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Optimization, Purification and Characterization of Extracellular Lipase Produced by *Serratia marcescens* EGHK-19

Heba K. Issa[(1)#], Mohamed I. Abou Dobara[(2)], Ahmed K.A. El-Sayed[(2)], Magdy I. El-Bana[(1)]

[(1)] Department of Botany, Faculty of Science, Port Said University, Port Said, Egypt;
[(2)]Department of Botany & Microbiology, Faculty of Science, Damietta University, Damietta ElGededa, Egypt.

LIPASES are hydrolytic enzymes which have significant potential for commercial applications, particularly in the breakdown of oil contaminants. *Serratia marcescens* EGHK-19 isolate exhibited considerable lipase activity. This study investigates the optimization, purification, and characterization of lipase from the *Serratia marcescens* EGHK-19 isolate. The optimized culture conditions revealed that maximal lipase activity was achieved after 24 hours at 30°C and pH 7, with continuous shaking at 150 rpm. Utilizing a 2% inoculum percentage with 1% diesel and 0.3% tryptone in the presence of Fe^{2+}, Ca^{2+}, Mg^{2+} salts, and Tween 80 resulted in the highest activity at 17.278 U/ml/min. The purification process involved acetone precipitation and DEAE-Sephadex column chromatography, revealing a molecular weight of approximately 60 kDa on SDS-PAGE. This method exhibited a 0.985-fold purification and the final yield was limited to 2.097% due to lipase aggregates. Characterization of the purified lipase indicated optimal activity (8.765 U/mL/min) at 40°C and pH 7. The Km and Vmax values were calculated as 6.89 mM and 65.79 µmol/min, respectively. The presence of SDS, Tween 80, and Triton X-100 surfactants resulted in the inhibition of lipase activity. Despite these inhibitors, the biochemical characteristics of the purified lipase suggest its potential as an excellent candidate for various industrial applications.

Keywords: Aggregates, DEAE Sephadex, SDS-PAGE, Submerged fermentation.

Introduction

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are a specific class of enzymes that catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at the lipid and water interface (Gupta et al., 2004). It is well known that the reaction is reversible, and this enzyme can catalyze various reactions such as interesterification, alcoholysis, acidolysis, esterification, and aminolysis (Tomke & Rathod, 2015). Also, they do not require cofactors, and they are active at organic interfaces (Yong et al., 2016). Thus, lipases are used in different industries such as detergents, food, bioenergy, flavors, pharmaceuticals, paper, textiles, leather, cosmetics, perfumery, and wastewater treatment (Hasan et al., 2006; Vivek et al., 2022). Globally, lipases rank third in total sales of industrial enzymes after proteases and carbohydrates (Dahiya & Purkayastha, 2011). The international lipase market is expected to cross USD 797.7 million by 2025, rising at a 6.2% compound annual growth rate from 2017 to 2025 (Fatima et al., 2021).

Lipases are widely distributed in animals, plants, and microorganisms (Sahu & Martin, 2011). Bacterial lipases are commercially more important due to the facility of their culture and optimization to obtain a higher yield and more stability than others (Hasan et al., 2006). The majority of microbial lipase products come in free forms like powders and liquids (Bhatia, 2018). Such important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas*, *Burkholderia*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Staphylococcus*, and *Chromobacterium* spp. The capacity of *Serratia*

#Corresponding author email: heba.kamal@sci.psu.edu.eg, Telephone: 01060700188
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spp. to generate lipase has also been investigated (Immanuel et al., 2008; Zaki & Saeed, 2012; Vivek et al., 2023).

On lipidic carbon sources, such as oils, fatty acids, or glycerol, lipases are often generated in the presence of an organic nitrogen source (Jaeger & Eggert, 2002). Bacterial lipases are mostly generated via submerged fermentation and are extracellular. Understanding the variables that affect the enzymatic activity of lipases, such as temperature, ions, and pH values, is necessary for the use of lipases in a variety of industrial activities (Aouf et al., 2014). Therefore, the current research aims to optimally produce lipase by *Serratia marcescens* isolate EGHK-19. Furthermore, we aim to fine-purify lipase and study its characteristics.

Materials and Methods

Microorganism

Serratia marcescens EGHK-19 was isolated from soil contaminated with oil in Port Said governorate, Egypt, situated at coordinates 31°10'56.1"N and 32°16'47.1"E. Its identification was carried out using Bergy's Manual of Systematic Bacteriology (Bergey & John, 2000) and confirmed through the analysis of the 16S rDNA sequence (unpublished data). The 16S rDNA sequence has been deposited in the Gene Bank with the accession number OR362347.

Optimization of lipase enzyme production factors

The production media for MSM (minimal salt medium) comprises the following components per liter: K_2HPO_4 1.73g, KH_2PO_4 0.68g, $MgSO_4.7H_2O$ 0.1g, $FeSO_4.7H_2O$ 0.03g, NH_4NO_3 1.0g, $CaCl_2.2H_2O$ 0.02g, and NaCl 4.0g, with a targeted final pH of 7.0 (Sekar et al., 2011). To initiate bacterial growth, loopfuls of a 24h bacterial culture were introduced into 50mL of nutrient broth (NB) supplemented with 1% diesel. This culture was then incubated for 24h in a shaking incubator at 30°C with 150rpm agitation. Following incubation, the culture was resuspended in a 0.9% (w/v) sodium chloride solution, achieving an initial cell density equivalent to 0.5 McFarland standards. A one-milliliter portion of the resulting bacterial suspension was employed as the starter culture (Ghazali et al., 2004).

