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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

A Preliminary Study on Purification and Characterization of Lipase(s) Produced by *Cryptococcus diffluens* D44

Esra BÜYÜK[1], Orkun PİNAR[1]

[1]*Marmara University, Faculty of Engineering, Department of Bioengineering, 34854, Maltepe, Istanbul, Turkey*

Abstract
In the present work, preliminary purification, and characterization of lipases from *Cryptococcus diffluens* D44, which was isolated from petroleum sludge, were performed. In the purification steps, subsequential to acetone precipitation, lipases from *C. diffluens* D44 were purified by DEAE Sepharose resulting in two different peaks, named Lip1 and Lip4. Sephadex G-100 size-exclusion chromatography was also performed for further purification of Lip1 and Lip4 and resulted in three different lipases as Lip1-1 (1.0 purification fold with 2.4% recovery), Lip1-2 (0.8 purification fold with 7.2% recovery), and Lip4-1 (1.2 purification fold with 4.5% recovery). As a result of characterization studies of these three lipases resulting from different peaks, optimum temperatures were found as 60 °C, 65 °C, and 65 °C for Lip1-1, Lip1-2, and Lip4-1, respectively. Furthermore, thermal stability studies were conducted at 50 °C, 60 °C, and 70 °C, and lipases of *C. diffluens* D44 maintained over 70% of their initial activity at 50 °C. The optimum pH for Lip1-1 and Lip1-2 was pH 9.0 although pH 5.0 was for Lip4-1. Considering the organic solvent effect on lipase activity, 10% methanol enhanced the relative activity of Lip1-1 and Lip4-1 while 10% ethanol caused a decrease in the relative activity of lipases except for Lip1-2. According to the indicated features based on the results, these different lipases from *C. diffluens* D44 could be promising candidates for industrial and biotechnological applications. To the best of our knowledge, this is the first study on the purification of lipases from C. *diffluens* D44.
Keywords: Lipase, *Cryptococcus diffluens* D44, purification, characterization

Öz
Bu çalışmada, petrol çamurundan izole edilmiş olan *Cryptococcus diffluens* D44'ten lipazların saflaştırılması ve karakterizasyonuna ait ön çalışmalar gerçekleştirilmiştir. Saflaştırma çalışmalarında, aseton çöktürmesinin ardından, *C. diffluens* D44'ye ait lipazlar, DEAE Sepharose ile saflaştırılarak iki farklı tepe noktası elde edilmiş, Lip1 ve Lip4 olarak isimlendirilmiştir. Sephadex G-100 boyut ayırma kromatografisi kullanılarak, Lip1 ve Lip4'ün saflaştırılması sonucunda da, Lip1-1 (%2,4 geri kazanım ile 1,0 saflaştırma katında), Lip1-2 (%7,2 geri kazanım ile 0,8 saflaştırma katında) ve Lip4-1 (%4,5 geri kazanımla 1,2 saflaştırma katında) olmak üzere üç farklı lipaz elde edilmiştir. Farklı piklerden elde edilen bu üç lipazın karakterizasyon çalışmaları sonucunda, Lip1-1, Lip1-2 ve Lip4-1 için belirlenen optimum sıcaklıklar sırasıyla 60 °C, 65 °C ve 65 °C olarak bulunmuştur. Ayrıca, termal stabilite çalışmaları 50 °C, 60 °C ve 70 °C'de yürütülmüş, *C. diffluens* D44'ten elde edilen lipazlara ait enzim aktivitesi 50 ºC'de başlangıç aktivitesinin %70'inin üzerinde korunabilmiştir. Optimum pH değerlerine bakıldığında, bu değerler, Lip1-1 ve Lip1-2 için pH 9.0 olarak bulunurken, Lip4-1 için pH 5.0 olarak belirlenmiştir. Ayrıca organik çözücülerin lipaz aktivitesi üzerindeki etkileri dikkate alındığında %10 metanol, Lip1-1 ve Lip4-1'in nispi aktivitesini arttırırken, %10 etanol ise Lip1-2 dışındaki lipazların nispi aktivitesini azaltmıştır. Çalışmadaki belirlenen özelliklere göre, *C. diffluens* D44'ten elde edilen bu farklı lipazların, endüstriyel ve biyoteknolojik uygulamalar için umut verici adaylar olabileceği düşünülmüştür. Bildiğimiz kadarıyla bu çalışma, *C. diffluens* D44'ten lipazların saflaştırılmasına ilişkin ilk çalışma niteliğindedir.
Anahtar Kelimeler: Lipaz, *Cryptococcus diffluens* D44, saflaştırma, karakterizasyon

I. INTRODUCTION

Lipases (EC 3.1.1.3) have the capacity to catalyze the hydrolysis of triacylglycerol to glycerol, and free fatty acids [1, 2]. The lipase enzyme was discovered in 1856 by Claude Bernard in pancreatic juice. Although pancreatic lipases are mostly used to treat digestive problems of humans, shortage of pancreas from animal sources and difficulty in collecting available material prevent the usage of pancreatic lipases in different industrial applications [3]. For that reason, researching other sources for lipases came to the fore in industry and since then, plants and various microbial sources consisting of yeast, fungi, bacteria, and archaea have been evaluated for lipolytic enzymes because of their rapid growth on cheap media giving high yield, more stable, higher yields, and ease of genetic manipulation. Moreover, their enzymes have high substrate specificity to various substrates with the capability of catalyzing diverse reactions including esterifications, transesterifications, and alcoholysis [4-11].

