 5 (a) SDS–PAGE of the partially purified lipase produced by *Acinetobacter* sp (lane 1, cell free culture supernatant sample; lane 2, ASP- sample; lane 3, HIC-sample), and (b) FT-IR spectra from the produced lipase after (black line (1)) and before (green line (2)) the partial purification