The impact of various factors on lipase activity was systematically investigated using the one-factor-at-a-time (OFAT) approach. To optimize lipase production, the starter culture was inoculated into 50mL of MSM production medium. Lipase activity was assessed at different incubation periods (12, 24, 48, 72, and 96h). The influence of diverse incubation temperatures (25°C–50°C) and initial pH ranges (5–10) was examined, with subsequent evaluation of pH values in the MSM medium following bacterial growth. Additionally, the study explored the effects of different agitation rates (120–250rpm) and initial inoculum percentages (1%–7% v/v of overnight seed culture). The fermentation medium was enriched with varying diesel concentrations (0.5%–4%) and 1% of different carbon cosubstrates (glucose, sucrose, and lactose) to identify the optimal conditions for lipase yield. The impact of various nitrogen sources on lipase activity was investigated by substituting the original nitrogen source, ammonium nitrate, with peptone, tryptone, yeast extract, urea, sodium nitrate, and ammonium sulfate. The concentration of the most effective nitrogen source was further fine-tuned within the range of 0.05% to 0.7%. Furthermore, the study explored the influence of trace elements on lipase activity by selectively eliminating $FeSO_4$, $MgSO_4$, and $CaCl_2$ from the production medium. A control was maintained by using the original BH medium without removing any ingredients. To assess the impact of surfactants, 50µL of Tween 80, Tween 20, and Triton X-100 were individually added, with a culture without surfactant serving as a control.

Estimation of lipase activity

To quantify lipase productivity, a cell-free culture was obtained, following the methodology outlined by Mahmood et al. (2017). The hydrolytic activity of the lipase was assessed using para-nitrophenyl palmitate (pNPP) as a substrate, employing a method detailed by Iqbal & Rehman (2015). The measurement criterion stipulated that one unit of lipase activity corresponds to the release of 1µmol of free p-nitrophenol per minute. Protein content was determined in accordance with the procedure outlined by Lowry et al. (1951), utilizing Bovine Serum Albumin (BSA) as a standard.

Purification of lipase

Acetone precipitation method

The 24-hour-old culture was subjected to centrifugation at 9,168 x g for 5min at 4°C. The resultant supernatant underwent filtration using a cellulose membrane filter with a pore diameter of 0.45µm, yielding a clear crude enzyme solution. This filtered lipase solution was subsequently

treated with 50% cold absolute acetone for 3h at 4°C. The resulting suspensions were then centrifuged at 20,627 x g for 15min in a cooling centrifuge at 4°C. The obtained pellets were reconstituted in a 50mM Tris-HCl buffer (pH 7) and assessed for both lipase activity and protein content. To concentrate the precipitated protein, dialysis tubing sacks (sigma cellulose membrane 3cm, cut-off 10 KDa) were employed, and lipase activity along with protein content were recalculated.

Ion exchange chromatography

For additional purification, the enzymatically active fraction underwent ion exchange chromatography using a DEAE-Sephadex anion exchanger column (3cm x 20cm). The column was initially equilibrated with a flow rate of 2mL/min using an equilibration buffer (50mM Tris-HCl buffer, pH 8). The concentrated protein was then loaded onto the column, and elution was carried out with a linear continuous gradient of NaCl in the equilibration buffer, ranging from 0 to 1000mM over 3h. Fractions, each with a volume of 2mL, were collected and subsequently screened for both lipase activity and protein content. The fractions exhibiting the highest lipase activities (18mL) were gathered, concentrated, and stored at -20°C for further procedures. Throughout each purification stage, the fold purification and the recovery of lipase were determined.

Gel electrophoresis

In accordance with the methodology described by Nam et al. (2016), with some modifications, SDS-PAGE was conducted using a 4% stacking gel and a 10% resolving gel under reducing conditions. Each protein sample (20µL) was combined with 2.5µL of SDS sample buffer. This 3X buffer consisted of 1M Tris HCl pH 6.8, 10% SDS, 0.3% Bromophenol blue, 1% β-mercaptoethanol, and 30% glycerol. A running buffer, comprising 0.1% (w/v) SDS dissolved in 25 mM Tris-HCL and 192mM glycine at pH 8.3, was employed at 120 V for 120min using a Mini-Protean Tetra Cell (Bio-Rad). The gels were stained for 20min using a solution composed of 0.1% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, and 10% (v/v) acetic acid. A PageRuler™ Prestained Protein Ladder served as a reference for molecular weights. The migration distances of standard proteins were plotted against their logarithmic molecular weights. The migratory lengths of potential lipase bands were measured, allowing for the estimation of their molecular weights.

In this study, chromogenic zymography was employed in conjunction with SDS-PAGE. Following electrophoresis, the SDS gel underwent a series of washes: first for 10min in 2.5% (v/v) Triton X-100 (50mM Tris-HCl pH 8), followed by another 10min with 1% (v/v) Triton X-100 in the same buffer. Subsequently, the gel was washed with 20% isopropanol for 20min, as detailed by Kwon et al. (2011). After three rinses with distilled water, the gel was incubated for 30min at room temperature in an activation buffer (50mM Tris-HCl, pH 8.0). These washing steps were crucial for removing denaturing agents and facilitating the refolding of lipases. The gel was then coated with a molten chromogenic substrate consisting of 1% (v/v) tributyrin (Sigma-Aldrich, USA), 0.01% (w/v) phenol red, 10mM $CaCl_2$, and agar (2%). The pH was adjusted to 7.3–7.4 using 0.1N NaOH. The chromogenic substrate solidified and was incubated at room temperature, following the approach outlined by Davis (1964). Lipase activity was visually indicated by the appearance of a yellow band in the gel, as described by Singh et al. (2006).