Corresponding Author: Orkun PİNAR, **Tel:** 0 216 777 3573, **e-posta:** orkun.pinar@marmara.edu.tr

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Although different types of microorganisms have been evaluated as lipase producers, yeasts are preferable for industrial applications compared to bacteria [12-15]. *Candida rugosa*, the best-known yeast, has been frequently used for lipase synthesis. Moreover, different yeast species such as *Rhizopus*, *Rhizomucor*, other *Candida species*, and *Saccharomycopsis lipolytica* from various sources, have been investigated for their ability to produce lipases [16-18]. For large-scale and commercial lipase production, *Geotrichum candidum* and species belonging to *Candida* genus (*Candida antarctica*, *Candida cylindracea*, *Candida lypolytica*, and *Candida rugosa*), and *Aspergillus* sp., *Rhizomucor* sp., *Rhizopus* sp., *Yarrowia lipolytica*, and *Pseudomonas* sp. are mostly preferred yeast species [17, 19-21].

Besides these species, in 2014, *Cryptococcus diffluens* D44 isolated from petroleum refinery sludge was reported as a newly found lipase producer [22]. After a year, Yılmaz and Sayar [23] studied the characterization of crude D44 lipase having a molecular weight of 45.7 kDa. The optimum pH and temperature of D44 lipase were reported as pH 9.0 and 45 °C, and D44 lipase requires metal ions as co-factors for its activity. Besides these properties, one of the excellent properties of D44 lipase is its extensive stability in organic solvents, mainly 10% methanol.

To understand the lipase synthesized by *C. diffluens* D44, in the present work, D44 lipase was purified by using conventional purification methods. To the best of our knowledge, this is the first study on the purification of lipases from *C. diffluens* D44.

II. MATERIALS AND METHODS

2.1. Strain and Chemicals

Cryptococcus diffluens (D44), were kindly supplied from Assoc. Prof. Dr. Hüsniye Tansel Yalçın (Ege University, Faculty of Science). All chemicals were used in analytical grade and purchased from either Merck (Darmstadt, Germany) or Sigma-Aldrich (St Louis, MO), unless otherwise noted.

2.2 Production of Lipase(s) from *C. diffluens* D44

Preculture medium, containing malt extract (0.3%, w/v), yeast extract (0.3%, w/v), peptone (0.5%, w/v), and glucose (1.0%, w/v), was prepared as 20 mL in 100 mL Erlen-Meyer flask with an initial pH of 6.2. *C. diffluens* D44 stored at -80 °C in glycerol stocks (15%) were first transferred to a preculture medium including 1.5% (w/v) agar with a pH of 6.2 and inoculated at 28 °C for 2 days. After that, the preculture medium was inoculated with a single colony of *C. diffluens* D44 from agar plates and incubated at 28 °C and 180 rpm for 16 h. Yeast cells were then harvested, and the wet cell pellet was used to inoculate the production medium.

For lipase production, basal medium including yeast extract (0.1%, w/v), peptone (3%, w/v), $MgSO_4.7H_2O$ (0.05%, w/v), KH_2PO_4 (0.1%, w/v), and $NaNO_3$ (0.3%, w/v) supplied with olive oil (2%, v/v) was used as the production medium [23]. The initial pH of the medium was adjusted to 7.0 using 5 M HCl. All the media were sterilized by autoclave (121 °C for 15 min). The olive oil, was first sterilized by dry heat for 60 min at 180 °C in a drying-oven, was added to the culture medium after cooling [22]. Then, the wet cell pellet was resuspended and transferred to the lipase production medium to adjust the initial optical density (OD) of 1.0 at 600 nm. After 6 days of incubation at 250 rpm and 28 °C, yeast cells were harvested and supernatant including extracellular crude lipase was used in the purification experiments.

2.3. Purification of D44 Lipase(s)

In the first step of the purification, proteins were precipitated by using acetone as an organic solvent. The precipitation with acetone was carried out by using the method of Thermo Scientific "Acetone Precipitation of Proteins" [24]. Firstly, cold (-20 °C) acetone was added to the crude lipase solution in four times volumes of enzyme solution and the mixture was incubated at -20 °C for 1 h. Then, the solution was centrifuged at 13000 g for 15 min and pellets were held at room temperature for 30 min to evaporate the acetone residue. After that, these pellets were dissolved in sodium acetate buffer (50 mM, pH 5.6), and dialyzed against same buffer to remove the acetone residue completely at 4 °C overnight. The dialyzed lipase was stored at 4 °C for further applications.

