MARMARA UNIVERSITY
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PURIFICATION AND CHARACTERIZATION OF LIPASE PRODUCED BY *Cryptococcus* STRAINS ISOLATED FROM PETROLEUM SLUDGE: ASSESSMENT OF ITS APPLICATION IN INDUSTRY

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ACKNOWLEDGEMENTS

First of all, I would like to express my special thanks of gratitude to my supervisor Prof. Dr. Dilek KAZAN for her endless support, patience, positivity and deepest knowledge during my Master of Science thesis. I am also sincerely grateful to my co-supervisor Dr. Orkun Pinar for sharing always his experiences and valuable knowledge with me during thesis experiments in a great patience. They always encouraged me whenever I desperate. It was a great opportunity and an honour to work with their guidance.

I would like to thank my lovely friends Gülnihal BOZDAĞ, İdil YEMİŞER and Kübra LAÇİN for their endless help, positivity, cooperation and friendship. They were always morale boosters during my thesis.

I am very grateful to my best friends Aycan KAYA, Bilge ŞENSAZLI and Büşra BİNİCİ for their endless friendship and limitless motivation. Also, I am very thankful to Selçuk BALSÜZEN for his endless support and patience always being with me.

My sincere thanks go to my beloved family, my mother Nigar BÜYÜK and my siblings Erhan BÜYÜK, Nagehan BÜYÜK and Ergün BÜYÜK. Also, I am grateful to my lovely cousin Nuray BÜYÜK. They always supported me in all my life being with me.

I would like to express my thanks to Marmara University BAPKO, Research Fund-the project number FEN-C-YLP-141118-0594 to provide me consumables used in my thesis. I am also grateful to Scientific and Technological Research Council of Turkey (TUBITAK) with KAMAG Project number 115G079 for providing me a unique opportunity to gain skills on fermentation technologies from optimization of small-scale bioprocess to pilot scale bioprocess for production of biological products.

September, 2020 **Esra BÜYÜK**

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Bu çalışmada, petrol çamurundan izole edilen maya suşları olan *Cryptococcus albidus* D24 ve *Cryptococcus diffluens* D44'ten lipazın saflaştırılması ve karakterizasyonu incelenmiştir. D24 ve D44'ten lipazın saflaştırma çalışmalarında ilk olarak amonyum sülfat ve aseton çöktürmesi birincil adım olarak uygulanmıştır. *C. albidus* D24'ten lipaz, her iki çöktürme yöntemine de etkili bir yanıt vermemiştir. Bu sonuçlar: %90 amonyum sülfatta %2.24 geri kazanım ve 0.44 saflaştırma katı verirken, aseton ile çöktürmede %0.64 geri kazanımla 0.01 saflaştırma katı vermiştir. Diğer yandan, *C. diffluens* D44'ten elde edilen lipaz, en yüksek saflaştırma katını aseton çöktürme sonunda 1.12 saflaştırma katı ve %84 ile enzim geri kazanımını verirken, amonyum sülfat ile verimli bir şekilde çöktürülememiştir. Sonuç olarak, saflaştırma adımına *C. diffluens* D44'ten elde edilen lipaz ile devam edilmiştir. Enzim çöktürüldükten sonra D44 lipazı, DEAE sefaroz anyon değişimi ile saflaştırılmış ve iki farklı pik (Lip1 ve Lip4) elde edilmiştir. Lip1 ve Lip4' ün Sephadex G-100 jel filtrasyonuna yüklenmesi sonucunda 3 farklı pik elde edilmiştir. Bunların saflaştırma katsayıları ve verimleri karşılaştırıldığında Lip1-1 %2.4 geri kazanımla ve 1.0 saflaştırma katsayısıyla, Lip1-2, %7.2 geri kazanımla ve 0.8 saflaştırma katıyla ve Lip4-1, 1.2 saflaştırma katı ve %4.5 verimle sonuçlanmıştır. Bu kısmen saflaştırılmış lipazların moleküler ağırlıkları, farklı bant aralıklarında SDS-PAGE jeli üzerinde gözlemlenmiştir.

Saflaştırma işleminin ardından karakterizasyon çalışmaları yapılmıştır. Lip1-1, Lip1-2 ve Lip4-1 için optimum sıcaklıklar sırasıyla 60ºC, 65ºC ve 65ºC olarak bulunmuştur. Lip1-1 ve Lip1-2 için optimum pH değeri 9.0 iken, pH 5.0 Lip4-1 için optimum olarak bulunmuştur. Termo stabilite analizi 50ºC, 60ºC ve 70ºC'de yapılmıştır ve sonuçlar lipaz enzim aktivitesinin 250. dakikadan sonra 50ºC 'de azalmaya başladığını ve bu üç tip lipazın 70ºC' de ise 30. dakikadan itibaren aktivitelerinin düştüğü belirlenmiştir.

%10 metanol, bu lipazların rölatif aktivitesini arttırırken, bunun tersine, %10 etanol, Lip1-2 dışındaki lipazların rölatif aktivitesini düşürmüştür.

Ek olarak, *C. albidus* D24 ve *C. diffluens* D44'ün saflaştırılmamış lipazından şeker esterlerinin sentezi incelenmiştir.

ABSTRACT

Thesis Title: Purification and Characterization of Lipase Produced by *Cryptococcus* Strain Isolated From Petroleum Sludge: Assessment of Its Application in Industry

In this thesis, purification and characterization of lipase from *Cryptococcus albidus* D24 and *Cryptococcus diffluens* D44, which are the yeast strains isolated from petroleum sludge, were investigated. In the purification studies of lipase from D24 and D44, firstly ammonium sulfate and acetone precipitation were applied as a primary step. Lipase from *C. albidus* D24 did not response to both precipitation methods giving 0.44 purification fold with 2.24% recovery at 90% ammonium sulfate and 0.01 purification fold with 0.64% recovery at acetone precipitation. On the other hand, lipase from *C. diffluens* D44 gave the highest purification fold and enzyme recovery as 1.12 and 84%, respectively at the end of acetone precipitation whereas it was not efficiently precipitated by ammonium sulfate. As a result, the purification step was continued with lipase from *C. diffluens* D44. After enzyme precipitation, D44 lipase was purified by DEAE sepharose anion exchange and it resulted in two different peaks (Lip1 and Lip4). Further purification of Lip1 and Lip4 by Sephadex G-100 gel filtration resulted in three types of lipases as Lip1-1 at 1.0 purification fold with 2.4% recovery, Lip1-2 at 0.8 purification fold with 7.2% recovery and Lip4-1 at 1.2 purification fold with 4.5% recovery and molecular weights of these partially purified lipases were observed on SDS-PAGE gel at different bands ranges.

Characterization of these three lipases was carried out and optimum temperatures were found as 60ºC, 65ºC and 65ºC for Lip1-1, Lip1-2 and Lip4-1 respectively. Optimum pH of Lip1-1 and Lip1-2 were determined as pH 9.0 while Lip 4-1 showed highest activity at pH 5.0. Thermo stability analysis was performed at 50ºC, 60ºC and 70ºC and results showed that lipase enzyme activity decreased after 250 minutes at 50ºC and decreased at 70ºC after 30 minutes for these three types of lipases.

10% methanol enhanced the relative activity of these lipases on the contrary 10% ethanol reduced the relative activity of lipases except Lip1-2.

Additionally, the synthesis of sugar esters from crude lipase of *C. albidus* D24 and *C. diffluens* D44 was evaluated.

SYMBOLS

% : Percentage

°C : Celsius

A : Absorbance

g : Grams

hr : Hour

kDa : Kilo Dalton

L : Liter

M : Molar

mL : Milliliter

mM : Millimolar

mg : Milligram

min : Minute

nm : Nanometer

rpm : Revolution per minute

v : Volume

V : Volt

w : Weight

U : Unit

µg : Microgram

µL : Microliter

µmol : Micromole

ABBREVIATIONS

APS	:	Ammoniumpersulfate
BSA	:	Bovine Serum Albumin
C. albidus	:	*Cryptococcus albidus*
C. diffluens	:	*Cryptococcus diffluens*
CV	:	Column volume
DEAE	:	Diethylaminoethyl
EDTA	:	Ethylene diamine tetra acetic acid
MW	:	Molecular weight
MWCO	:	Molecular weight cut off
OD	:	Optical density
p-NP	:	p-nitrophenol
p-NPP	:	p-nitrophenyl palmitate
Rf	:	Retention factor
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecylsulfate polyacrylamide gel
TLC	:	Thin layer chromatography

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1. INTRODUCTION

Enzymes are biological polymers including different amino acids as monomers which bind by amide bonds. They catalyse specific and wide variety of reactions, and their catalytic sites determine the specificity of them to their substrates. Recently, processes carried out by enzymatic catalysis have been gained great interest because of the requirement of eco- and environmentally friendly processes to create a sustainable world (Li et al., 2012). After 1940, depending on advancement in biochemistry and enzyme technology, nearly 4000 of the predicted 25,000 and more enzymes present in nature are determined, and among these enzymes, about 200 are commercialized and applied on different industries (Sharma et al., 2001; Divakar and Manohar, 2007). Since enzymes catalyse the different type of specific reactions, Enzyme Commission (EC) classify enzymes into six groups as oxidoreductases, transferases, hydrolases, lyases, ligases, and isomerases, based on catalytic reactions (Singh et al., 2016).

Within these groups, hydrolases are one of the most popular enzymes used in an industrial scale. Lipases, lipolytic-water soluble enzymes, belong to the hydrolytic enzymes group and hydrolyse the lipids digestion reactions (Reis et al., 2009). Lipases also catalyse the esterification reaction in the presence of organic solvents. Since the esterification reactions carried out at a low water environment, water amount in the reaction media determines the direction of the catalytic reactions (Paiva et al., 2000; Akoh and Min, 2002; Salleh et al., 2006). Due to their ability of ester bond formation, lipases also catalyse the transesterification reactions including acidolysis, interesterification and alcoholysis reactions.

Lipolytic enzymes are single-domain molecule and have a mobile lid domain on their active site (Saxena et al., 2003; Gilham and Lehner, 2005; Somashekar et al., 2007). The working mechanism of the lid depends on the reaction media. For example, in the media containing a high amount of aqueous solution, the lid is generally closed, whereas in the presence of a hydrophobic layer, it is partially opened so that substrates can reach the the enzyme’s active site (Saxena et al., 2003; Barbe et al., 2009; Dave and Madamwar, 2010; Khan et al., 2017).

For that reason, lipases possess the ability to utilize a wide range of substrates – nature or synthetic substrates -, to perform high performance at extreme conditions and chemo-, regio-, enantioselectivity. Especially, their chemo-, regio- and enantioselective properties provide the usage of them in pharmaceuticals as raw materials at high optical purity (chirally pure). Additionally, they are used in cosmetics, foods and biodiesel production. Thus, these enzymes occupy a considerable place of prominence among the industrial enzymes (Barros et al., 2010; Kumar et al., 2016).

1.1. Industrial Application of Lipases

As known that; due to the higher reaction rates, good performance in milder reaction conditions, higher reaction specificity, enzymes are used in a remarkable number of industrial applications (Hasan et al., 2006). With the discovery of microbial enzymes, their importance in industrial sectors has gained a consolidated acceleration by increasing the demand from suppliers. Subsequently, for enzyme extraction and purification methods protein engineering and genetic engineering have enhanced and developed this trend. Also, heightened awareness of consumers against food quality, population explosion and ecological problems are considered to be the main factors promoting the enzymes market growth. The prominent companies, including Novozymes (Denmark), DuPont (US), and DSM (Netherlands), have over 75% of the market share in the worldwide. According to the last statistical data, the enzymes market is valued at USD 9.9 billion in 2019 and is reached USD 14.7billion by 2025, recording a CAGR of 6.7%. Also, with a compound annual growth rate (CAGR) of 7.1% from 2020 to 2027 it is projected to reach more value in 2027 than one in 2025 (*Enzymes Market by Product Type (Industrial Enzymes and Specialty Enzymes), Source (Microorganism, Plant, and Animal), Type, Industrial Enzyme Application, Specialty Enzymes Application, and Region – Global Forecast to 2025*, n.d.; *Enzymes Market Size, Share | Global Industry Report, 2020-2027*).