Characterization of purified lipase enzyme

Determination of temperature optima

To assess the enzyme's sensitivity to temperature, experiments were conducted at different temperature settings, encompassing 30°C, 40°C, 50°C, and 60°C. The relative activity (%) was then determined under standard enzyme testing conditions.

Determination of PH optima

To ascertain the optimal pH for the lipase enzyme, tests were conducted across a range of pH levels. The reaction mixture was dissolved in different buffer systems, including potassium phosphate buffer (50mM, pH 6), and Tris-HCl buffer (50mM, pH 7-9), and the relative activity (%) was determined through a standard assay technique.

Effect of different detergents on purified lipase activity

The impact of various detergents, including SDS, Tween 80, and Triton X-100 at a concentration of 1%, was examined. The purified lipase was pre-incubated with these compounds at a refrigerated temperature (4°C) for 15min to allow for stabilization. Residual activity was then assessed by comparing it to the lipase activity without any pre-incubation, considered as 100%.

Determination of Km and Vmax of purified lipase

The impact of different concentrations of pNPP (4, 8, 1.2, 1.6mM) in 0.05 M Tris-HCl buffer at pH 8.0 was studied at 50°C concerning the rate of hydrolysis catalyzed by the purified lipase (100µL). The Michaelis-Menten constant *(Km)* and maximum reaction rate (*Vmax*) were determined through Lineweaver-Burk plots (Lineweaver and Burk, 1934), where the slope equals Km / Vmax.

Statistical analysis

The obtained data underwent significant statistical analysis using one-way ANOVA ($P< 0.05$) with Tukey's test for post hoc comparisons. Mean values, when applicable, were presented as the mean ± standard error. Statistical analyses were performed using SPSS software for Windows (SPSS Inc., Chicago, IL, USA; Version 16.0).

Results

Optimization of lipase producing media

After 24 hours of incubation, the *S. marcescens* EGHK-19 isolate demonstrated its peak lipase activity at 5.364 ± 0.257U/mL/min, which subsequently declined with prolonged incubation (Fig. 1A). Notably, the lipase activity reached a substantial maximum productivity of 4.191 ± 0.189U/mL/min at 30°C, with the lowest activity observed at 50°C (0.467 ± 0.054U/mL/min) (Fig. 1B). Maximum lipase synthesis occurred at a neutral pH of 7 (4.665U/mL/min), diminishing at both acidic and alkaline pH levels (Fig. 1C). A significantly higher lipase activity of 4.541 ± 0.054 U/mL/min was recorded at 150rpm (Fig. 1D). Figures 1E and 2A illustrated that an inoculum size of 2% (v/v) with 1% supplemented diesel resulted in the highest lipase production, reaching an activity of 4.109U/mL/min. However, the addition of 1% cosubstrate in the fermentation medium led to a notable reduction in lipase activity compared to the control, as depicted in Fig. 2B. Substituting NH_4NO_3 with organic nitrogen tryptone significantly increased lipase productivity, reaching a maximum of 7.874 ± 0.319U/mL/min, whereas the lowest activity was observed with $(NH_4)_2SO_4$ (Fig. 2C). Fig. 2D indicated that the maximum enzyme activity of 10.323U/mL/min was achieved with 0.3% (w/v) tryptone. Lipase activity suffered a negative impact when any elements were removed, as shown in Fig. 2E. Additionally, Fig. 2F highlighted the lipase activity with the addition of various surfactants, with Tween 80 demonstrating the maximum enhancement after 24 hours of incubation, reaching a significant activity of 17.278 ± 0.163 U/mL/min.

Purification of lipase enzyme

The lipase enzyme derived from *S. marcescens* EGHK-19 underwent purification to achieve homogeneity. This purification process involved consecutive steps of acetone precipitation and ion exchange chromatography (anion). The outcomes of the purification studies are summarized in Table 1. Acetone precipitation, followed by concentration on sucrose, yielded 41.599%, leading to a 9.731-fold purification and a specific activity of 164.992U/mg. Subsequently, the concentrated precipitate underwent DEAE Sephadex anion exchange chromatography, revealing two distinct peaks in the elution profile. The first peak was observed at fraction 23, exhibiting an activity of 13.21 U and a specific activity of 13.858 U/mg protein (Fig. 3). The second peak emerged in active fractions 63–71, within the 0.6–0.7 M NaCl gradient. This peak demonstrated a total yield of 2.097%, accompanied by a 0.985-fold purification and a specific activity of 16.708 U/mg protein.

Figures 4A and B present the SDS-PAGE analysis of the purification process along with zymogram analysis. The findings illustrate that the lipase underwent purification to homogeneity, evident from the presence of a single band in the well corresponding to DEAE Sephadex. In the gel, the precipitated crude lipase by Acetone was observed confined within the stacking gel (Fig. 4, lane 2). Zymogram analysis corroborated the SDS-PAGE results, revealing active lipase bands that corresponded to pure lipase. The molecular weight of the purified lipase was estimated to be around 60 kDa.