2.3.1 Ion-exchange chromatography

DEAE Sepharose, a weak anion exchanger, was used to separate proteins based on ionic charges. For ion-exchange chromatography, the column was equilibrated with 5 column volumes (CV) of the binding buffer (Tris-HCl, 50 mM, pH 7.5) and then 10 mL of the concentrated enzyme was loaded onto the DEAE sepharose column and then washed with binding buffer to remove unbound proteins. After that, NaCl gradient elution was applied at a 3 mL/min flow rate using the binding buffer supplemented with 0.1 - 0.5 M of NaCl. During the elution, the fractions were collected, and the protein concentration of the fractions was measured at 280 nm spectrophotometrically. Fractions having higher 280 nm absorbance were applied to the lipase activity assay and active fractions were pooled and dialyzed against sodium acetate buffer (50 mM) at pH 5.6 to remove the NaCl from the enzyme solution. After ion-exchange chromatography, size exclusion chromatography was applied using Sephadex G-100 as the matrix for further purification.

2.3.2 Size exclusion chromatography

Sephadex G-100 was equilibrated with sodium acetate buffer (50 mM) at pH 5.6 for 24 h, and 2 mL of the concentrated enzyme obtained by anion exchange chromatography was loaded onto the size exclusion chromatography column. Fractions were collected at a 0.4 mL/min flow rate and the protein concentration of

the fractions was measured at 280 nm spectrophotometrically. Fractions having higher 280 nm absorbance were applied to the lipase activity assay and active fractions were pooled.

2.4. Determination of Lipase Activity and Protein Concentration

The lipase activity was determined by the method described by Yalçın et al. [22]. For the preparation of pNPP (p-nitrophenyl palmitate) substrate, 30 mg of pNPP was dissolved in 10 mL propan-2-ol which was emulsified in 90 mL of sodium acetate buffer (50 mM and pH 5.6) including 500 mg Triton X-100. 100 µL enzyme solution and 2 mL of the pNPP containing emulsion were mixed well, and incubated at 37 °C for 3 min, and then, the reaction was stopped using sodium carbonate solution (150 µL, 1 M). For the enzyme activity calculation, the absorbance was measured at 410 nm spectrophotometrically against the enzyme-free substrate solution as blank. Activity assay was performed with duplicate measurements for each sample. The definition of one unit of lipase activity (U) was the amount of enzyme that liberates one µmol p-nitrophenyl for one min under the assay conditions. Bradford method [25] was applied for the determination of the protein concentration via developing a curve using series BSA (Bovine Serum Albumin) standards.

2.5. Characterization of Purified Lipase(s)

2.5.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed at 100-110 V for 120 min to represent the protein band(s) belonging to different fractions according to the method of Laemmli [26], using a pair of gels composed of stacking gel and resolving gel with 5% (w/v) and 12% (w/v) monomer concentration, respectively. BIO-RAD Precision Plus Protein Standards All Blue (161-0373) was used as the protein marker. Finally, Coomassie R250 staining was applied to stain the gel in order to visualize proteins in the resolving gel.

2.5.2 Effect of temperature on the activity and stability of purified lipase(s)

For the determination of the optimal temperature of the purified enzyme, the enzyme activity was determined at temperatures ranging between 30 – 70 °C and at pH 5.6. The thermal stability of purified enzymes was determined because of the great importance for industrial applications. The enzyme solution was incubated at 50 °C, 60 °C, and 70 °C, and samples were taken at certain time intervals. These samples were used to determine the residual lipase activity respecting the initial activity, which was taken as 100%, and then the enzyme was assayed.

2.5.3 Effect of pH on purified lipase activity

The optimum pH of the purified lipases was determined at the pH ranges between 4.0 – 10.0. For this pH range, citrate buffer (50 mM) at pH 4.0, acetate buffer (50 mM) at pH 5.0, phosphate buffer (50 mM) between pH 6.0 and 8.0, glycine-NaOH buffer (50 mM) between pH 9.0-10.0 were used to prepare the substrate solution. The pH, at which the highest lipase activities were obtained, was considered as 100% and the other activities were shown as relative activity.

2.5.4 Effect of organic solvents on lipase activity

The effects of organic solvents on purified lipase(s) were investigated with the addition of ethanol and methanol to the reaction mixture at both 10% and 20% concentrations. Lipase activity assay was performed as 100 µL enzyme solution and 2 mL of pNPP substrate solution prepared in sodium acetate buffer (50 mM, pH 5.6) containing 10% and 20% organic solvents in concentration were mixed well and reactions were incubated for 3 min at 40 °C. A control, not including organic solvents, was used considering as 100% activity, and activities were calculated as the relative activity.

III. RESULTS AND DISCUSSION

3.1. Purification of *C. diffluens* D44 Lipase(s)

Production, purification, and characterization of new lipases to enhance industrial microbial lipase candidates are crucial since they have extensively been used as biocatalysts in industrial and biotechnological applications [10, 27, 28].