Lipases having ubiquitous properties can be used in many of industrial processes; in detergent industry as fat stain removers, in medical applications as digestive aids, in textile to remove of lubricants, pharmaceuticals such as anticholesterolemics, anti-inflammatories and thrombolytics (Divakar and Manohar, 2007). Some of applications areas of lipases are shown in **Figure 1.**

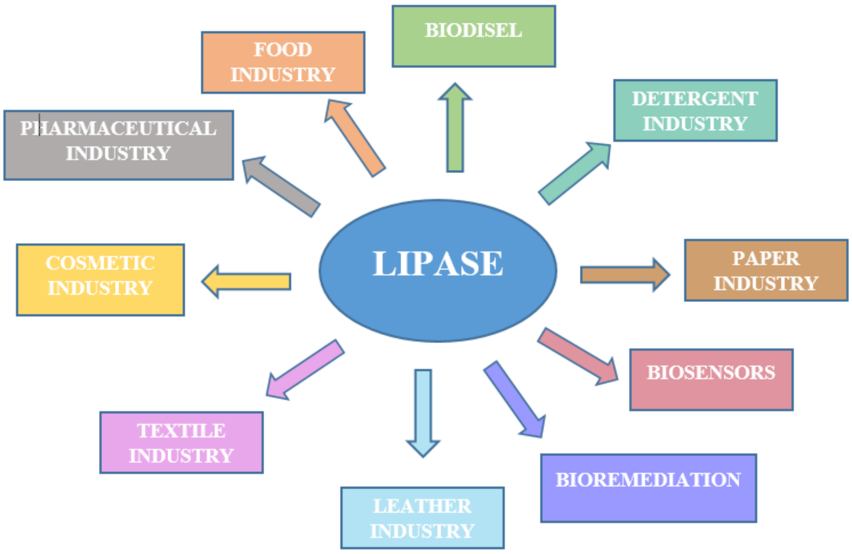

Figure 1.1. Industrial applications area of microbial lipases.

Thanks to the wide usage area of lipases, they take place in these popular industrial enzymes in the global market as the third largest group after carbohydrases and proteases (Guerrand, 2017). World's top lipase producer company is Novozymes from Denmark, US-Based Du Pont and Swiss company Roche follow it, respectively as the second and third producers of lipase (Brígada et al., 2014; Gupta et al., 2015).

1.1.1. Lipases in food applications

Due to the ability to catalyze fats and oil, lipases possess a great importance for food industry such as bakery, beverages, flavour development, cheese flavouring, etc. in diary industry, they mainly accelerate the ripening cheeses by hydrolysing milk fat to flavour enhancement agents for cheese-like products (Arora et al., 2020). *Rhizomucor miehei* lipases can be given an example for hydrolysis fat in milk (Adrio and Demain, 2014; Arora et al., 2020). Also Karra-Châabouni et al., (2006) studied the immobilized lipase produced by *Staphylococcus simulans* for the synthesis of ethyl valerate as flavouring agent. In 2010, Ahmed et al., were studied that an alkaline lipase produced from *Acinetobacter* sp. EH28 that is organic solvent-tolerant bacteria can be used for the production of ethyl caprylate**.**

In bakery, lipases produced by *Candida cylindracea*, *Aspergillus niger* and *Rhizopus oryzae* provide to increase the loaf volume, softness and masses and allow to enhance the shelf-life of baked products (Guerrand, 2017). Additionally, they are used for processing the coffee to enhance the whiteness & flavour and processing of egg yolk to synthesize mayonnaise (Pérez et al., 2019).

1.1.2. Lipases in biodiesel production

Recently, one of the biggest environmental problems is global warming caused by rising levels of greenhouse gases due to the enormous fossil fuel consumption. This continuous usage of fossil fuels has also led to the reduction of resources. Therefore, there is a need for renewable biofuels, as an example biodiesel derived from vegetable oils (soybean oil, rapeseed oil, palm oil, sunflower oil, corn oil and canola oil used cooking oil, etc.) and renewable resources such as algae and plants, serve as a potential candidate for the same (Arora et al., 2020). This process happens by transesterification of vegetable oils resulting in a mixture of fatty acid alkyl esters (biodiesel) and glycerol shown in Figure 1.2, in the presence of a catalyst (acid, base or enzyme) and also their characteristics are very similar to the conventional ones. Synthesis of biodiesel by chemical catalyst bring some drawbacks such as high energy consumption, difficulty in recovering glycerol and releasing high amount of alkaline in the wastewater. However, enzymatic synthesis is environmentally friendly and also a low energy requiring process that takes place in mild reaction conditions (Tan et al., 2010; Jain and Mishra, 2015).

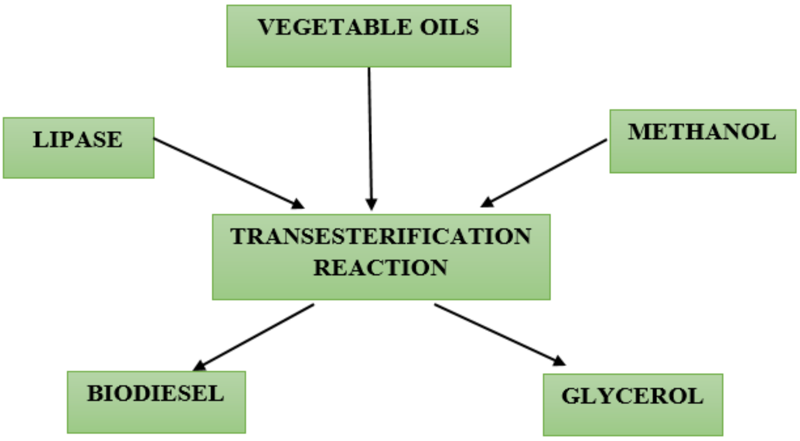

Figure 1.2. Biodiesel production by transesterification reaction.

The enzymatic synthesis of biodiesel is obtained by esterification of fatty acids or transesterification of oils and fats with alcohols such as ethanol and methanol. For example, biodiesel was synthesized by the transesterification using a lipase produced by *Acinetobacter venetianus* (Arora et al., 2020). Among the alcohols, methanol is mostly preferred because of its low cost. For this reason, fatty acid methyl ester is the most commonly used product. Continuously, biodiesel production is being researched by many scientists for further development and for industrial sectors in order to catch the best feasibility (Tan et al., 2010).

1.1.3. Lipases in pharmaceutical industry

The high selectivity and specificity of lipases make them valuable catalysts especially for the synthesis of pharmaceuticals and fine chemicals, where the demand for regio- and enantiomerically pure molecules is continuously increasing. Lipases used in the pharmaceutical industry, resolving the ability of racemic mixtures by the synthesis of single enantiomers is crucial for the pharmaceutical industry.

Several drugs and active pharmaceutical ingredients (API) are currently used in medicine for the treatment of cardiovascular disease, digestion problems, anxiety, Alzheimer disease, etc. (Noumara and Casiada, 2016). Anciently, lipases have been used for digestive aid. As an example, Yang et al., (1997) studied the synthesis of lovastatin that is used to lower the cholesterol levels by a lipase from *Candida rugose*. Also, lipases are used to production of Polixatel (taxol 1), which is given for the treatment of ovarian cancer (Fukaya et al., 2016).

1.1.4. Lipase in bioremediation

In the past years, waste has traditionally been disposed of by digging a pit and filling it with waste materials. This waste disposal method was difficult to maintain due to the lack of new places to dump each time. Developed new technologies for waste disposal using high temperature incineration and chemical separation. Due to the drawbacks of these technologies such as non-ecofriendly and expensive, bioremediation using microorganisms by secreting enzymes in order to utilize the contaminants has gained currency as a suitable alternative. At first time, lipases from *C. rugosa* used to remove oil from the surface (Bailey and Ollis, 1986). Then, it was found that lipases can be utilized in wastewater treatment including household waste, poultry waste and industrial wastes (Arora et al., 2020).

1.1.5. Lipases in detergent industry

Due to their ability of hydrolysis of fats (lipids) at extreme temperature and pH even at low concentrations, lipases are used as a crucial additive in the detergents formulations and they have placed into the second most important group of detergent enzymes to remove oil stains after proteases. Based on that, Lipex® and Lipolase® from Novozymes are two commercial examples of lipases in the detergent industry. Detergents formulated by lipases are biodegradable that they are not harmfull for human health and the environment (Pérez et al., 2019; Arora et al., 2020).

1.1.6. Lipases for paper and pulp production

The presence of triglycerides and waxes in feedstock as wood is unfavourable situation in paper and pulp industries, and they are needed to remove in the process of pulping and refining process (Gutiérrez et al., 2001). Additionally, organic solvents used in the process is another drawbacks such as lack of environmentally friendly usage. For this reason, lipases are the best choice in order to hydrolysis of lipophilic compounds to more hydrophilic and less sticky substances. This method has been used commercially in industries all over the world. For example, Demuner et al., (2011) reported that *Pseudomonas* lipases (KWI-56) gained an enhance in bleaching applications (Demuner et al., 2011).

1.1.7. Lipases in cosmetics and perfumery industry

Lipases have the ability of catalysing the important products as ingredients in cosmetics and perfumery formulations. As an example, menthol is one of the most popular fragrance in cosmetics for cooling effect and it is derived from methyl benzoate by lipolytic catalysis and also, butyl butyrate production for odor is carried out by microbial lipases (Pérez et al., 2019; Vishnoi et al., 2020). Commercially, lipase from *Candida antarctica* is used to produce ester benzyl propionate as an odor for cosmetic sectors (de Meneses et al., 2019).

1.1.8. Lipases in textile and leather industry

In the textile sector, lipases are preferred to remove machine lubricants residue and also, they are used to give the textile tissues softness without using blemishes during releasing hydrophilic chemical compounds in the reaction. Also, they can be used for the desizing cotton fabrics in the combination with alpha amylase enzyme (Perez et al., 2019).

In the processing of animal skins and hides, fat removal present in the tissue is critical and earlier, this process was performed by organic solvents and surfactants. However, this conventional method causes environmental contamination, lipases are being used for dehairing of animal hides and skin in the leather industry (Sanchez and Demain, 2017).

1.1.9. Lipase in ester synthesis

Nowadays, lipases have been commonly used for ester synthesis reactions. While esters derived from short chain fatty acids are generally used for flavoring agents, esters derived from long chain fatty acids (methyl or ethyl esters) are preferred to use in biodiesel production as enriching agents (Sharma et al., 2001). Additionally, they are used as anti-microbial agent, bio-lubricant, biosurfactant, emulsifier in a wide range of industrial sector. Lipase –catalysed esters are biodegradable and eco-friendly compared to the traditionally synthesized ones. As an example, trimethylolpropane esters were synthesized by lipase as lubricants. Also, lipases can be used to synthesize esters by using organic solvent because of the stability in organic solvents (Pérez et al., 2019).

Lipase catalysed ester synthesis is typically carried out with an acyl donor (fatty acids or fatty acid esters) and an acyl acceptor (carbohydrate, sugar alcohol) by transesterification reactions in organic solvents (Gumel et al., 2011).

Sugar esters are the most popular used nonionic and nontoxic biosurfactant also called as glycolipids having a wide range of hydrophilic-lipophilic balance (HLB) values. Since they have biodegradable, non-toxicity, non-irritant to skin and no-odour properties, they can be used in the food, pharmaceutical and cosmetic industries. Among them, sucrose esters have been commercially approved by FDA and are used in the food industry, such as wheat products, confectioneries, and dairy products, etc. (Ren and Lamsal, 2017).

In the study of Staron et al., (2018), medical applications of lactose esters in cancer treatment and in diagnostic tools as bionsensors were investigated. Siebenhaller et al., (2017) reported that sugar esters synthesis by Novozyme 435 lipase was achieved utilizing beechwood lignocellulosic extracts as a renewable sugar resource. Moreover, in the study of Siebenhaller et al., (2018), honey and agave syrup consisting of the high amount of fructose and glucose and a low amount of water was used as substrate and also media for the production of sugar esters without using any organic, ionic liquids or DES solvents.

In the following section, the microbial productions of lipolytic enzymes are summarized.

1.2. Production of Lipases

1.2.1. Microorganisms used for lipase production

Lipase was discovered for the first time in 1856 by Claude Bernard in pancreatic juice. Although pancreatic lipases are mostly used to treat digestive problems of humans, the shortage of pancreas from animal source and difficulty of collecting available material prevents the usage of pancreatic lipase in different industrial applications (Hasan et al., 2006). For that reason, researching other sources for lipases came to the fore in industry and since then, plants and a large number of microbial resources consisting of bacteria, yeast, fungi and archea and also a few insects are evaluated for lipolytic enzymes (Singh et al., 2019; del Hierro et al., 2020).

Among these sources mentioned above, microorganisms are the most favourable and the more useful resources and they are widely used to produce lipases by fermentation thanks to the rapid growth on cheap media giving high yield, more stable, ease of genetic manipulation. Moreover, their enzymes have high substrate specificity to various substrates (Al-Zuhair, 2011; Anbu, 2013; Priji et al., 2015). Generally, most of the commercial lipases are obtained from bacteria and fungi, including yeasts. Moreover, lipases from bacteria and fungi are attracting special interest because of their relatively moderate production conditions. Some important lipase producer microorganisms are shown in Table 1.1.

Table 1.1. Common bacterial, fungal and yeast sources of lipases.