Characterization of purified lipase

Temperatures between 30 and 60°C were used to investigate lipase activity; the maximal activity (8.765 U/mL/min) was found at 40 °C (Fig. 5A), where there was 346.306% relative activity. The lipase activity decreased at elevated temperatures. Over a wide pH range of 6 to 9, the purified enzyme was active. The maximum lipase activity occurred at pH 7 with 129.277% of relative activity (Fig. 5B). In this study, the

addition of three tested surfactants minimized the lipase activity (Fig. 5C). The maximum reduction of lipase activity was observed in the presence of SDS, recording 23.526% of relative activity. Tween 80 was found to be the least effective surfactant, where the enzyme retained 79.416% of its initial activity. The Km and Vmax values of lipase were 6.89 mM and 65.79µM/min, respectively (Fig. 5D).

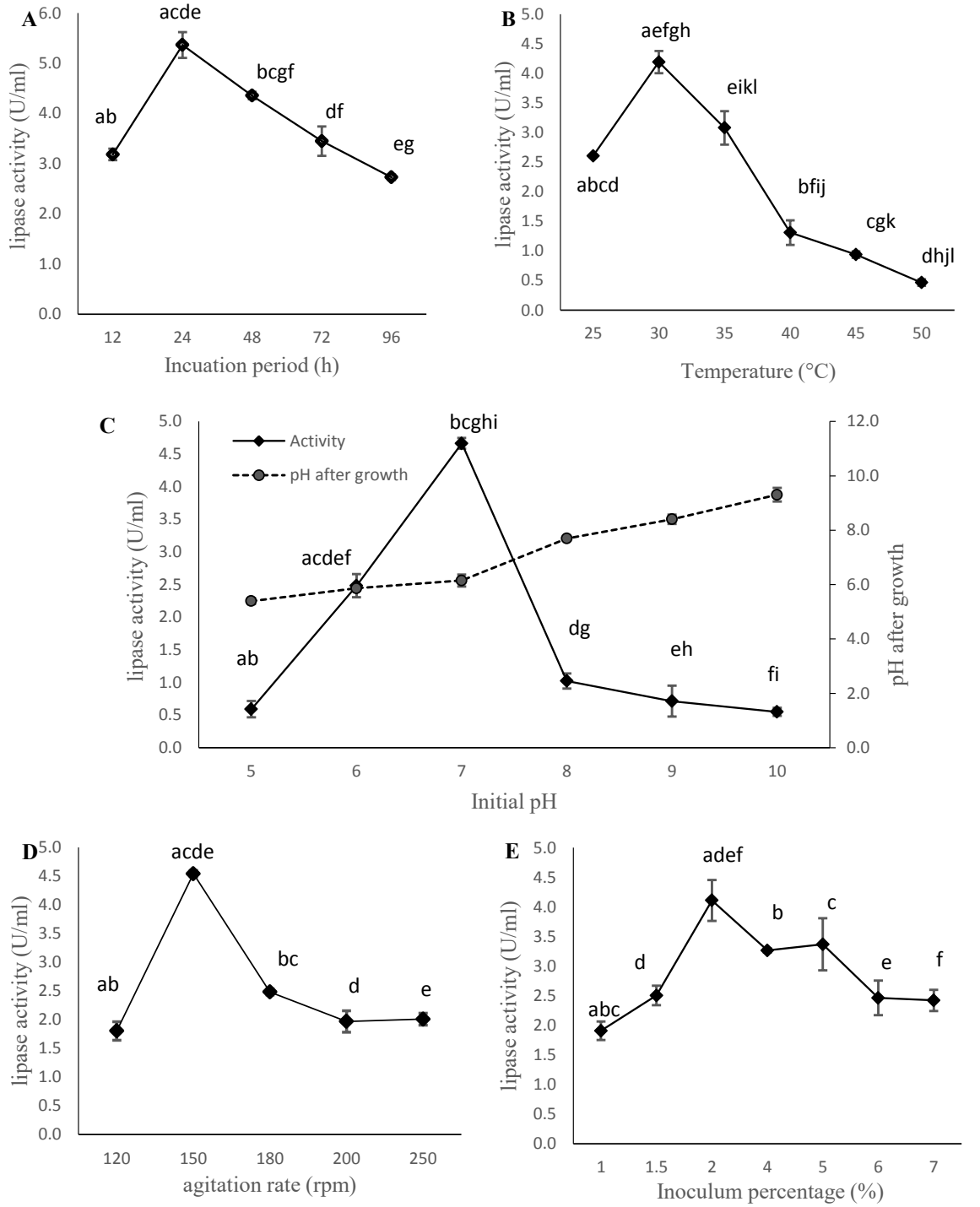

Fig. 1. Different factors affecting lipase production by *Serratia marcescens* EGHK-19 isolate [A: Incubation period, B: Incubation temperature, C: Initial pH, D: Agitation rate (rpm), and E: Inoculum percentage (%). Similar lowercase letters indicate significant differences according to Tukey's test (P<0.05)]