In the present work, for the purification of the extracellular D44 lipase (198 U/mg, 7.9 mg/mL protein), cell-free supernatant was first subjected to acetone precipitation followed by dialysis against sodium acetate buffer (50 mM) at pH 5.6. As seen from Table 1, the lipase produced by *C. diffluens* D44 was precipitated by using acetone with 76.9% yield, 0.9 purification fold, and 170 U/mg specific activity. D44 lipase(s) was enriched by using ion-exchange chromatography and size exclusion chromatography, sequentially.

3.1.1 Ion-exchange chromatography

When DEAE Sepharose chromatographic separation (Figure 1 and Table 1) was applied, two sharp peaks having lipase activity were obtained. The first peak denoted as Lip1, had the highest total activity as 440 U, belonging to the unbound fractions and the second peak denoted as Lip4, had second-highest total activity as 145 U, was obtained after 300 mM NaCl elution. Since DEAE Sepharose is a positively charged resin, this result suggested that D44 lipase in the unbound fraction (Lip1) may carry a net positive charge while the lipase present in Lip4 has negatively charged groups. Elution was also achieved by using 100 mM and 200 mM NaCl solutions. However, these peaks were excluded since enzyme activities in these fractions were low compared to 300 mM NaCl elution. Comparing the total activity and total protein content through Lip1 to Lip4 with

crude enzyme, the purification yield, and fold of the ion exchange column is 44.45% and 0.45, respectively.

As seen in Table 1, Lip1 has 28.1% purification yield and 122 U/mg specific activity while the sharp peak of bound fraction obtained after 300 mM NaCl elution (Lip4) resulted in 9.2% yield and 177 U/mg specific activity. Similar to our work, Syihab et al. [29] studied the purification of lipase from *Pseudoxanthomonas* sp. by acetone precipitation following DEAE Sepharose fast flow. They obtained two different peaks eluted in sodium phosphate buffer (20 mM) containing 0.4 M and 0.6 M NaCl respectively, after DEAE Sepharose separation. In that study, specific activity, and enzyme recovery of the first peak was 1.7 and 55%, while the second peak gave 2.9 U/mg specific activity and 46% yield. In another study, Jermsuntiea et al. [30] reported that lipase purification of *Mortierella alliacea* resulted as 24% yield and 137 U/mg specific activity as a result of DEAE Sepharose separation.

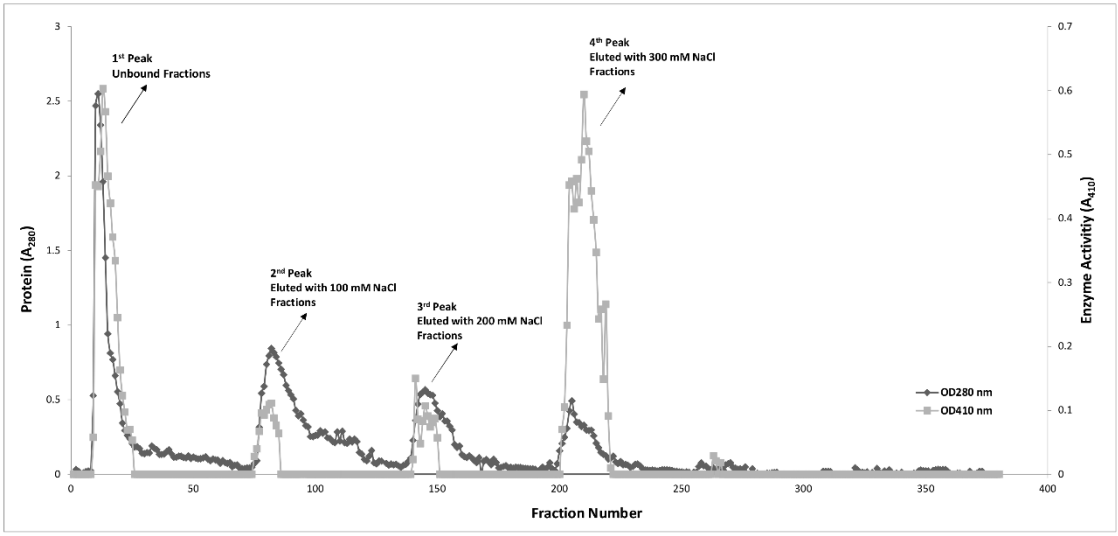

Figure 1. Elution profile of lipase on DEAE Sepharose column

In the study of Ai et al. [31], DEAE Sepharose was used the last purification step as polishing, giving 6.2% recovery with 18.657 U/mg specific activity. Comparing our results with the results obtained by Syihab et al. [29], Jermsuntiea et al. [30], and Hambarliiska et al. [32], our results are very close to their results. Moreover, Das et al. [9] studied the purification of lipase from *Aspergillus tamarii* JGIF06 by using DEAE Sepharose after ammonium sulphate precipitation. Although the purification yield reported for JGIF06 lipase [9] as 43.1% is close to our work, specific activity (260.21 U/mg) of purified lipase from strain JGIF06 is higher than Lip1 and Lip4 of D44 strain.