Source	**Name**	**References**
Bacterial	*Staphylococcus aereus*	Tyski et al., (1983)
	Pseudomonas fragi CRDA 037	Schuepp et al., (1997)
	Bacillus subtilis 168	Leusisse et al., (1993)
Fungal	*Rhizopus oryzae*	Hiol et al., (2000)
	Aspergillus niger	Sugihara et al., (1988)
	Penicillium cyclopium	Chahinian et al., (2000)
Yeast	*Cryptococcus albidus*	Tiwari et al., (2011)
	Geotrichum candidum	Lotti et al., (1998)
	Candida rugosa	Lotti et al., (1998)

In the 20th century, lipases from bacterial origin have been worked on *Bacillus prodigiosus, Bacillus pyocyaneus* and *Bacillus fluorescens* (Hasan et al., 2006). On the other hand, yeasts have been used in food, detergent and other industrial sectors for years. Due to that yeasts are easier to handle and grow, they are more preferable for industrial applications in comparison with the bacterial ones (Kademi et al., 2003; Vakhlu, 2006). *C. rugosa* is the best known yeast that they have been used frequently 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 (Patel, 2008; Singh and Mukhopadhyay, 2012). Lipases producer yeasts are illustrated in Table 1.2. Among them, *Geotrichum candidum* and species belonging to *Candida* genus (*C. antarctica*, *C. cylindracea*, *Candida lypolytica*, and *C. rugosa*) and *Aspergillus* sp., *Rhizomucor* sp., *Rhizopus* sp., *Yarrowia lipolytica* and *Pseudomonas* sp. are mostly preferred yeast species for large scale and commercial lipase production (Satyanarayana and Kunze, 2009; Guerrand, 2017).

Table 1.2. Lipase producing yeasts.

Source	**Reference**
Candida albicans	Hube et al., (2000)
Trichosporon asahii MSR 54	Kumari and Gupta, (2012)
Yarrowia lipolytica	Pignede et al., (2000)
Cryptococcus albidus D24	Yalçın et al., (2014)
Cryptococcus albidus D44	Yalçın et al., (2014)
Rhodotorula slooffiae D38	Yalçın et al., (2014)
Candida davisiana D40	Yalçın et al., (2014)
Cryptococcus uzbekistanensis D57	Yalçın et al., (2014)
Candida antarctica	Rotticci et al., (2001)
Kurtzmanomyces sp. I-11	Kakugawa et al., (2002)

Recently, Yalçın et al., (2014) isolated yeasts from petroleum refinery sludge. In order to identify the isolate D24 and D44, they used molecular techniques as PCR amplification of ribosomal RNA genes, RFLP analysis of the DNA and the non-coding internal transcribed spacers (ITS) region. Additionally, they analyzed D1/D2 region of 26S rRNA which is used to recognize ascomycetous yeasts. According to their results, a size of PCR product of D1/D2 domain of 26S rRNA was 571 bp for isolate D24 and D44. Comparison of their sequence with GenBank database showed that isolate D24 identified as *Cryptococcus albidus* belongs to bacidiomycetous while isolate D44 identified as *Cryptococcus diffluens* belongs to ascomycetous group. The RFLPs of the ITS1-5.8-ITS2 of *C. albidus* D24 and *C. diffluence* D44 were determined as 540 and 530 bp. The molecular size of the PCR product of the 18S rRNA (1800 bp) was similar for both strains used to produce lipase enzyme (Yalçın et al., 2014).

1.2.2. Lipase production processes

Microbial lipases are generally extracellular so, their production is affected by medium components, temperature, pH, and dissolved oxygen amount. There are many research to increase lipase production, which focused on carbon and nitrogen sources, inoculation concentration, agitation, media pH and the growth temperature (Rajendran et al., 2008;

Kishan et al., 2013; Bindiya and Ramana, 2014). Medium components especially carbon sources, are the most significant nutrients due to that they are the main sources for the energy requirements of microorganisms. For this reason, they highly affect the growth and metabolite production (Singh et al., 2017). Also, production of extracellular lipases generally occurs in the presence of a lipid component in the medium as an inducer (Salihu and Alam, 2012). For this reason, lipids are essential carbon sources to obtain high yield of lipases (Salihu and Alam, 2012). Fatty acids are good inducers on extracellular lipase production such as olive oil, etc. As an example, Kamini et al., (2000) reported that triolein was good inducer for lipase production by a *Cryptococcus* species. Besides, nitrogen sources have an essential effect on lipase production from microorganisms. There are various nitrogen sources such as yeast extract, urea, tryptone, sodium nitrate, ammonium sulfate and peptone but peptone is the most favourable one to lipase production at high yield (Bharathi and Rajalakshmi, 2019). In the optimization of lipase from *Bacillus* sp. DM9K3, Singh et al., (2017) reported that 1% olive oil (v/v) and 1.5% peptone (w/v) exhibited the highest lipase activity as C and N sources.

Apart from the medium contents, environmental conditions such as pH and temperature play also a critical role in lipase secretion. Microorganisms having the ability of lipase production can grow at mild temperatures between 20 and 45 °C (Gupta et al., 2004). As an example, Huang et al., (2015) reported that *R. miehei* was highly produced lipase at 30°C whereas the bacteria strain *Bacillus* sp. DM9K3 was preferred to produce lipase at 50°C in high yield (Singh et al., 2019). Also, pH has an important parameter for lipase production and microbial growth. Fungal microorganisms have ability to grow at high pH values ranged from 4.0 to 12.0 (Mahmoud et al., 2015). Mukhtar et al., (2016) determined that the pH value of 6.5 achieved the highest lipase production from *R. oryzae*. Turati et al., (2019) reported that lipase activity and production from *Penicillium* sp. section Gracilenta CBMAI 1583 was enhanced when the medium was adjusted to pH of 4.0 while Singh et al., (2019) observed high lipase activity from a bacterial source at pH of 9.

There are two popular techniques for microbial lipase production: submerged fermentation and solid state fermentation. Submerged fermentation presents liquid broth medium for microorganism cultivation. However, solid state fermentation uses solid substrate media for microorganism growth. Solid state fermentation is generally preferred for the production of lipase from fungal and yeast microorganisms in industry (Bharathi and Rajalakshmi, 2019).

1.2.3. Lipase purification

Microbial enzymes can perform their catalytic activity apart from the natural environment when sufficient conditions are provided at the reaction media, and therefore enzymes can be used in a wide variety of application areas. Based on that, it is of great importance to identify the tissues or cell parts in which enzymes are located, to reveal their biochemical reaction functions, to examine their mechanisms of action and their kinetic properties in detail, and to obtain enzymes by purifying them.

Commercial microbial lipases are generally secreted to the extracellular environment so that the purification processes of lipases are easier than intracellular ones. The cell-free solution which is accepted as the crude enzyme is subjected to firstly concentration steps and then chromatographic steps. Besides that, new strategies such as aqueous two phase partitioning, three phase partitioning, immunopurification and reverse micellar system have been applied to purify lipases in high purification fold with fewer purification steps (Melani et al., 2020).

While the enzymes used in the pharmaceutical industry require a high degree of purity, they do not require such high purity in the biodiesel industry. For this reason, purification methods should be determined according to the application area of enzymes, so that unnecessary cost and time loss are avoided.

1.2.3.1. Methods used to concentrate proteins

Traditional techniques for lipase extraction and purification are initially applied for the first recovery, which is also called as preliminary purifications. There are several concentration techniques consisting of adding dry matrix polymers to enzyme solution (Sephadex G-25), ultrafiltration, lyophilisation, which is not frequently used method due to causing denaturation of enzymes, precipitation methods with ammonium sulfate or organic solvents such as cold acetone or ethanol (Erarslan and Kazan, 1998; Palmer and Boner, 2008) The choice of an appropriate method depends on the biochemical characteristics of lipases such as hydrophobic-hydrophilic structure (Saxena et al., 2003).

The common purification method (concentration or precipitation) has part in 80% of all purification strategies and 60% of these procedures apply ammonium sulfate precipitation. Following ammonium sulfate precipitation, acetone and ethanol are the other common methods (Saxena et al., 2003). Precipitation techniques depend upon the modification of the solvent solution by adding a specific amount of organic solvents or salts to change the solubility of proteins and form the precipitated protein. However, to avoid the loss of the biological activity of lipases during this process, the whole process is performed at around 4°C (Melani et al., 2020). Ayaz et al., (2015) used ammonium sulfate precipitation at 80% saturation following with size exclusion chromatography for purification of lipase by *Streptomyces* sp. OC 119-7 and obtained 5.52-fold purified lipase enzyme. Also different saturation concentration of ammonium sulfate was applied for the precipitation of lipase originated different microorganisms. As an example, Gururaj et al., (2016) purified a lipase from *Acinetobacter* sp. AU07 with the 60% saturation of ammonium sulfate while Ayaz et al., (2015) performed the precipitation 80% saturation of ammonium sulfate. Sultan et al., (2020) studied lipases from bacterial strains isolated from rotten fruits and vegetables and partial purified by ammonium sulfate fractionation. They reached the highest enzyme activity at 60% ammonium sulfate saturation. Also, Boran et al., (2019) characterized lipase from *Streptomyces violascens* OC125-8 by partially purification with ammonium sulfate at 80% saturation and found lipase enzyme purified at 3.28 fold. Anggriani et al., (2020) isolated bacteria producing lipases from fish fermented products and purified these lipases by ammonium sulfate at 40% and 100% saturation at 4.79 and 7.49 folds with recovery of 28.16% and 36.33%, respectively. However, Massadeh et al., (2012) purified lipase from *Bacillus stearothermophilus* HU1 and reported that ammonium sulfate precipitation was not an appreciated method in terms of purification yield since they obtained 9.66% recovery and 1.03 purification fold after chromatographic step.

Protein precipitation with organic solvents is also needed to be performed at around 0°C to avoid protein denaturation and apart from that insolubility of proteins in organic solvents such as acetone at this temperature value. As a result, impurities having the ability to be soluble in an organic solvent are easily removed from the precipitated proteins. Syihab et al., (2017) studied the purification of thermostable and alcohol tolerant lipase from *Pseudoxanthomonas* sp. firstly precipitated acetone fractioning (0-40%) and obtained 2.4 fold purified enzyme. Also, lipase from *Rhizopus arrhizus* was purified by 50% acetone precipitation at 3.6 fold with 70% high enzyme recovery (Zhekova et al., 2019). Addition to that, Singh et al., (2019) studied on the purification of lipase from *Bacillus* sp. DM9K3 and they obtained 15.5 fold purified lipase with 70% high recovery.

Another type of concentration method is ultrafiltration that separates according to their particle size. Based on molecular weight of the interested proteins ultrafiltration is performed to concentrate the protein and remove the impurities (Charcosset, 2011).

1.2.3.2. Chromatographic methods

After the extraction and the concentration procedures of enzymes, they become a more suitable state for further purification by chromatography. This method works the principle of differential distribution of interested enzymes in a mobile phase carried on the stationary phase. Chromatographic separation of enzymes depends on the enzyme interaction with the material of stationary phase, such as molecular weight, ionic charge, hydrophobicity and affinity. Generally, in enzyme purification, there are three main chromatographic methods using ion-exchange, affinity and molecular size properties of the enzyme (Saxena et al., 2003).

The most commonly employed one is ion exchange chromatography and its frequently used anion and cation exchangers are the diethylaminoethyl (DEAE) as a cationic group and carboxymethyl (CM) as an anionic group. The best known ion exchangers are Q-Sepharose that are used for unknown ısoelectric point of the enzyme, DEAE-Sepharose that are used for known isoelectric point of the enzyme (Kumar et al., 2005) and CM-cellulose (Sangeetha et al., 2011).

The second most used one is size exclusion chromatography or gel filtration with the other name. It is constructed by pouring gel matrix material having a defined pore size into the column and packed. Therefore, enzymes move through the column along different paths depends on their sizes. Summarily, its working principle depends on the pore size. Also, it has some practical advantages including easy operation and scaling up, isocritical elution and use of mild conditions (Sun et al., 2011).

Hydrophobic interaction chromatography (HIC) is another known purification technique. It is performed to separate enzymes through their hydrophobic interaction with hydrophobic ligands onto the resin. This resin consists of weak ligands, such as short-chain phenyl, octyl and butyl (Ünlüer et al., 2014). Affinity chromatography as an expensive method depends on specific interactions with ligands on the resin, such as monoclonal antibodies, that bind highly specifically to antigens so that this chromatography type gives extremely high selectivity (Saxena et al., 2003; Sun et al., 2011).

Recently, to overcome using multiple purification steps and to gain time, new strategies were developed. The aqueous two-phase system, reverse micellar system and immunopurification are the prominent methods. The aqueous two-phase system takes advantage of two immiscible solutions separation. Enzyme solution is mixed with salts such ammonium sulfate and polymer solution (PEG) is added as a result aqueous two-phase system is formed. The frequently used the polymers combined with salts are PEG/potassium phosphate and PEG/magnesium sulfate (Show et al., 2015).

Reverse micellar system is a method that used for extraction of enzymes. This system consists of an organic phase, aqueous phase in the droplet form and surfactant molecules the inner side as a stabilizer. It forms a convenient aqueous environment for enzymes' inner side of the micelles and it prevents contact between enzymes and solvents (Rajashekharappa, 2017). Immunopurificaiton is one of the highest affinity techniques using specific antibodies and so can tackle with purification difficulties that other methods will not resolve. However, it is not a feasible method in large-scale (Saxena et al., 2003).