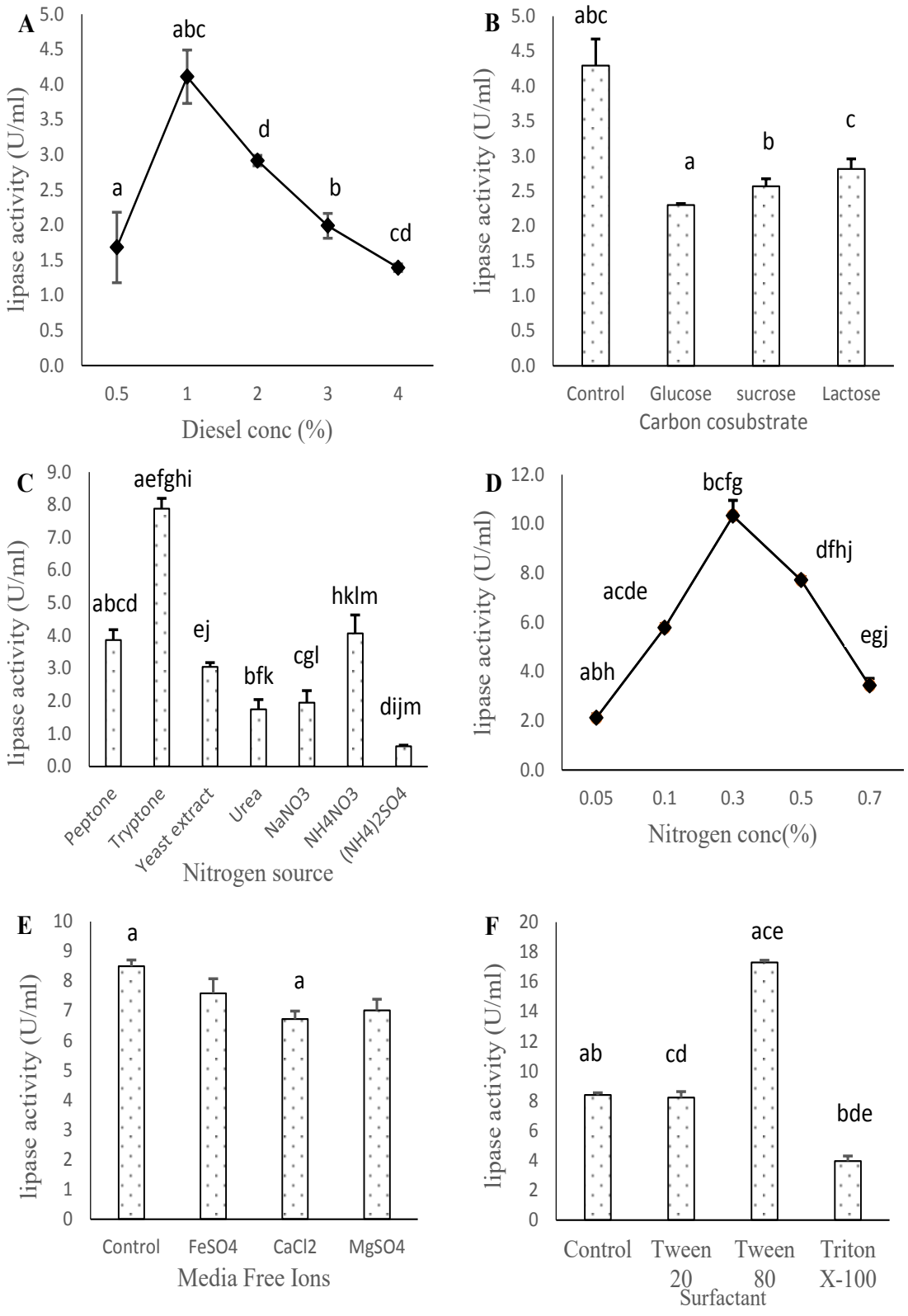

Fig. 2. Different factors affecting lipase production by *Serratia marcescens* EGHK-19 isolate [A: Diesel conc (%), B: Carbon cosubstrate, C: Nitrogen source, D: Nitrogen conc (%), E: Media Free Ions and F: Surfacant. Similar lowercase letters indicate significant differences according to Tukey's test (P<0.05)]

TABLE 1. Summary of Purification Studies on lipase Produced by *Serratia marcescens* EGHK-19

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Volume (mL)	Recovery (%)	Fold purification
Crude enzyme	1589.29	93.73	16.956	515	100	1
Acetone precipitation (50%)	1555.56	3.6	432.1	30	97.878	25.484
Concentration on sucrose	661.122	4.007	164.992	1.260	41.599	9.731
Ion-exchange chromatography	33.333	1.995	16.708		2.097	0.985