3.1.2 Size exclusion chromatography

To increase the purity of the lipases, Lip1 and Lip4 were further subjected to size exclusion chromatography (SEC). Results for Sephadex G-100 purified lipase are shown in Figure 2 and Table 2. As seen in Figure 2, proteins in Lip1 were not separated effectively on Sephadex G-100 chromatography and were separated into two different peaks (indicated as Lip1-1 and Lip1-2 fractions) (Figure 2 (a)) however, the purification of Lip4 by Sephadex G-100 resulted in a single sharp peak (indicated as Lip4-1 fraction) having 243.3 U/mg specific activity, 1.2 purification fold, and 4.5% yield was obtained (Figure 2 (b), Table 2).

Similar to our work, Jermsuntiea et al. [30] and Edupuganti et. al. [10] applied size exclusion chromatography followed by acetone precipitation and ion-exchange chromatography for the purification of lipase from *M. alliacea* and *Staphylococcus epidermidis* (MTCC 10656), respectively. As the result of gel filtration chromatography, the specific activity obtained for both *M. alliacea* (179 U/mg) [30] and strain *S. epidermidis* MTCC 10656 (123.95 U/mg) [10] is lower than that obtained for Lip4-1 and Lip1-1, while the specific activity of Lip1-2 is higher than strain MTCC 10656 (123.95 U/mg) [10].

Table 1. Purification Table of *C. diffluens* D44 lipase using anion exchange chromatography

Purification Steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield Recovery (%)	Fold
Crude extract	1568	7.9	198	100	1
Precipitated by Acetone & Dialyzed & Ultrafiltrated	1206	7.09	170	76.9	0.9
DEAE 1st peak-Unbound fractions (Lip1)	440	3.60	122	28.1	0.6
DEAE 4th peak -Eluted by 300 mM NaCl (Lip4)	145	0.82	177	9.2	0.9

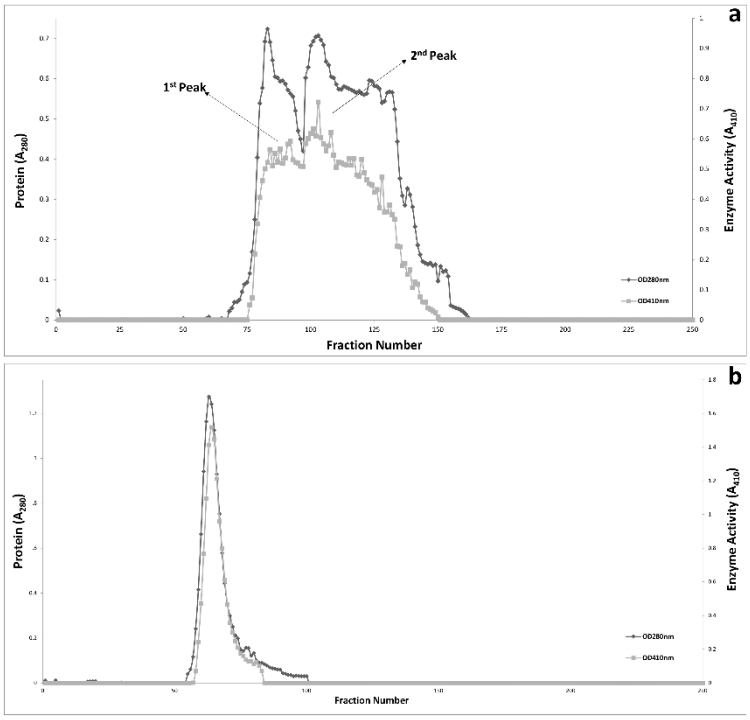

Figure 2. Elution profile of a) Lip1 and b) Lip4 on Sephadex G-100

For the purification yield of SEC, 4% yield reported for *M. alliacea* lipase [30] is close to our results while SEC yield of lipase from MTCC 10656 (10) (7.5%) is higher than that obtained for both Lip1-1 and Lip4-1 but very close to Lip1-2. However, considering the purification process, purification folds of Lip1-1, Lip1-2, and Lip4-1 are lower than that obtained for *M. alliacea* [30] as 6.2 and *S. epidermidis* (MTCC 10656) [10] as 18.5.

Table 2. Purification of *C. diffluens* D44 lipase after anion exchange chromatography

Samples	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield Recovery (%)	Fold
Lip1	440	3.6	122	28	0.6
Fraction Lip1-1	38	0.2	190	2.4	1.0
Fraction Lip1-2	113	0.74	153	7.2	0.8
Lip4	145	0.82	177	9.2	0.9
Fraction Lip4-1	70	0.29	243.4	4.5	1.2

3.2. Characterization Studies

3.2.1 SDS-PAGE analysis

To check the purity of the protein bands obtained after SEC, Lip1-1, Lip1-2, and Lip4-1 were analyzed by SDS-PAGE electrophoresis (Figure 3). As shown in Figure 3, there were 2 different protein bands in lane 2 (Lip4-1) that one of them was around 75 kDa and the other one was around 50 kDa. Moreover, for unbound fractions subjected to SEC, three different bands were visualized between 50 and 75 kDa in lane 3 (Lip1-1), and five different bands were detected at 25, 37, and 50-75 kDa in lane 4 (Lip1-2). Although the determination of lipase activity in both unbound (Lip1) and bound (Lip4) fractions made us think that D44 has more than one gene that encoded lipases, this result requires further clarification.