Sivaramakrishnan and Incharoensakdi (2016) studied the purification of lipase from *Bacillus* sp. and they used ammonium sulfate precipitation and ion-exchange chromatography as purification steps respectively. As a result, the lipase was obtained at 5.1-purification fold with a yield of 10.5%. Also, Singh et al., (2019) purified lipase from *Bacillus* sp. DM9K3 by ammonium sulfate precipitation following anion exchange chromatography DEAE cellulose at 39.1 fold with 40% recovery. Cao et al., (2020) have been recently purified lipase from *Trichosporon* sp. by 50% ammonium sulfate and DEAE sepharose –anion exchange chromatography column - (1.6 cm × 5 cm) which was equilibrated with 20 mM Tris-HCl buffer at pH 8.3. The target proteins were eluted with the buffer. As a result, lipase was purified 3.96 fold with 36.6 recovery. Also, Rehman et al., (2020) purified lipase from *Pseudomonas mandelii* HTB2 using acetone precipitation and gel filtration Sephadex G-75 in phosphate buffer.

However, a single chromatographic step is not sufficient to obtain the desired high purity. Thus, mostly more than one chromatographic step is combined. As an example to that, lipase from *Mortierella alliacea* was purified in three steps; acetone precipitation, ion exchange chromatography with DEAE sepharose and gel filtration with Superdex G-100 and as a result, lipase was obtained with a yield of 4% and 6.2 purification fold (Jermsuntiea et al., 2011). However, lipase from the yeast *Cryptococcus* sp. S-2 was purified at 17.1 factor by performing ultrafiltration and SP-5PW cation exchange chromatography (Kamini et al., 2011). Moreover, Das et al., (2016) purified lipase produced from *Aspergillus tamarii* JGIF06 by 3 steps. Firstly, lipase was precipitated with ammonium sulfate and then was loaded onto anion exchange chromatography DEAE sepharose column (2 x 10 cm) and equilibration was achieved with 20 mM Tris–HCl buffer at pH 6 and the enzymes were eluted with gradient flow of (0–200 mM) NaCl. After that, the last step- gel filtration was performed onto the Sephadex G-200 column (2 cm x 20 cm) equilibrated with same buffer 20 mMTris–HCl buffer at pH 6. It resulted in 43.1 % enzyme recovery with 7.9 purification fold. As similar to this study, lipase from *Bacillus* sp. was purified by two step chromatographic methods as DEAE sepharose equilibrated with 50 mM Tris–HCl buffer (pH 8) by a gradient of 0.1–1M NaCl elution and Sephadex G-100 with same buffer-50 mM Tris–HCl buffer (pH 8). It has given 13.01 purification fold with 20% recovery (Saraswat et al., 2018).

1.2.4. Lipase characterization

In order to understand the structural and conformational integrity, optimum catalysis environment with the purpose of their application in industrial areas, properties of enzymes must be determined. Optimum temperature, pH of the enzyme, its stability at different pH and temperature values, organic solvent stability, the effect of metal ions are some of the important parameters to apply enzymes in different industrial applications. The biochemical properties of some selected yeast lipases are illustrated in Table 1.3.

Table 1.3. Biochemical properties of yeast lipases.

Yeast	**Molecular Weight (kD)**	**Optimum pH**	**Optimum Temperature**	**References**
Arxula adeninivorans	50	7.5	30ºC	Boer et al., (2005)
Candida rugosa DMS 2031	Lip A-64 LipB- 62 LipC- 60	7.8 7.8 7.8	35 - 40ºC	Benjamin and Pandey, (2001)
Kurtzmanomyces sp. I-11	49	1.9 - 7.2	75ºC	Kakugawa et al., (2002)
Trichosporon fermentans WU-C12	Lip 1-53 Lip2- 55	5.5	40ºC	Arai et al., (2006)
Cryptococcus sp. S-2	22	7.0	37ºC	Kamini et al., (2000)
Cryptococcus diffluens D44 crude	45.7	9.0	45ºC	Yılmaz and Sayar, (2014)

One of the original properties of enzymes is their molecular weights. As seen in Table 1.3, the molecular weights of lipases changes between 19 and 60 kDa (Chandra et.al., 2020). However, lipase having molecular weight higher than 60 kDa has been reported. Moreover, the molecular weight of lipase from *Virgibacillus alimentarius* LBU20907 is reported as 100 kDa (Dueramae et al., 2017).

Lipases are stable at a wide range of pH values. *Chromobacterium viscosum, A. niger* and *Rhizophus* sp., produced acidic lipases, while lipase from *Penicillum nitroaeducens* is active at pH 11.0. Besides pH, temperature is another important parameter that affects enzyme activity. Chandra et al., (2020) reported that lipolytic enzymes are generally more stable at low temperatures.

When the cofactor requirement is examined, lipases do not need any cofactors for their activity. However, metal ions enhance and/or inhibit their activity. Turati et al., (2019) showed that $MnCl_2$, and $CoCl_2$ activated the lipase enzyme from *Penicillium* sp. section Gracilenta CBMAI 1583. Contrary to results obtained by Turati et al., (2019), lipase from *Acinetobacter* sp. K5b4 was inhibited by $CoCl_2$ and $MnCl_2$ (Al Limoun et al., 2018).

Generally, calcium ion induce the lipase activity, while Ni^{2+}, Hg^{2+}, Sn^{2+} Zn^{2+}, Mg^{2+}, EDTA and SDS inhibits the lipase enzyme (Chandra et al., 2020).

Besides these properties of lipases explained above, effect of organic solvent on stability of lipases are also important. Al Limoun et al., (2018) reported that the activity of purified lipase enzyme from *Acinetobacter* sp. K5b4 increased remarkably in the presence of methanol, DMSO and acetone at 20, 40 and 60% (v/v) concentrations while, ethanol, acetonitrile and propanol decreased the enzyme activity. Similarly, Singh et al., (2019) investigated the effect of organic solvents on *Bacillus* sp. DM9K3 lipase and they found that the enzyme activity was decreased in the presence of immiscible organic solvents like ethanol and acetone. However, the presence of hydrophobic solvents such as benzene, toluene, hexane, etc. increased lipase activity. Therefore, they explained that enzyme activity loss in the presence of hydrophilic organic solvents resulted from that these organic solvents move away from the surface of the enzyme.

Recently, Yalçın et al., (2014) firstly reported the lipase producing strain *C. diffluence* D44. Then, Yılmaz and Sayar (2014) studied the production and characterization of crude D44 lipase and, lipase showed the excellent stability at 10% methanol. Besides *C. diffluens* D44 lipase, Yalçın et al., (2014) also reported the *C. albidus* D24 as another lipase producing strain.

Because of their excellent properties, this work aimed to purify the lipase from *C. albidus* D24 and *C. diffluens* D44 to understand the function and structure of these proteins**.** Additionally, crude D44 and D24 lipases were evaluated for fatty acid sugar ester synthesis. To our knowledge, this is the first study of the purification of lipases from *C. albidus* D24 and *C. diffluens* D44 purification.

Materials and methods applied through this thesis are explained in the following section.

2. MATERIALS AND METHODS

2.1. Materials and Equipments

2.1.1. Yeast strains and chemicals

C. albidus (D24) and *C. diffluens* (D44), were kindly supplied from Assoc. Prof. Dr. Tansel Yalçın (Ege University, Faculty of Science, Department of Biology, Basic and Industrial Microbiology Section).

All chemicals given in Table 2.1 were of analytical grade and purchased from either by Merck (Darmstadt, Germany) or Sigma Aldrich (St Louis, MO) unless otherwise noted.

Table 2.1. Chemicals used throughout the experiments.

Chemical	Supplier
Acetic Acid	Merck
Acetone	Merck
Acrylamide	Biofroxx
Anis-aldehyde	Sigma Aldrich
Agar	Sigma Aldrich
Ammonium Sulfate	Merck
Ammonium persulfate	Biofroxx
Bromophenol blue	Sigma Aldrich
Chloroform	Merck
Citric acid monohydrate	Merck
Coomassie Brilliant Blue R-250	Sigma Aldrich
Coomassie Brilliant Blue G-250	Amresco
D- Glucose Monohydrate	Merck
DEAE Sepharose	GE Healthcare
Ethanol	Sigma Aldrich
Ethyl Acetate	Merck
D-Fructose	Sigma Aldrich
Glycerol	Merck
Glycine	Merck

Table 2.1. *continued.*

Chemical	**Supplier**
Hydrochloric Acid (37%)	Merck
Isopropanol	Merck
Magnessium Sulfate Heptahydrate	Merck
Malt Extract	Sigma Aldrich
Methanol	Merck
N,N-methylen bis-acrylamide	Biofroxx
TEMED	Biofroxx
Olive Oil	Komili
p-Nitrophenyl Palmitate	Sigma Aldrich
p-Nitrophenol	Sigma Aldrich
Peptone	Merck
Potassium Dihydrogen Phosphate	Merck
Potassium Phosphate Dibasic	Merck
Sephadex G-100	GE Healthcare
Sodium Acetate	Merck
Sodium Carbonate	OMNI
Sodium Chloride	Merck
Sodium Dodecyl Sulfate (SDS)	Sigma Aldrich
Sodium Hydroxide	Merck
Sodium Nitrate	Carlo Erba
Sulfuric Acid	Sigma Aldrich
TGS Buffer -10X	Bio Rad
Trisodium citrate dihydrate	Sigma Aldrich
Triton X-100	Amresco
Trizma Base	Sigma Aldrich
Vinyl Decanoate	Sigma Aldrich
Vinyl-Octanoate	Sigma Aldrich
Yeast Extract	Merck
β-mercaptoethanol	Sigma Aldrich

2.1.2. Equipments

Equipments used in the thesis were listed below.

Table 2.2. List of equipments used.

Equipment	**Supplier**
Amicon Stirred Ultrafiltration Cell 8050	Millipore
Analytical Balance	Denver Instrument
Autoclave	Nuve OT 032
Automatic pipettes	Thermo Scientific
Centrifuge	Sigma 2-16KL
Chemidoc Imaging System	Bio Rad
Deep Freezer (-20ºC)	Arçelik No Frost
Deep Freezer (-80ºC)	Thermo Scientific 88400V
Electrophoresis System and Power Supply	Bio Rad
Fraction Collector 2110	Bio Rad
Fume Hood	Hedlab X-Pro, EN
Heating Magnetic Stirrer	Velp Scientifica
Ice Machine	Bar Line
Incubator	Nuve FN400
Laminar Air Flow Cabinet Bio Class II	Esco Lab
Microplate Reader	Biotek
Microtube Shacking Incubator	Labnet Vortemp 56 EVC
Mini Spin Centrifuge	Bio Rad
Orbital Shaker	Zhicheng
Peristaltic Pump	Cole Parmer
pH Meter	Hanna Instruments
Refrigerator	UĞUR
Spectrophotometer	Bio Rad SmartSpec Plus
Ultrasonic Bath	Bandelin
Vortex	Capp CRV-45X
Water Bath	LabO SM3

2.1.3. Buffers and solutions

Table 2.3. Compositions of buffers.

Buffers/Solutions	**Composition**
5X SDS-PAGE Sample Buffer	250 mM Tris-HCl pH 6.8, 10 % SDS, 30% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue
5X SDS-PAGE Running Buffer	15 g Tris, 72 g Glycine, 5 g SDS were dissolved in 1 L distilled water.
SDS-PAGE Staining Buffer	One gram Coomassie Brilliant Blue R-250, 500 ml methanol, 100 ml acetic acid were mixed and completed to 1 L solution with distilled water
SDS-PAGE Destaining Solution	50 ml methanol, 100 ml Glacial Acetic acid were dissolved in 1 L of distilled water.
10% APS	0.1 g APS were dissolved in 1 ml distilled water
10% SDS	10 g SDS were dissolved in 100 ml distilled water
30% Acrylamide solution	58.4 g Acrylamide and 1.6 g Bis-acrylamide were dissolved in 100 ml distilled water and stored at 4°C in the dark
30% glycerol solution	30% (v/v) glycerol and 70% (v/v) distilled water
1,5 M Tris, pH 8.8	18.15 g Tris dissolved in 100 ml distilled water and pH was adjusted to 8.8 with HCl
0.5 M Tris, pH 6.8	6.0 g Tris-HCl dissolved in 100 ml distilled water and pH was adjusted to 6.8 with HCl
Citric acid- sodium citrate buffer pH 4.0	0.05 M, 8.0 ml sodium citrate was mixed with 0.05 M, 13 ml citric acid
Dipotassium phosphate-monopotassium phosphate buffer pH 6.0	0.05 M, 2.0 ml dipotassium phophate is mixed with 0.05 M, 18 ml monopotassium phosphate

Tablo 2.3. *continued.*

Buffers/solutions	**Composition**
Dipotassium phosphate- monopotassium phosphate buffer pH 6,5	2.937 gram dipotassium phophate and 4.51 gram monopotassium phosphate were added to 1 L of distilled water
Dipotassium phosphate- monopotassium phosphate buffer pH 7.0	0.05 M, 22 ml dipotassium phophate was mixed with 0.05 M, 6 ml monopotassium phosphate
Dipotassium phosphate- monopotassium phosphate buffer pH 7,5	6.406 gram dipotassium phophate and 1.799 gram monopotassium phosphate were added to 1 L of distilled water
Dipotassium phosphate- monopotassium phosphate buffer pH 8.0	0.05 M 47 ml dipotassium phophate was mixed with 0.05 M, 3 ml monopotassium phosphate
Glycine- sodium hydroxide buffer pH 9.0	0.05 M, 17 ml glycine was mixed with 5.0 ml sodium hydroxide
Glycine- sodium hydroxide buffer pH 10	0.05 M, 12.5 ml glycine was mixed with 8.0 ml sodium hydroxide
Sodium acetate- acetic acid buffer pH 5.0	0.05 M, 8.0 ml sodium acetate was mixed with 0.05 M 32 ml acetic acid
Sodium acetate- acetic acid buffer pH 5.6	0.05 M, 3640 ml sodium acetate was mixed with 0.05 M, 360 ml acetic acid
Sodium Carbonate 1 M	10.59 gram of sodium carbonate was dissolved with distilled water

2.2. Methods

2.2.1. Storage and maintenance of strains

C. albidus D24 and *C.diffluens* D44 were stored at -80°C in 15% glycerol stocks. Glycerol stock samples were transferred to agar medium consisting of 0.3% malt extract (w/v), 0.3% yeast extract (w/v), 0.5% peptone (w/v), 1% glucose (w/v), 1.5% agar (w/v) with the pH: 6.2 and inoculated at 28°C for 2 days. After that, agar mediums were stored at +4°C.