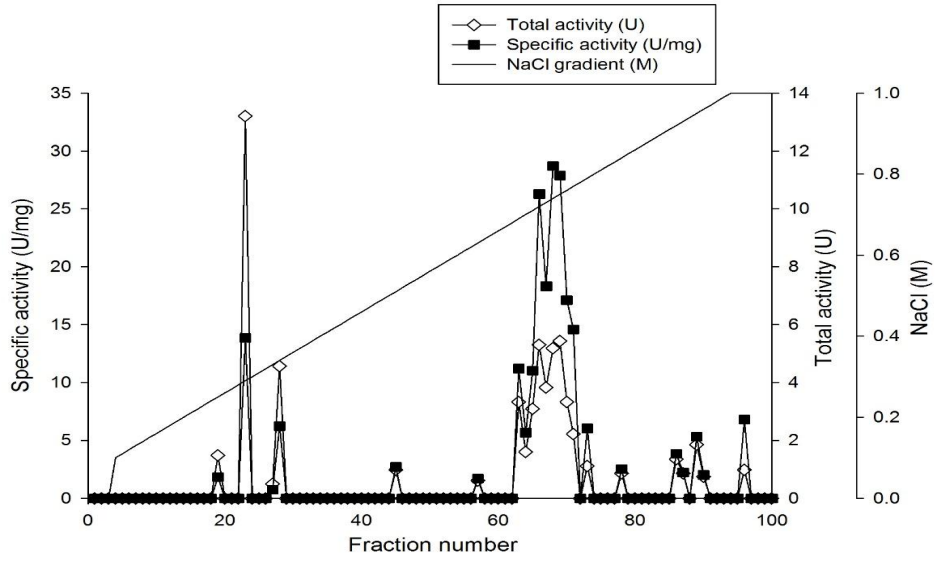

Fig. 3. Elution profile of lipase on the DEAE Sephadex column, produced by *Serratia marcescens* EGHK-19 isolate.

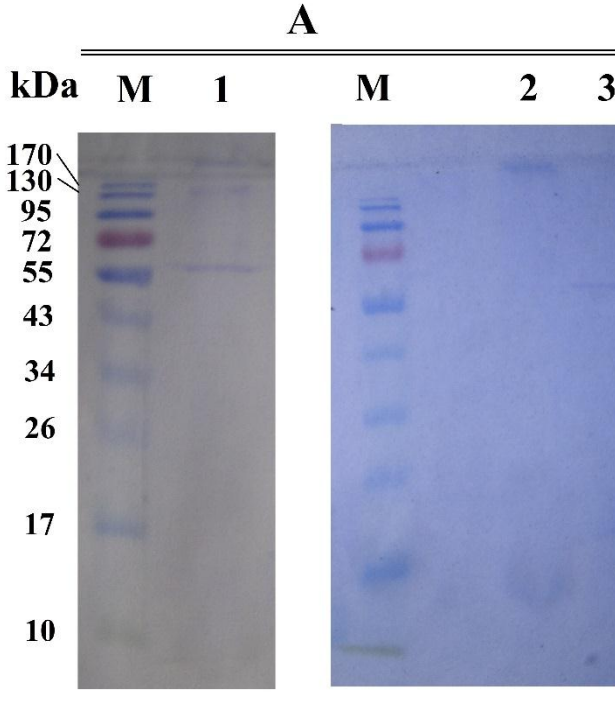

Fig. 4. SDS-PAGE of purified lipase by *Serratia marces*
Brilliant Blue and (**B**) Activity staining with phenol red tr
lane2: precipitated lipase with acetone, and lane 3: purified l

Discussion

The production of lipase is notably influenced by both the bacterial strain and the composition of the medium, which play pivotal roles in governing enzyme synthesis and activity. To determine the maximal lipase production of *S. marcescens* EGHK-19, the investigation focused on examining the relationship between crude lipase activity and various controlled variables. The results indicated that the peak lipase activity was observed after a 24-hour period, suggesting the essential role of lipase during the initial stages of bacterial growth. Prolonged incubation periods were associated with a potential decomposition of the enzyme due to interactions with other components in the medium (Ramesh and Lonsane 1987). This finding contrasts with observations in *S. marcescens* isolated from the Serra of Ouro Branco State Park in Brazil, where lipase production continued to increase up to 96 hours of bacterial growth (Luz et al. 2021).

The incubation temperature plays a crucial role in microbial lipase production, and the findings suggest that 30°C is the optimal temperature for achieving the highest lipase production, indicating that the investigated strain is a mesophilic bacterium. Researchers have revealed that a slight increase in temperature, up to 38°C, stimulates lipase production. Conversely, lower temperatures have been observed to decrease lipase production, and higher temperatures can adversely affect its activity (De Souza et al. 2019). In a study by Prasad (2013), optimal lipase activity of 6.102 U/ml was achieved using *S. marcescens* isolated from industrial effluent after 45 hours at 30°C and pH 7, in a medium enriched with olive oil, glucose, and starch. The pH of the medium also influences the metabolic activities of microorganisms. The observed maximum activity at pH 7 indicates that the isolate is a neutrophilic bacterium. This finding aligns with a previous study by Devaraj et al. (2018), where bacterial lipases exhibited either alkaline or neutral pH preferences. Following bacterial growth, the pH of the medium decreases due to the production of fatty acids during diesel degradation.

The results indicated an inverse relationship between agitation rate and lipase activity, with the highest lipase activity (4.541 ± 0.054 U/ml/min) recorded at an agitation rate of 150 rpm, while the lowest activity (approximately 2 U/ml/min) occurred at 200 and 250 rpm. This phenomenon is attributed to increased turbulence, which can lead to cell rupture. The disruption of cells can result in the release of intracellular enzymes, such as proteases, which may break down the liberated lipase enzymes (Rajesh et al., 2010). Additionally, at higher shaking rates, the mass transfer limitation that typically controls lipase production might shift toward reaction control. Nahas (1988) reported that elevated shaking speeds could lead to the denaturation of the enzyme.