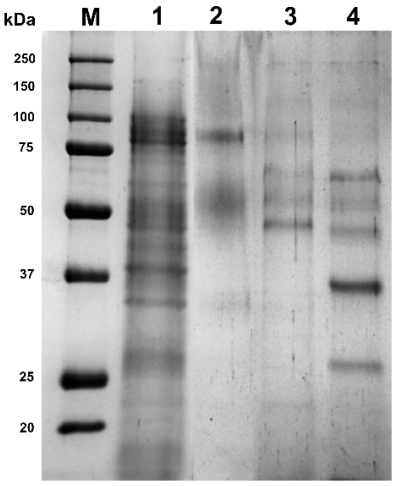

Figure 3. SDS-PAGE gel image represents the bands of lipases after purification steps. **M:** Protein Marker, **Lane 1:** Crude lipase, **Lane 2:** Lip4-1 after size exclusion chromatography, **Lane 3:** Lip1-1 after size exclusion chromatography, and **Lane 4:** Lip1-2 after size exclusion chromatography

3.2.2 Effect of temperature on the activity and stability of purified lipase(s)

As seen in Figure 4, the purified lipases of *C. diffluens* D44 (Lip1-1, Lip1-2, and Lip4-1) were active between 30-70 °C. According to these experiments, the optimum temperature for Lip1-2 and Lip4-1 was found as 65 °C, while the optimum temperature for Lip1-1 was 60 °C. They showed thermophilic behaviour under these conditions. Similar to this study, Rade et al. [33] found the optimal temperature of lipase from *Rasamsonia emersonii* at around 65 °C and Syihab et al. [29] reported 70 °C and 50 °C for the optimal temperature of two lipases from *Pseudoxanthomonas sp.*

The effect of temperature on the stabilities of Lip1-1, Lip1-2, and Lip4-1 are shown in Figure 5. When the thermal stability of Lip1-1 (Figure 5 (a)), Lip1-2 (Figure 5 (b)), and Lip4-1 (Figure 5 (c)) was investigated, a 20% decrease in the activity of Lip1-1 was observed at 50 °C during the first 30 min. Then, Lip1-1 keeps its activity for 4 h. However, at 60 °C, and 70 °C, Lip1-1 lost 60% of its activity at 60 °C after 2 h of incubation. Therefore, Lip1-1 is more stable at 50 °C, and the thermal stability is reduced with increasing temperature. Lip1-2 and Lip4-1 showed a similar stability profile to Lip1-1 as they are stable at 50 °C, and their stabilities were decreased at higher temperatures. At 50 °C and 60 °C, Lip4-1 lost 25-30% of its initial activity, then the activity remained constant at a prolonged incubation time. However, the enzyme maintained only 30% of its activity for 30 min of incubation period at 70 °C. According to the thermal stability results of a lipase from *Aspergillus oryzae* ST11 by Paitaid et al. [34], the lipase activity was dramatically decreased after 55 °C, while the residual activity was found as 23% at 65 °C. Moreover, Ayinla et al. [35] reported that a small purified lipase from *Rhizopus oryzae* ZAC3 loses 45% and 78% of its activity after incubation for 90 min and 120 min at 65 °C, respectively. These results are also similar to the thermal stability characteristics of Lip1-1, Lip1-2, and Lip4-1 lipases of *C. diffluens* D44.

3.2.3 Effect of pH on lipase activity

According to optimal pH determination, the optimal pH of Lip4-1 was determined at a pH between 4.0-10.0. The optimum pH for Lip1-1 and Lip1-2 was found as 9.0, while the optimum pH for Lip4-1 was 5.0 (Figure 6). Generally, the optimal pH range of yeast lipases is variable between 4.0 – 8.0, however, there are few examples having optimal pH values greater than 9.0 [13]. In the literature, different optimal pH values of lipases from yeast strains were reported. Rade et al. [33] reported a novel lipase from the thermophilic fungus *Rasamsonia emersonii* having higher enzyme activity at acidic conditions as pH 3.5 whereas, five different isolates of *Aspergillus niger* from oilseed had an optimal enzyme activity at pH 7.5 were introduced. Additionally, Syihab et al. [29] studied the effect of pH on two different lipases from *Pseudoxanthomonas sp.* and they found variable optimal pH values between these enzymes as pH of 10.0 and pH of 8.0 for Lip1 and Lip2, respectively.