2.2.2. Inoculum preparation

Preculture medium which is composed of 0.3% malt extract (w/v), 0.3% yeast extract (w/v), 0.5% peptone (w/v), 1% glucose (w/v) with an initial pH of 6.2 was prepared in 100 mL. Erlenmeyer flasks with the final working volume of 20 mL. It was sterilized in autoclave at 1.06 bar and 121°C for 15 minutes. Sterilized preculture medium was inoculated with a single colony of *C. albidus* D24 and 3 colonies of *C. diffluens* D44 from agar plates. Incubation was carried out in an orbital shaker at 180 rpm and 28ºC. After 16 h inoculation, cells were harvested by centrifuge at 4000 rpm for 6 min for *C. albidus* D24 and 7000 rpm for 6 min for *C. diffluens* D44 under the biosafety LAF cabin. Then, wet cell pellet was used to inoculate production medium.

2.2.3. Lipase production

For lipase production, basal medium including (w/v) 0.1% yeast extract, 0.05% $MgSO_4.7H_2O$, 3% peptone w/v, 0.3% $NaNO_3$ and 0.1% KH_2PO_4 supplied with olive oil at a concentration of 2% v/v was used as production medium. In order to increase lipase production, the basal medium was supplemented with 1 % glucose. The initial pH of the medium was adjusted 7.0 using 5M HCl. All media were sterilized by autoclave (121 °C for 15 min). After cooling, the olive oil, previously sterilized by dry heat (180 °C for 60 min) in drying-oven, was added to the culture medium. Then, wet cell pellet was resuspended in medium and transferred to lipase production medium to adjust initial optical density (OD) of 1.0 at 600 nm. In all experiments, initial optical density (OD) was kept at the constant value of 1.0. Cells were permitted to grow at 250 rpm and 28ºC for 6 days. At the end of the cultivation period, cells were harvested and centrifuged at 12000 rpm for 15 min at 4ºC and supernatant was used as extracellular crude lipase.

2.2.4. Lipase activity assay

The lipase activity was determined by using the method described by Yalçın et al., (2014). For substrate preparation, 30 mg of pNPP was dissolved in 10 mL propan-2-ol which were emulsified in 90 mL of 0.05 M sodium acetate, pH 5.6, containing Triton X-100 at appropriate amount. 0.1 mL of enzyme solution was mixed with 2 mL of the pNPP containing emulsion and incubated at 37°C for 3 min. The reaction was stopped by adding 0.15 mL of 1 M sodium carbonate solution and the absorbance was measured at 410 nm spectrophotometrically against enzyme-free substrate solution as blank.

In order to obtain reproducible data, substrate solution was supplemented with different amounts of Triton X-100 as 300 mg, 400 mg and 500 mg in 90 ml of 50 mM sodium acetate buffer.

For the calculation of enzyme activity, a calibration curve was prepared by plotting different 4-nitrophenyl concentrations against OD_{410} (Appendix A). "One unit of lipase activity (U) was defined as the amount of enzyme that liberates 1 μmol p-nitrophenyl for 1 minute under the assay conditions". Activity assay was performed with dublicate measurement for each sample.

2.2.5. Determination of protein concentration

The protein concentration was determined by Bradford method (Bradford, 1976). The samples including enzymes was mixed with Bradford reagent at 1:1 (v/v) and then incubated in the dark at room temperature for 5 min. The absorbance of samples was measured at 595 nm and protein concentration was calculated by using bovine serum albumin as standard (Appendix B).

2.2.6. Purification of Lipase

2.2.6.1. Ammonium sulfate precipitation

This process was applied by adding the ammonium sulfate at 90% saturation slowly to the solution including crude lipase. The ammonium sulfate precipitation was processed for 16 hours by continuously stirring at 4 ºC. After 16 hours, the mixture was centrifuged at 15000 rpm for 1 hour and pellet was resuspended in 0.05 M sodium acetate buffer at pH 5.6 and dialysed (cellulose membrane 12000 Da MWCO, Sigma Aldrich), based on Dialysis Methods for Protein Research by Thermo Scientific, against the same buffer to remove the ammonium sulfate residue at +4ºC overnight. The dialysed lipase was stored at 4 ºC for further applications.

2.2.6.2. Acetone precipitation

This precipitation was carried out by using the method of Thermo Scientific "Acetone Precipitation of Proteins". Firstly, cold (-20ºC**)** acetone was added to the crude lipase solution in four times volumes of enzyme solution. The mixture was vortexed for around 5 minutes and incubated at -20ºC for 1 hour. After incubation, the solution was centrifuged at 13000 xg for 15 minutes and pellets were hold at room temperature for 30 minutes to evaporate the acetone residue. As a final step, pellets were dissolved in 0.05 M sodium acetate buffer at pH 5.6 and dialysed against the same buffer to remove the acetone residue completely at 4ºC overnight. The dialysed lipase was stored at 4ºC for further applications.

2.2.6.3. Concentration by ultrafiltration method

After enzyme precipitation, lipases were concentrated to appropriate concentration value due to that the ion exchange chromatography works in best at around its dynamic protein binding capacity. In this process, lipase solution was ultrafiltered against 10 MWCO cellulosic membrane filter in Amicon stirring cell at 300 rpm and 4ºC applying 0.5 bar nitrogen gas until the desired protein concentration was obtained. Before the chromatographic method, concentrated enzyme solution was dialysed in binding buffer for buffer exchange.

2.2.6.4. Anion exchange chromatography by DEAE sepharose

Firstly, to find the most proper buffer and pH for DEAE Sepharose, 6 different buffer at different pH values (50 mM sodium acetate pH 5.6, 50 mM potassium phosphate pH 6.0, 50 mM potassium phosphate pH 6.5, 50 mM potassium phosphate pH 7.0, 50 mM potassium phosphate pH 7.5 and 50 mM Tris-HCl pH 7.5) in a batch system were used to determine the highest fold and the amount of proteins bound to the resin.

DEAE Sepharose resin was packed into the column (10 cm x 1.35 cm) and equilibrated with 5 column volume (CV) of the binding buffer. After equilibration, 10 ml of concentrated enzyme was loaded onto DEAE sepharose column and then washed with binding buffer to remove unbound proteins. After that, NaCl gradient elution was applied at a flow rate of 3 ml/min using the binding buffer supplemented with 0.1 – 0.5 M of NaCl (0.1, 0.2, 0.3, 0.4 and 0.5 M). During the elution, the fractions were collected and protein concentration of the fractions was measured at 280 nm specctrophotometrically. Fractions having higher 280 nm absorbance were applied to lipase activity assay and active fractions were pooled and dialysed against the 50 mM sodium acetate buffer at pH 5.6 to remove the NaCl from enzyme solution. Protein concentration and enzyme activity of pooled fractions were determined using the method explained in section 2.3.4 and 2.3.5. Dialysed enzymes were concentrated with ultrafiltration again.

2.2.6.5. Gel filtration chromatography by Sephadex G-100

Sephadex G-100 was swelled with 50 mM sodium acetate buffer pH 5.6 at room temperature for 3 days. After swelling, the resin was packed into the column (72 cm x 0.75 cm) and equilibrated with 50 mM sodium acetate buffer pH 5.6 for 24h. Then 1.5 ml of concentrated enzyme obtained from anion exchange chromatography was loaded onto the column and fractions were collected at a flow rate of 0.4 ml/min. Protein concentration of the fractions was measured at 280 nm spectrophotometrically. Fractions having higher 280 nm absorbance were applied to lipase activity assay and active fractions were pooled. Protein concentration of pooled fractions were measured using Bradford method.

2.3.7. Characterization of lipase

2.3.7.1. Molecular weight of purified lipase by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of purified lipase according to the method of Laemmli, (1970), using stacking gel (5%, w/v) and resolving gel (12%, w/v). Electrophoresis was carried out at 110 V for 120 minutes. After that, gel was stained by using Coomassie R250 to visualize proteins. BIO-RAD Precision Plus Protein Standards All Blue (161-0373) containing seven proteins (10–250 kDa range) was used for determination of the protein's molecular weight.

2.3.7.2. Effect of temperature on purified lipase activity

To observe the effect of temperature on purified lipase and to determine the optimum temperature of purified enzyme, 2 ml substrate solution as prepared by disolving pNPP in 50 mM sodium acetate buffer at pH 5.6 was mixed with enzyme solution (0.1 ml). The reaction mixture was incubated for 3 minutes at temperatures between 30 – 70ºC ranges. Optical density of solution was measured at 410 nm wave length. Temperature at which the highest lipase activity obtained was evaluated as 100% and the others were stated as relative activity.

2.3.7.3. Optimum pH of purified lipase

The optimum pH of the purified enzyme was determined at he pH ranges between 4.0 – 10.0. Buffers as citrate buffer, acetate buffer, phosphate buffer and glycine-NaOH buffer at 50 mM concentration were used for pH 4.0, pH 5.0, pH 6.0-8.0 and pH 9.0-10.0, respectively. 2 ml substrate solution as prepared by disolving pNPP in buffers given above was mixed with enzyme solution (0.1 ml) and incubated at 37ºC. Optical density of solution was measured at 410 nm wave length. pH at which the highest lipase activity obtained was evaluated as 100% and the others were stated as relative activity.

2.3.7.4. The stability of purified lipase at different temperatures

In order to investigate the stabilitiy of purified lipase at 50, 60 and 70ºC, enzyme solution was incubated at 50, 60 and 70ºC for different time intervals. Then, the activity of the samples were determined and the residual lipase activity was measured with respect to initial activity. The initial activity was taken as 100% and percent residual activity relative to initial activity was calculated.

2.3.7.5. Stability of purified lipase in the presence of ethanol and methanol

According to previous results reported by Yılmaz and Sayar (2015), ethanol and methanol at 10 and 20% concentrations were used. Similar procedure introduced by Yılmaz and Sayar (2015) was applied and relative lipase activity was calculated by considering the activity in the absence of organic solvents as 100%.

2.3.8. Fatty acid sugar ester synthesis

Based on Siebenhaller et al., (2018), sugar ester synthesis was carried out by crude lipase enzymes from *C. albidus* D24 and *C. diffluens* D44. 2.5 ml of concentrated crude lipases were incubated with 1.66 gr fructose and 200 µl of vinyl decanoate and vinyl octanoate in a 100 ml Erlenmeyer flask at 50 rpm and 50ºC for 48 hours. Absence of vinyl ester in the reaction media was used as a control. After incubation, 2 ml of hot water was added to each flask to stop the reaction. Then, 3.5 ml of ethyl acetate was added to separate the organic phase which was further subjected TLC to analyze the synthesis of sugar esters.

Least 10 µl of the crude extracts spotted on a TLC silica gel 60 F_{254} plate (20 cm x 20 cm, Millipore) as a stationary phase. After that, sugar esters were separated in the mobile phase including chloroform:methanol:acetic acid, 65:15:2 (v/v/v). To detect the different sugar esters, TLC plate is dipped into the dyeing solution including anisaldehyde:sulphuric acid:acetic acid, 0.5:1:100 (v/v/v). After that, the TLC plate is heated at 200ºC hot air in a drying oven for 5 minutes. The migration (R_f) of visualized bands on the plate were calculated to compare to the study of Siebenhaller et al., (2018).

3. RESULTS AND DISCUSSION

3.1. The Effect of Triton X-100 on Lipase Activity

In order to determine lipase activity in the cultivation medium, p-Nitrophenyl Palmitate (pNPP) was generally used as a substrate. Since, the solubility of pNPP in isoprophanol is very important to obtain reproducible results, in order to increase the dispersion of fatty acids, Triton X-100, a nonionic surfactant, is added to the reaction medium. For that reason, in the present work, lipase activity was determined in the presence of 200 mg Triton X-100 (Yalçın et al., 2014). However, turbidity problem together with precipitation was encountered during the preparation of substrate solution in sodium acetate buffer using stock pNPP solution prepared in isopropanol.