On an industrial scale, it is preferable to generate maximal enzyme activity with lower inoculum concentrations. Additionally, the large initial inoculum size would deplete nutrients in the culture medium and oxygen (Ismail et al., 2018). Also, excessive concentrations of oil substrate may be cytotoxic and typically slow down the oxygen transfer rate to the fermentation medium, which would reduce the specific growth rate and thus lipase production (Ismail et al., 2018). These findings are aligned with the obtained results for *S. marcescens*, where the lipase activity recorded its highest level of 4.109 U/mL/min with 1% supplemented diesel and 2% (v/v) inoculum size. However, *Pseudomonas gessardii* gave the best production of lipase, depending on the 6% (v/v) inoculum size (Veerapagu et al. 2013). Also, Vivek et al. (2023) used 5% for higher lipase productivity. The obtained results of lower lipase activity accompanied by the addition of carbon cosubstrate matched other experimental results (Abdel-Fattah 2002; Kaushik et al. 2006). Lipase synthesis is stimulated by lipid carbon sources, notably natural oils. Gao et al. (2004) concluded that lower activity was obtained in media supplemented with glucose during the optimization of *S. marcescens* ECU1010 for lipase production. Glucose supplementation to the basal production medium suppresses lipase production, perhaps by catabolic repression (Dharmsthiti and Kuhasuntisuk 1998). Contrary to Prasad (2013), applying *S. marcescens* isolated from industrial effluent had an optimum activity of 6.102 U/mL in a medium supplied with olive oil, glucose, and starch.

A nitrogen source is indispensable for microbial growth and the augmentation of microbial lipase production. Our findings align with previous studies asserting that tryptone (an organic nitrogen source) proved to be the most effective nitrogen source for lipase production. Gupta et al. (2004)

reported a similar preference for organic sources over inorganic ones, emphasizing that ammonium salt can create an acidic environment by releasing free acids after the utilization of ammonium ions. This acidic environment may potentially hinder or disrupt the secretion of lipase.

Removal of some elements (Ca^{2+}, Mg^{2+}, and Fe^{3+}) from the lipase production medium decreased the lipase activity, as shown in Fig. 2E. Divalent cations can either promote or prevent the synthesis of enzymes in bacteria. Rathi et al. (2001) noted an increase in lipase synthesis from *Burkholderia* sp. in the presence of Ca^{2+} and Mg^{2+}. The majority of other metal ion salts, however, prevented the formation of lipase. Iron was discovered to be essential for *Pseudomonas* sp. G6's ability to produce lipase (Kanwar et al., 2002). However, several studies produced incompatible findings (Ma et al., 2006; Tembhurkar et al., 2012; Mahmood et al., 2017). By oxidizing the -SH groups on the lipase enzyme molecule, metal ions will decrease lipase enzyme activity. Consequently, the tertiary structure of the enzyme will change, which will change the active site's shape and cause the enzyme to become inactive (Liebeton et al., 2001; Açıkel et al., 2011).

Diesel oil, being hydrophobic and immiscible with water, presents a challenge for microbial utilization. The addition of a surfactant addresses this issue by rendering the production medium more soluble. This, in turn, enhances the bioavailability of the substrate to bacteria, consequently promoting the production of the lipase enzyme (Immanuel et al., 2008; Kaczorek et al., 2018). The use of Tween 80 to augment lipase production aligns with this observation. Montpas et al. (1997) similarly emphasized the essential role of a surfactant, such as Tween 80, in facilitating rapid degradation. In summary, the results highlight that different microorganisms exhibit varying maximum lipase activity under distinct conditions.

Lipase purification was carried out to obtain the desired protein and to get rid of any extraneous ones. According to the results, acetone precipitation followed by ion exchange chromatography is sufficient for lipase purification. The sharp appearance of the first peak in the elution profile fraction number (23) could be due to a problem in handling. This related to some troubles with manual work and was hence discarded. The second peak was further purified by ion exchange chromatography. Although we obtained a fine, pure lipase that appeared as a single active lipase band on SDS-PAGE gel, the purification yield was only 2.097%. This is attributed to the formation of lipase aggregates that were lost during the separation steps. Several microbial lipases have been reported for their self-aggregation in solution due to their protein surface hydrophobic amino acids (Sugimura et al. 2000). Lipase protein's self-aggregation represents a challenge to their purifications (Sztajer et al. 1992). Aggregates could also reduce the homogeneity of enzyme solutions, thereby preventing accurate determination of protein concentrations. Thus, we attribute the unexpected increase in protein concentrations after the third purification step to the formation of aggregates. Moreover, we think that these inaccurately high protein concentrations resulted in a decrease in the calculated specific activities and folds along the purification steps. Thus, the final fine pure lipase band is accompanied by a purification fold of less than 1 (0.985) due to aggregations; this represents a limitation of our purification protocol.

The solubility of proteins is a critical factor in any protein purification process, influenced by the solvent and the physicochemical properties of the proteins (Middelberg, 2000). Our protocol incorporated both an appropriate pH and a high relative ionic strength of solutions, in accordance with recommendations by Lodish et al. (2000), to enhance the solubility of proteins with solvent-exposed hydrophobic amino acids. However, despite these efforts, further refinement of the purification protocol is warranted. In future studies, we aim to enhance this protocol by identifying additives that can prevent aggregation without compromising enzyme activity.