As the most important part in the study of Yılmaz and Sayar [23], the crude lipase enzyme from D44 has an optimal pH of 9.0, which is similar to that obtained for Lip1-1 and Lip1-2 possesses similar optimal pH and completely different to that obtained for Lip4-1. This is another indication of the synthesis of more than one lipase from *C. diffluens* D44.

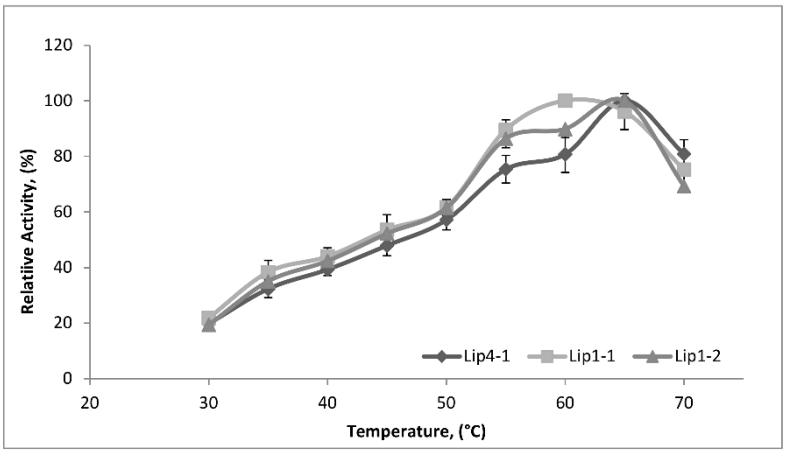

Figure 4. Optimal temperature of Lip1-1, Lip1-2, and Lip4-1 lipases

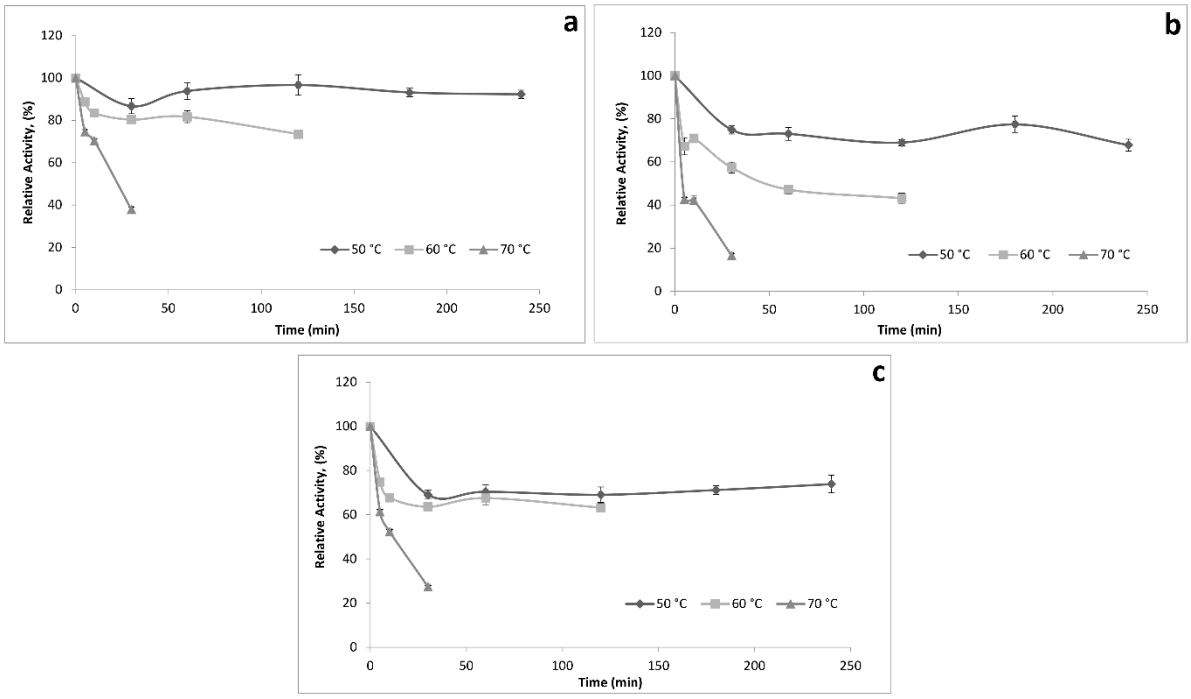

Figure 5. Thermal stability of lipases; a) Lip1-1, b) Lip1-2, and c) Lip4-1

3.2.4 Effect of organic solvent on lipase activity

Since the intense stability of crude *C. diffluens* D44 lipase in ethanol and methanol up to 20% [23] has been proven, the effects of ethanol and methanol on purified D44 lipase(s) were investigated by adding indicated organic solvents to the reaction mixture at 10% and 20% concentrations.