These problems caused the variation of absorbance values for the same enzyme samples in spectrophotometric readings. It was thought that the turbidity was caused by the immiscibility of pNPP in water. To overcome this problem and to obtain reproducible results of the lipase activity, the substrate solution was supplemented with different amount Triton X-100 as 200 mg (A), 300 mg (B), 400 mg (C) and 500 mg (D) (Gupta, 2002). The results are tabulated in Table 3.1 and the appearance of the turbidity and precipitation was shown in **Figure 3.1.**

Table 3.1. Enzyme activities of crude lipase at different amount of Triton X-100.

Amount of Triton X-100, (mg)	Enzyme Activity, (U/mL)				Standard Deviation
	Exp.- 1	Exp.- 2	Exp.- 3	Average	
200	1.3484	1.0787	1.5102	1.3120	± 0.2180
300	1.8554	1.6181	1.8231	1.7655	± 0.1287
400	2.3085	2.4271	2.5027	2.4128	± 0.0978
500	3.6569	3.7648	3.7216	3.7144	± 0.0543

As seen in **Table 3.1**, increasing Triton X-100 concentration caused to increase lipase activity and the reproducible results were obtained by using 500 mg Triton X-100 in the substrate solution. This was proved that high amount of Triton X-100 extinguished the immiscibility problems between the synthetic fatty acid (pNPP) and water.

According to these results, substrate solution was supplemented with 500 mg Triton X-100 throughout the whole study.

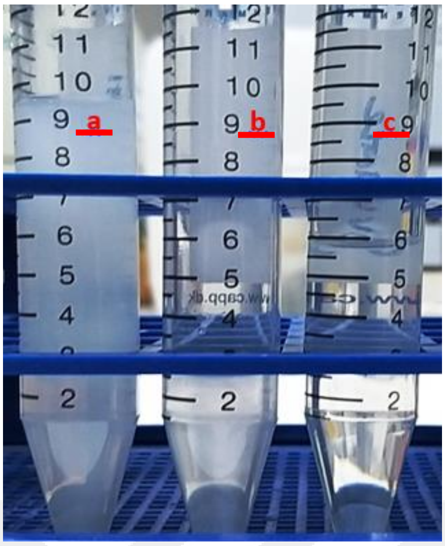

Figure 3.1. pNPP solution containing Triton X-100 at a) 200 mg, b) 400 mg and c) 500 mg.

3.2. Effect of Glucose on Lipase Production from *C. albidus* D24 and *C. diffluens* D44

It is well known that the production of biomaterials from microbial sources depends on various induction situations like medium compositions, environmental conditions (temperature, dissolved oxygen, pH, etc.). In the case of lipolytic enzymes, lipids are unique to induce extracellular lipase expression during cultivation (Darvishi et al., 2015) and expression level changes according to the nature of yeast strain (Geoffry et al., 2018). Apart from lipids, various carbon sources such as glucose, sucrose, glycerol, etc. have been used for the production of lipase (Sumarsih et al., 2019).

In our previous work, lipase production from *C. albidus* D24 and *C. diffluens* D44 was achieved by using olive oil as a carbon source. To increase lipase activity, the culture medium including olive oil was supplemented with 1% glucose (**Figure 3.2** and **Figure 3.3**). After 6 days of incubation, the cells were removed and extracellular lipase activity in the culture medium was determined according to the method described in section 2.2.4 and results are given in Table 3.2.

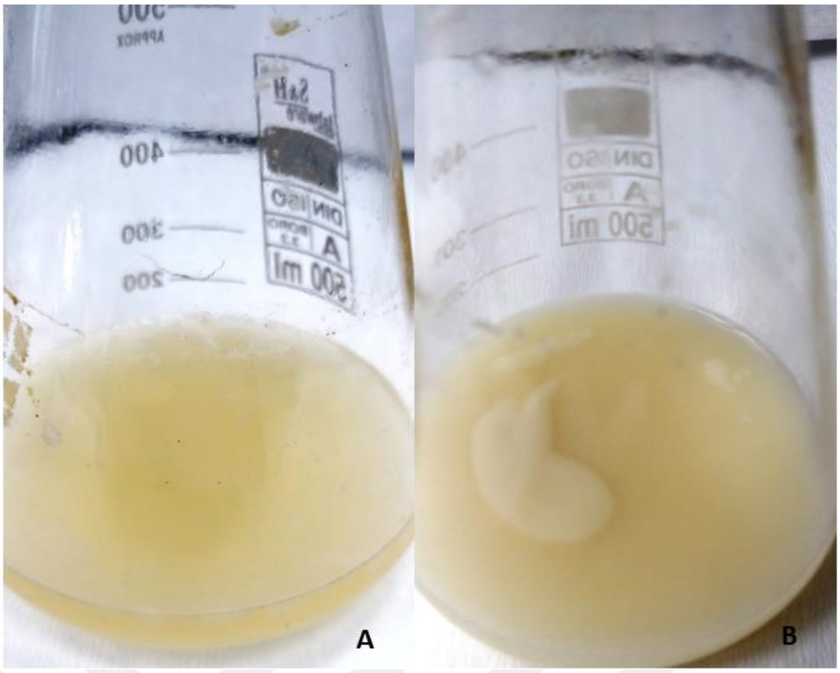

Figure 3.2. *C. albidus* D24 cultivation at 6th day, by using medium including A) 1% glucose and 2% olive oil B) 2% olive oil.

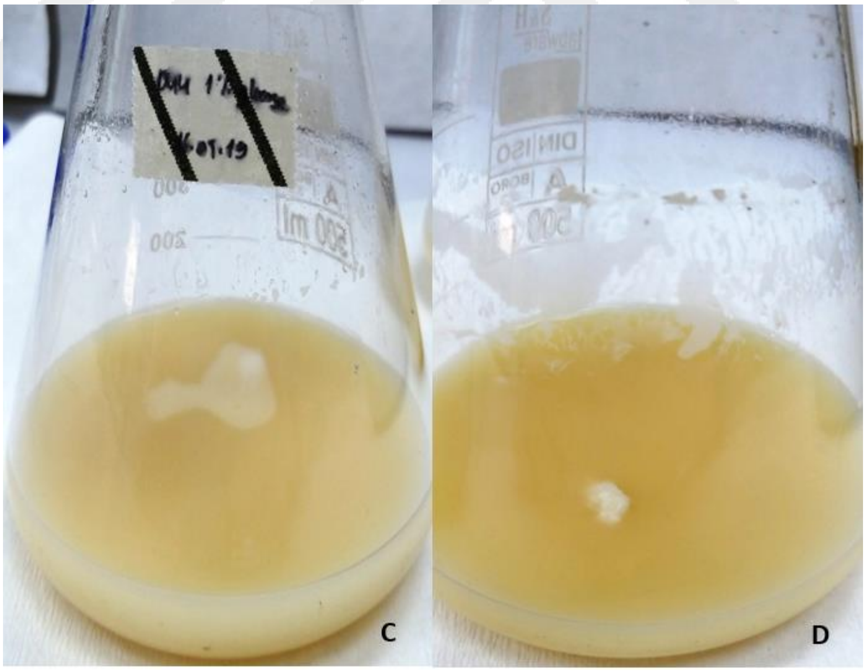

Figure 3.3. *C. albidus* D44 cultivation at 6th day by using medium including C) 1% glucose and 2% olive oil D) 2% olive oil.

Table 3.2. Effect of carbon source on lipase activity during the growth of microorganisms.

Microorganism	Presence and absence of 1% glucose	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)
C. albidus D24	Presence	2.7	648	240
	Absence	3.8	44	12
C. diffluens D44	Presence	3.5	50	14
	Absence	2.3	290	126

According to results, lipase production from *C. albidus* D24 was induced in the presence of glucose while lipase activity obtained from *C. diffluens* D44 was decreased in the presence of glucose. When specific activities of two conditions were compared (Table 3.2), the specific activity as 240 U/mg obtained in the presence of glucose was 20 fold higher than that of obtained in the absence of glucose. This can be commented that *C. albidus* D24 yeast strain secretes lipase at a high level in the presence of olive oil and glucose.

Unlike *C. albidus* D24, high yield of lipase activity from *C. diffluens* D44 was observed without glucose supplementation. As shown in Table 3.2, the highest specific activity was obtained from the culture media containing only 2% olive oil (as carbon source) as 126 U/mg which is nine times higher than that obtained with glucose addition. Summarily, lipase production from *C. albidus* D24 was enhanced in the presence of glucose, while supplementation of production medium with %1 glucose reduced lipase production *C. diffluens* D44 in six fold. Similar to our results obtained for *C. diffluens* D44, Ameri et al., (2019) found that maximum lipase secretion was obtained with the culture media including glucose and olive oil.

Generally, glucose has been known as a repressing effect on lipase production. Niaz et al., (2013) and Dalmau et al., (2000) reported the induction effect of glucose for extracellular lipase production from *C. rugosa* and *A. niger* NCIM 1207, respectively. In another study, the effect of carbon source on lipase production from *Trichoderma harzianum* isolated from oil contaminated soil was investigated and it was resulted in the negative effect of glucose on lipase production (Rihani and Soumati, 2019).

Based on these results, *C. albidus* D24 was grown in the medium including olive oil and glucose while D44 lipase was cultivated in the absence of glucose. After 6 days of the incubation period, D24 and D44 lipases were purified to characterize D24 and D44 lipase.

3.3 Purification of Lipase Produced by *C. albidus* D24 and *C. diffluens* D44

In order to purify proteins from its bulk solution, a common fundamental stage is precipitation of proteins by using ammonium sulfate [$(NH_4)_2SO_4$] and miscible organic solvents. Therefore, $(NH_4)_2SO_4$ and acetone were used to precipitate extracellular proteins from cultivation medium after cell harvesting.

3.3.1. Preliminary purification

In the present work, ammonium sulfate at 90% saturation and cold acetone were used to precipitate whole proteins expressed by *C. albidus* D24 and *C. diffluens* D44. At the end of precipitation step (Figure 3.4), samples were centrifuged to separate precipitated proteins and then they were dialysed against to 50 mM sodium acetate buffer pH 5.6.

As seen in Figure 3.4, ammonium sulfate precipitation resulted in two different phase. At the top phase an oily layer and at the bottom phase precipitated protein pellets were observed and both of them gave lipase activity. In acetone precipitation, proteins were precipitated at the same point and the supernatant has been shown a clear appearance.

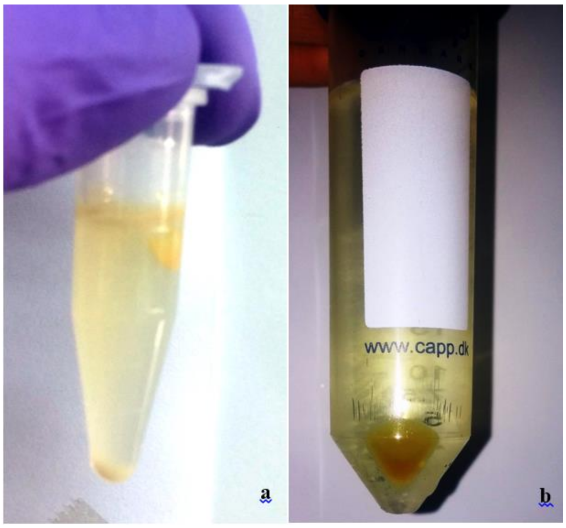

Figure 3.4. Precipitation of lipase from *C. diffluens* D44 by a) ammonium sulfate and b) acetone.

After dialysis step, lipase activity was determined and, yield and purification fold were calculated. Purification table are given in Table 3.3 and Table 3.4.

Table 3.3. Precipitation of proteins using 90% ammonium sulfate.

Sample	Purification Step	Total Protein mg	Total Activity U	Specific Activiy U/mg	Yield %	Fold
C. albidus D24	Crude extract	11	1305	117	100	1
	Precipitated and Dialysed	0.57	29	52	2.24	0.44
C. diffluens D44	Crude extract	2.32	174	75	100	1
	Precipitated and Dialysed	1.09	50	46	27	0.61

As seen in Table 3.3, the lipases produced by *C. albidus* D24 and *C. diffluens* D44 were not precipitated by using ammonium sulfate. For *C. albidus* D24, the ammonium sulfate precipitation resulted the precipitation of 83.3% of whole water soluble proteins. However, $(NH_4)_2SO_4$ affected D24 lipase activity and it lost almost whole activity. In the case of the precipitation of proteins of *C. diffluens* D44 proteins, 75% of whole proteins were precipitated and 84% of lipase activity remained.