SDS-PAGE and zymogram analysis were used to assess the purity and molecular weight of the active lipase samples that were produced after each step of purification. The lipase from *S. marcescens* has often been discovered as a high molecular weight protein, typically in the 52–65 kDa range (Abdou, 2003; Begam, et al. 2012; Vivek, et al. 2023). Other *Serratia* species had lipases with molecular weights of 65 kDa for *S. liquefaciens* and 67 kDa for the *Serratia* sp. W3 Tunisian strain (Eddehech et al. 2019; Salgado et al. 2020). The results of earlier studies are

consistent with the molecular weight of lipase from *S. marcescens* EGHK-19. A report from *S. marcescens* VITSD2-35 kDa provided a contrary opinion (Mohanasrinivasan et al. 2018).

Due to the presence of aggregates, the lipase was unable to penetrate the stacking gel and instead aggregated within the gel well, as evident in the zymography (data not shown). The inclusion of SDS detergent in the sample buffer proved crucial for solubilizing these aggregates. The zymogram of crude lipase confirmed the dissociation of lipase aggregates with varying molecular weights. Ne'eman et al. (1971) highlighted that potent ionic detergents like sodium dodecyl sulfate (SDS), which form distinct detergent-polypeptide complexes and effectively solubilize proteins, often denature and deactivate the biological activity of proteins. Therefore, SDS couldn't be employed in the purification process of lipase enzymes.

Every enzyme has a certain optimal temperature and pH for catalysis, which depends on the source and molecular structure. The nature of the coded amino acids in the fundamental structures may vary, which might explain the variance in the physical and chemical characteristics. Temperature affects the three-dimensional structure of the enzymes, which in turn affects the catalytic site. Previous studies reported that the optimum temperature of *S. marcescens* lipase is between 30 and 50 °C (Begam et al., 2012; Mohammadi et al., 2016). Our result of a 40 °C optimum is consistent with previous findings.

Enzymes, being proteins, experience changes in their activity based on alterations in their operating environment. The optimal pH range for the lipase of *S. marcescens* was determined to fall between 7 and 8, a finding consistent with prior research by Mohammadi et al. (2016) and Vivek et al. (2023). The current study aligns with and reinforces these earlier observations.

Surfactants can alter the tension between the substrate, lipid, and water, increasing the catalytic interfacial area, causing conformational changes, and altering the adsorption-desorption equilibria, which can increase or decrease lipolysis (Javadi et al., 2020). In this study, SDS was found to be the most effective surfactant, where the enzyme lost about 75% of its initial activity, as occurred with Eddehech et al. (2019). Javadi et al. (2020) found that lipase might interact with ionic surfactants like SDS because of its dominantly negatively charged and partly positively charged surfaces. As indicated in the study conducted by Patel et al. (2014), ionic surfactants have the ability to create complexes that, at higher concentrations, can fully denature the tertiary structure of proteins. Alternatively, at lower concentrations, these surfactants may modify the conformational stability and hydrophobicity of the protein surface. The *Serratia* sp. W3 Tunisian strain and *S. marcescens* ES2 lipase were shown to respond better to the Triton-Xs range of surfactants (Bae et al., 2006; Eddehech et al., 2019). However, Tween 80 and triton-X100 in the current study reduced the lipase activity. Surfactants, depending on their concentration and nature, are known to promote or suppress lipase activity.

Enzyme kinetics involved the determination of Km and Vmax values through Lineweaver-Burk plots, utilizing various concentrations of the pNPP substrate. Industrial biocatalysts are typically reported to exhibit Km values ranging between 10^{-1} and 10^{-5} M (Ali et al., 2022). The lower Km value (6.89 mM) observed in this study indicates a higher affinity of the lipase from *S. marcescens* EGHK-19 for pNPP, while the higher Vmax (65.79 µM/min) signifies greater lipase activity. In a study using pNPP as a substrate, the partially purified *S. marcescens* SCL1 lipase demonstrated Km and Vmax values of 3.349 mM and 0.568 U/ml, respectively (Ali et al., 2022). Notably, the Km value of *S. marcescens* SCL1 lipase was lower than that of *S. marcescens* EGHK-19 lipase, and the Vmax value was higher in the latter case. A similar trend was also observed with SMVT-1 lipase (Vivek et al., 2023). These findings suggest that our enzyme exhibits increased activity in lipolysis applications. It's worth noting that, apart from the substrate, various reaction variables, including temperature, pH, inhibitors, and ionic strength, can influence the Km and Vmax of lipase.

Conclusions

This study focused on optimizing the production of lipase from the isolated S. marcescens EGHK-19. Varied culture conditions were investigated, revealing that the maximum lipase production occurred after 24 hours at 30°C and pH 7.0, with continuous shaking at 150 rpm. Utilizing anion exchange chromatography following acetone

precipitation proved to be effective for enzyme purification. However, it was observed that lipase formed aggregates, impacting both recovery and the fold of purification. Electrophoresis analysis indicated an approximate molecular weight of 60 kDa for the lipase. The purified enzyme demonstrated activity over a broad range of temperatures and pH values, with an optimum at 30°C and pH 7. The distinct Vmax value suggests the potential scalability of this lipase for future commercial applications.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: Heba Kamal Issa conducted the experiments and drafted the manuscript; Mohamed I. Abou Dobara contributed to the review and editing process; Ahmed K.A. El-Sayed conducted gel electrophoresis with documentation; and Magdy I. El-Bana performed the statistical analysis and contributed to the review and editing of the manuscript. All authors have read and approved the final version of the manuscript for publication.

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