Table 3 represents the organic solvent tolerance of Lip1-1, Lip1-2, and Lip4-1 activity. According to the results from this table, the presence of ethanol decreased the Lip1-1 activity to 83% relative activity at 10% concentration, and at 20% concentration, the activity was completely inhibited. However, in the presence of methanol, it gave a higher relative enzyme activity than ethanol. At 10% methanol concentration, Lip1-1 had 130% relative activity and at 20% methanol concentration, it decreased to 115% relative activity with elevated methanol concentration, but it still had a higher relative activity than the control. When comparing the effects, it could be said that Lip1-1 is

more resistant to methanol than ethanol. Moreover, Lip1-2 is stable in the presence of 10% ethanol and methanol. However, increasing ethanol at a concentration from 10% to 20% caused to loss of whole Lip1-2 activity, while 27% of activity lost was observed by increasing methanol concentration from 10% to 20%. Lip4-1 lost 30% of its activity at a 10% concentration and 61% at a 20% concentration of ethanol. Nonetheless, the presence of methanol did not affect as low as ethanol affected. 10% methanol gave 146% relative activity while 20% methanol resulted in 138% relative activity (Table 3).

Table 3. Effect of organic solvent on Lip1-1, Lip1-2, and Lip4-1

Organic Solvent	Relative Activity (%)		
	Lip1-1	Lip1-2	Lip4-1
0 %, Control	100	100	100
10% Ethanol	83	119	70
20% Ethanol	0	0	29
10% Methanol	130	147	146
20% Methanol	115	73	138

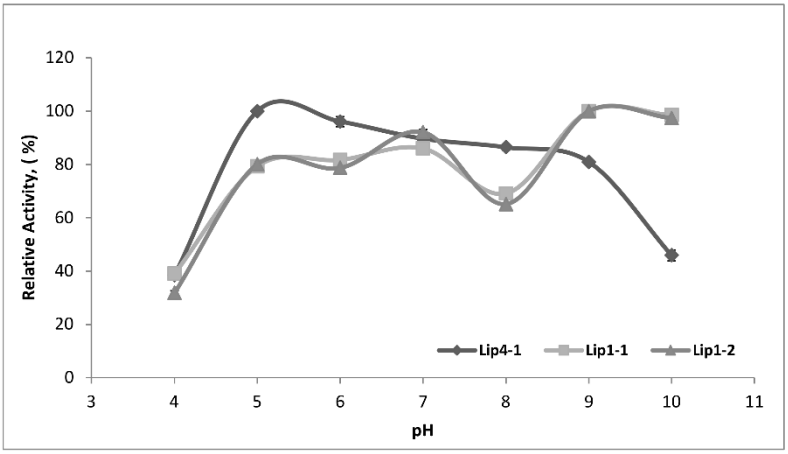

Figure 6. Effect of pH on Lip1-1, Lip1-2, and Lip4-1 lipases

D44 lipases were also found to have more tolerance to methanol especially Lip4-1 but, they are not resistant to ethanol at high degrees except Lip1-2 at a 10% concentration of ethanol. Organic solvents generally have inhibitory effects on enzyme activity by denaturation of amino acid sequences and this was proved by Hernández-Rodríguez et al. [36] that lipase activities from *Rhizopus sp.* were decreased in the presence of isopropanol and ethanol. Comparing our results with the results reported for crude D44 lipase, contrary to Lip4-1, 10% and 20% ethanol enhanced the activity of crude D44 lipase [23].

IV. CONCLUSION

Recently, microbial lipases have become popular, and they are widely used in various industrial areas due to their regio-, chemo-, and enantioselectivity characteristics to different substrates. With the development of biochemistry and enhanced engineering applications on biology, the usage of lipases has opened remarkable opportunities in biotechnological applications for both research and industry. To develop an enzyme for industrial applications, it is necessary to know the structure of the enzyme. However, for known and new lipases, there is limited information available about their characteristics.

Therefore, in the present work, lipase(s) produced by *C. diffluens* D44 were purified and analyzed. Our results suggested that *C. diffluens* D44 might produce at least two different lipases having negatively (Lip4) and positively charged (Lip1) groups since lipase activity was observed in both bound and unbound fractions of DEAE Sepharose chromatography. Three purified lipases from *C. diffluens* D44, Lip1-1, Lip1-2, and Lip4-1 are strongly resistant to high temperatures between 50 °C and 60 °C. Thus, this property makes these enzymes promising candidates not only for the detergent and food industry but also for biodiesel production because of their enhanced relative activity in the presence of methanol. Although relatively lower purity and yield were obtained compared to literature data, our results proposed that *C. diffluens* D44 produced different types of lipases having negatively and positively charged groups since lipase activity was observed in both bound and unbound fractions. Additionally, according to size-exclusion chromatography results, *C. diffluens* D44 has different genes that can encode different lipases (isozymes).

Nonetheless, D44 lipases and peptides that belong to these three lipases should be examined with additional detailed work to gain information about the structure and function of these superior methanol-stable D44 lipases. To the best of our knowledge, a limited number of studies have been achieved in terms of the structural characterization of lipases however this is the first research where the lipases from *Cryptococcus diffluens* D44 were purified, which is the first step in understanding the structural and biochemical characteristics of the enzyme.

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