In the literature, there are few studies obtained similar outcomes from ammonium sulfate precipitation method. Similar to our results, Massadeh et al., (2012) studied the purification of lipase from *B. stearothermophilus* HU1 and reported that ammonium sulfate precipitation was not an appreciated method in terms of purification yield since they obtained 9.66% recovery and 1.03 purification fold after chromatographic step. Additionally, Iftikhar et al., (2011) presented that ammonium suphate precipitation applied to purify lipase from wild type *Rhizopus oligosporus* resulted 90.24% yield with 1.74 fold. These findings are close to that obtained for the precipitation of proteins of *C. diffluens* D44. Also, Boran et al., (2019) characterized lipase from *S. violascens* OC125-8 by partially purification with ammonium sulfate at 80% saturation and found lipase enzyme purified at 3.28 fold. Anggriani et al., (2020) isolated bacteria producing lipases from fish fermented products and purified these lipases by ammonium sulfate at 40% and 100% saturation at 4.79 and 7.49 folds with 28.16% and 36.33% recovery, respectively.

Table 3.4. Precipitation of proteins using acetone.

Sample	**Purification Step**	**Total Protein mg**	**Total Activity U**	**Specific Activity U/mg**	**Yield %**	**Fold**
C. albidus D24	Crude extract	3.72	781	210	100	1
	Precipitated and Dialysed	3.10	5	1.6	0.64	0.01
C. diffluens D44	Crude extract	1.40	175	125	100	1
	Precipitated and Dialysed	1.05	147	140	84	1.12

When results of acetone precipitation of lipases were investigated (Table 3.4), lipase from *C. albidus* D24 was evaluated with 0.64% activity recovery and 0.01 purification fold. On the other hand, lipase from *C. diffluens* D44 gave 84% recovery with 1.12 purification fold that was the one having highest enzyme activity recovery and purification fold between concentration methods in this study. Jermsuntiea et al., (2011) purified lipase from *M. alliacea* fungus with acetone precipitation as first step and they found lipase activity recovery 52% and 3.7 purification fold. In addition to that, lipase from *R. arrhizus* was purified by 50% acetone precipitation at 3.6 fold with 70% high enzyme recovery while purification of the enzyme by ammonium sulfate was found inappropriate method due to the low yield and purification fold (Zhekova et al., 2019). Singh et al., (2019) studied the purification of lipase from *Bacillus* sp. DM9K3 and they obtained 15.5 fold purified lipase with 70% high recovery by acetone precipitation. When compared our findings to the results reported by Jermsuntiea et al., (2011), it has a higher purification fold than acetone precipitation of lipase from *C. diffluens* D44 but it has a lower recovery value than *C. diffluens* D44. Moreover, Syihab et al., (2017) reported similar research on lipase concentration methods that they compared acetone and ammonium sulfate precipitation. As a result, they explained that acetone precipitation of lipase gave higher yield and purification fold as 79 and 2.5, respectively.

Since the purification of lipase from *C. diffluens* D44 lipase has not been purified previously, it was decided to continue with *C. diffluens* D44 for further purification steps. Then purified lipases were characterized and compared with the results reported by Yılmaz et al., (2015).

3.3.2. Column Chromatography

In order to obtain high purity different chromatographic methods are used depending on molecular weight, ionic strength of proteins and the affinity of proteins to their ligands.

In the present work, D44 lipase was enriched by the anion-exchange chromatography and then purified by the gel filtration chromatography.

3.3.2.1. Anion exchange chromatography

Determination of binding buffer and pH value

To find the most proper buffer and pH for DEAE Sepharose column material, 6 different buffers at different pH values were tested in a batch system to determine how much protein was bound to the resin. In this study, it was aimed to find the best pH value that lipase enzyme can be bind to the column material based on the idea that lower enzyme recovery percentage in the prepared solution taking place in the column material defines lipase enzyme bind to the resin.

Table 3.5. Determination of DEAE Sepharose binding buffer.

Buffer Type	Sample	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)
50 mM pH:5.6 sodium acetate	Crude	9	0.95	9.5	100
	Purified	2	0.2	10	22
50 mM pH:6.0 potassium phosphate	Crude	5	0.5	12	100
	Purified	1	0.04	25	20
50 mM pH:6.0 potassium phosphate	Crude	6	0.59	36.8	100
	Purified	2	0.06	33	33.3
50 mM pH:6.5 potassium phosphate	Crude	4	0.6	6.6	100
	Purified	1	0.15	6.6	25
50 mM pH:7.0 potassium phosphate	Crude	4	0.4	10	100
	Purified	1	0.13	7.7	25
50 mM pH:7.5 Tris HCl	**Crude**	**4.1**	**0.55**	**7.3**	**100**
	Purified	**0.8**	**0.05**	**20**	**19.5**

According to Table 3.5, the higher yield was obtained in potassium phosphate pH 6.0 buffer media as 33.3% while the lower yield as 19.5% in Tris-HCl pH 7.5 buffer was found. Since the highest binding was obtained with Tris HCl buffer, we used it to load lipase enzyme to column including DEAE Sepharose matrix**.**

Anion exchange chromatography by DEAE Sepharose

After acetone precipitation and dialysis, D44 lipase was concentrated by 10 kDa cellulose membrane disc in Amicon ultrafiltration cell due to the remove low molecular weight proteins. In the meantime, DEAE Sepharose resin was packed into the column (10 cm x 1.35 cm) and equilibriated with 5 column volume (CV) of 50 mM Tris-HCl at pH 7.5 (**Figure 3.5)**. After equilibriation, 10 ml of the concentrated enzyme was loaded onto DEAE sepharose column. Gradient elution of bounded enzyme was achieved by using NaCl at a concentration ranged between 0-500 mM salt. Elution buffer fed to the column at a flow rate of 3 ml/min and, protein concentrations and lipase activity of the collected fractions (**Figure 3.6**) were measured spectrophotometrically. The elution profile of the fractions was shown in **Figure 3.7**.

Figure 3.5. Packing of DEAE sepharose column.

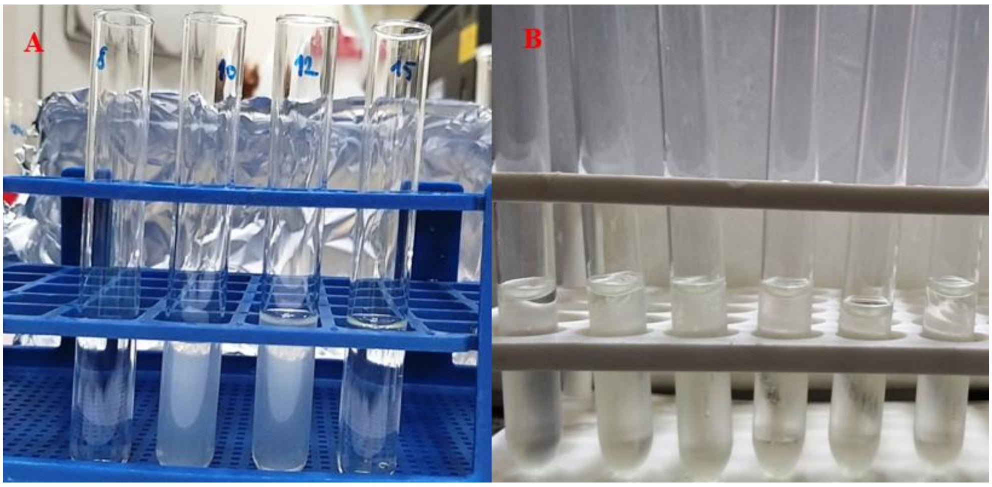

Figure 3.6. Collected fractions from ion exchange chromagraphy A) unbound fractions, B) eluted fractions with 300 mM NaCl.

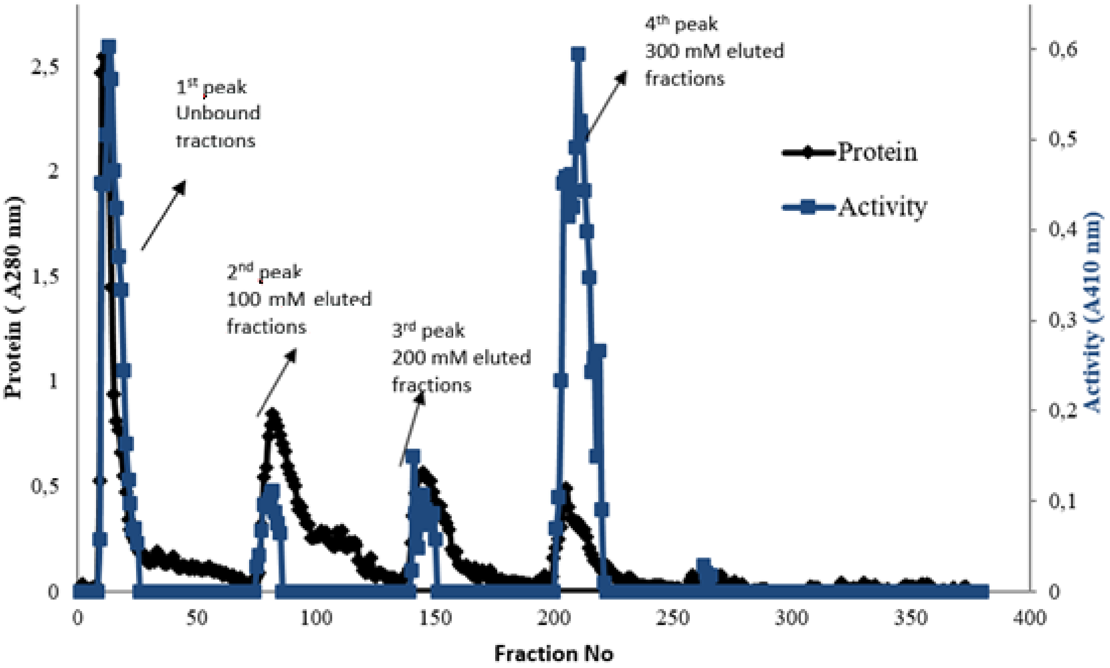

Figure 3.7. Elution profile of lipase on DEAE Sepharose column.

As seen in Figure 3.7, after DEAE sepharose chromatographic separation, four different peaks were obtained. The first peak, which is one of the highest peaks, belongs to the unbound fractions due to that they came without any sodium salt in the equilibration buffer. The second highest peak (last peak) belongs to fractions eluted with 300 mM NaCl in equilibration buffer. Second and third peaks were caught as intermediate peaks having less lipase activity and protein amount than the others.

The total lipase activity, specific activity, recovery and purification fold values at all purification steps are summarized in Table 3.6. According to Table 3.6, while the highest specific activity was obtained from the fourth peak as 177 U/mg and the second highest specific activity belongs to the first peak giving 122 U/mg value, the second and the third peaks gave 32 U/mg and 55 U/mg specific activity values, respectively. Although lipase enzyme was purified at the largest fold 0.9 from the third peak, its enzyme recovery percentage was less than the first peak, which it gave the highest recovery as 28.1%. Because second and third peaks had lower purification fold and recovery, they were eliminated for further gel filtration purification step.

Table 3.6. 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 &Dialysed & Ultrafiltrated	1206	7.09	170	76.9	0.9
DEAE 1st peak-Unbound fractions (Lip1)	440	3.60	122	28.1	0.6
DEAE 2nd peak- Eluted by 100 mM NaCl (Lip2)	52	1.64	312	3.3	0.2
DEAE 3rd peak- Eluted by 200 mM NaCl (Lip3)	60	1.09	55	3.8	0.3
DEAE 4th peak -Eluted by 300 mM NaCl (Lip4)	145	0.82	177	9.2	0.9

In the literature, when studies having similar lipase purification steps with this thesis were investigated, it was seen that Syihab et al., (2017) studied the purification of lipase from *Pseudoxanthomonas* sp. by acetone precipitation following DEAE sepharose fast flow.

They achieved that 2 different peaks eluted in 20 mM sodium phosphate buffer containing 0.4 M NaCl and 0.6 M NaCl respectively after DEAE sepharose separation. In that study, while DEAE first peak gives 3.1 purification fold and 55% enzyme recovery, the second peak gives 5.2 purification fold and 46% yield. Moreover, Jermsuntiea et al., (2011) reported that lipase purification of *M. alliacea* was resulted as 24% yield and 4.7 purification fold at the end of DEAE sepharose separation. According to the study of Ai et al., (2018), DEAE sepharose was used last purification step as polishing giving 6.2 recovery with 19.2 purification fold. Das et al., (2016) also reported higher purification fold (7.9) and yield (43.1%) for the purification of lipase from *A. tamarii* JGIF06 by using DEAE sepharose after ammonium sulfate precipitation.

To increase the purity of the enzyme, it was decided to continue with DEAE 1st peak and DEAE 4th peak for the next purification step using gel filtration chromatography.

3.3.2.2 Gel filtration chromatography

In order to increase the purity of the enzyme, size exclusion (gel fitration) chromatography was applied. After swelling process of gel filtration resin as Sephadex G-100, the resin was packed into the column (72 cm x 0.75 cm) and equilibrated with 50 mM sodium acetate buffer pH 5.6 for 24 hours (**Figure 3.8).** Lipase enzymes obtained from DEAE 1st peak denoted as Lip1 and the DEAE 4th peak denoted as Lip4 were concentrated by ultrafiltration (Amicon Millipore- UFC8010 Ultra-15 Centrifugal Filters) and then fed to the column.

Figure 3.8. Gel filtration Sephadex G-100 column system.

1.5 ml of concentrated Lip1 and Lip4 were loaded onto the column separately. After that, fractions were collected at a flow rate of 0.4 ml/min. Protein concentration of the collected fractions was measured at 280 nm spectrophotometrically. Fractions having higher 280 nm absorbance were applied to lipase activity assay and active fractions were pooled. Results were given in **Figure 3.9** and **Figure 3.10**.

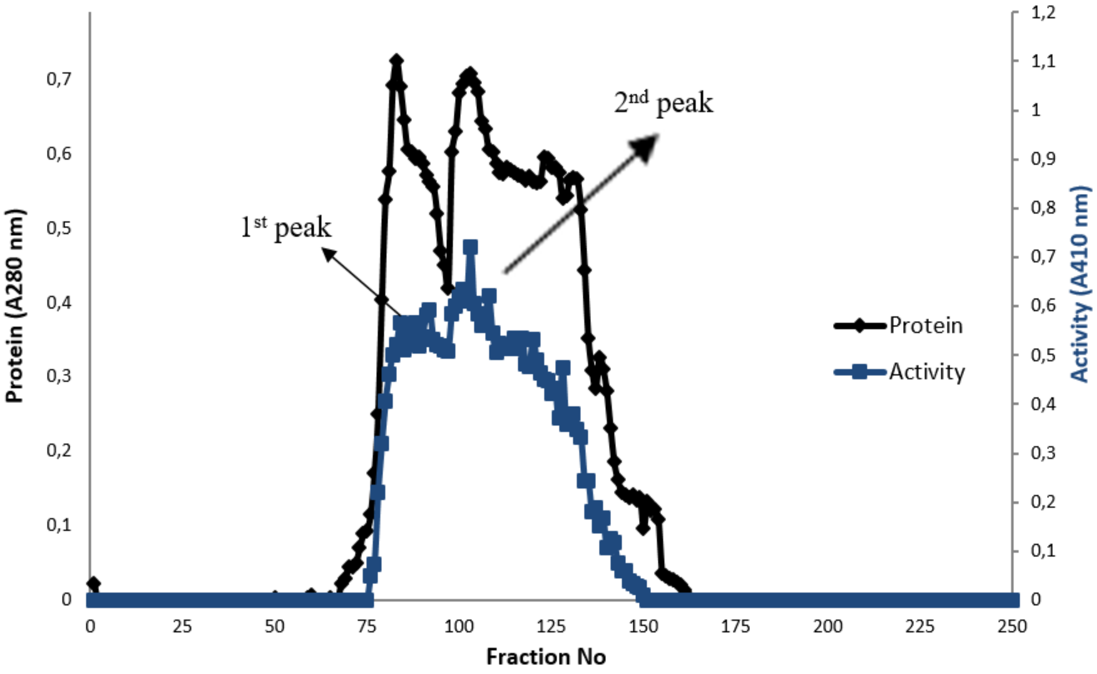

Figure 3.9. Elution profile of Lip1 on Sephadex G-100.

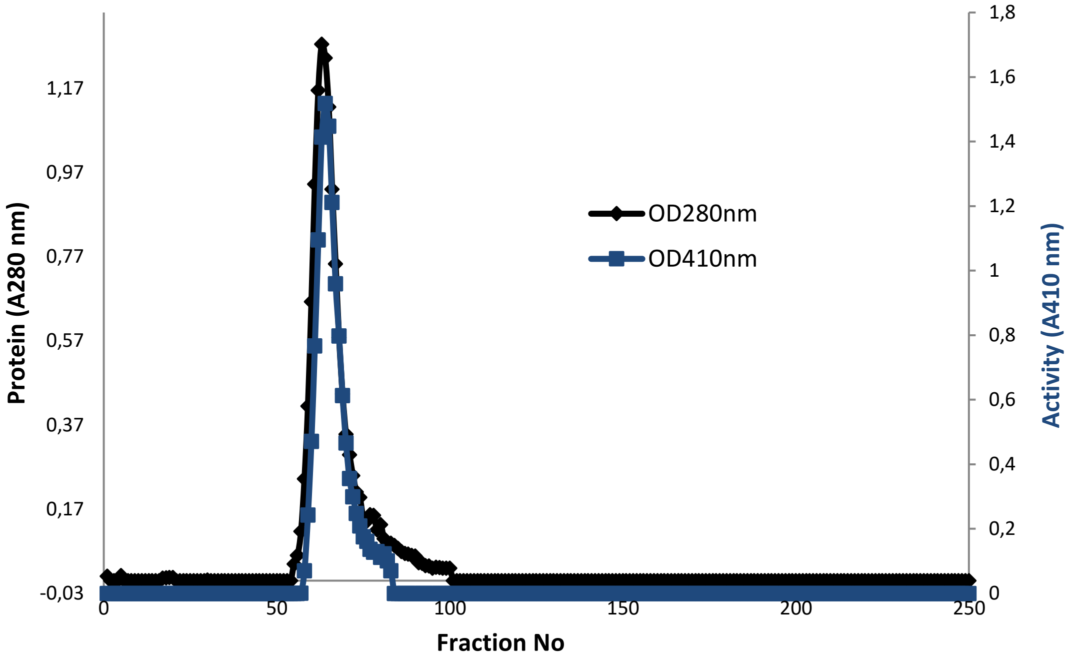

Figure 3.10. Elution profile of Lip4 on Sephadex G-100.

Lip1 on Sephadex G-100 chromatography was separated into two different peaks (denoted as Lip1-1 and Lip1-2). The first peak was between fractions 75-100, and second peak was between fractions 100 to 150. According to the enzyme activity and protein concentration of these two peaks, yields and purification folds were evaluated and results are given in Table 3.7. While the first peak from Sephadex G-100 gave 2.4% yield and 1.0 purification fold, the second peak demonstrated 7.2% yield and 0.8 purification fold. Compared to DEAE sepharose, enzyme activity recovery obviously decreased but purification fold increased.

Lip4 on Sephadex G-100 chromatography was observed in a single sharp peak (denoted as Lip 4.1). According to the enzyme activity and protein concentration of pooled fractions, yields and purification folds were evaluated (Table 3.7). Enzyme mixture coming from DEAE sepharose gave increased purification fold as 1.2 and lower enzyme activity recovery as 4.5%. After each purification step, purification was analysed by SDS-PAGE.

Table 3.7. Purification of *C. diffluens* D44 lipase after gel filtration chromatography.

Lipases Purified from DEAE Sepharose Column	**Lipases Purified from Sephadex G-100 Column**	**Total Activity U**	**Total Protein mg**	**Specific Activity U/mg**	**Yield Recovery %**	**Fold**
Unbound Fraction	Lip1	440	3.6	122	28	0.6
	1st peak (Lip1-1)	38	0.2	190	2.4	1.0
	2nd peak (Lip1-2)	113	0.74	153	7.2	0.8
4th peak	Lip 4	146	0.8	183	9.3	0.9
	Purified enzyme (Lip 4-1)	70	0.29	243.4	4.5	1.2

3.4. Partial Characterization of D44 Lipase

3.4.1. SDS-PAGE analysis

The SDS-PAGE analysis of D44 lipases after each purification step was shown in **Figure 3.11**. It can be seen from the figure that there are 2 different protein bands in lane 4 that one of them was around 75 kDa and the other one was around 50 kDa. The second band has similar MW as crude lipase from D44 which its molecular weight was found as 45.7 kDa (Yılmaz and Sayar, 2015). In lane 6, three different bands were visualized between

50-75 kDa and in lane 7, five different bands were detected at 25, 37 and 50-75 kDa. These three partially purified enzymes may be different lipases (isozymes) encoded by different genes from D44. Similar to our work, Syihab et al., (2017) reported two different lipases from *Pseudoxanthomonas* sp. having same molecular weights as 50 kDa.

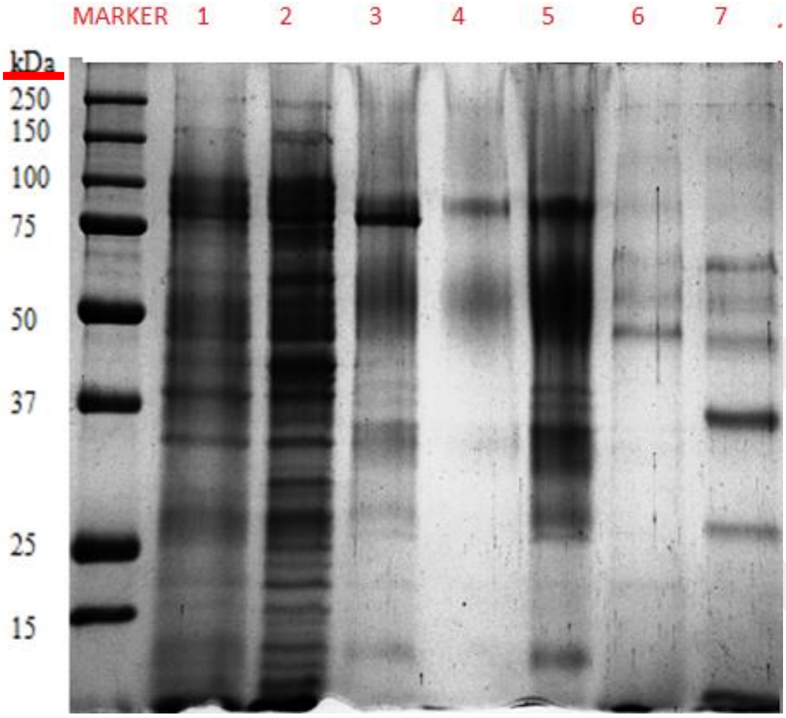

Figure 3.11. SDS-PAGE gel image represents the bands of lipases after purification steps 1) crude lipase, 2) lipase after acetone precipitation and dialysis, 3) Lip4 from DEAE sepharose chromatography, 4) Lip4-1 after gel filtration, 5) unbound fractions after DEAE sepharose chromatography, 6) Lip1-1 after gel filtration and 7) Lip1-2 after gel filtration.

Although molecular weights of yeast lipases from various strains usually vary between 33-65 KDa and yeast generally produces lipases in various isozymes (Vakhlu and Kour, 2006), molecular weight of one of the lipase purified from crude D44 lipase was 100 kDa (line 4). In literature, similar to our work, lipase produced by *V. alimentarius* LBU20907 exhibited a single band on SDS-PAGE at high molecular weight as 100 kDa (Dueramae et al., 2017).

3.4.2. Effect of temperature on lipase activity

It can be seen from Figure 3.12 that the purified lipases of *C. diffluens* D44 (Lip1-1, Lip 1-2 and Lip 4-1) were active between 30-70°C and the results revealed that the optimum temperature for Lip1-2 and Lip 4-1 was found as 65°C, while the optimum temperature for lip1-1 was 60°C (**Figure 3.12**). They showed thermophilic behaviour at these conditions. Similar to the present study, Rade et al., (2020) found the optimal temperature of lipase from *Rasamsonia emersonii* at around 65°C and Syihab et al., (2016) reported 70°C and 50°C for optimal temperature of lipases denoted as Lip1 and Lip2.

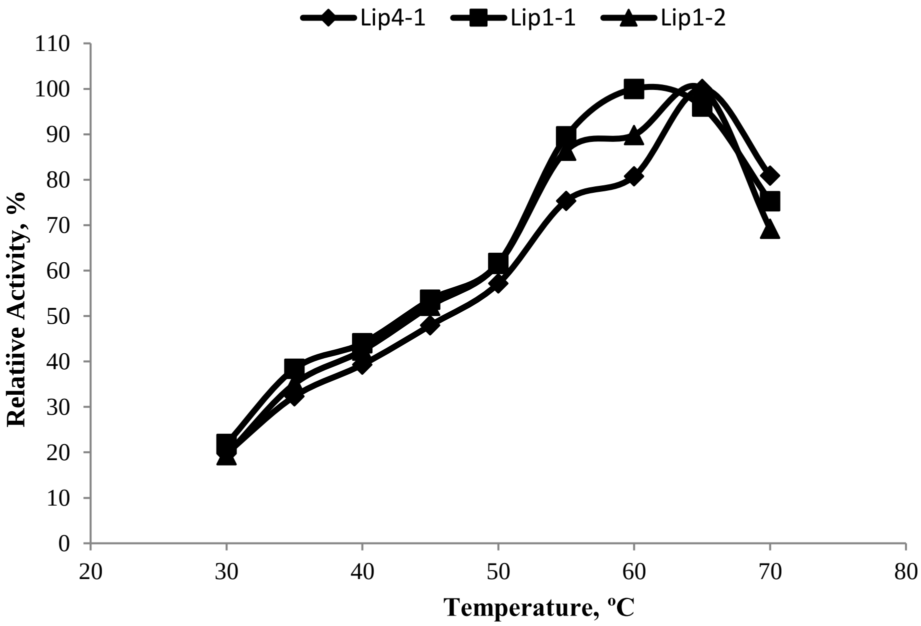

Figure 3.12. Effect of temperature on Lip1-1, Lip1-2 and Lip 4-1.

Compared the purified D44 lipases with crude D44 lipase, the optimum temperature of D44 crude lipase was 45°C which is lower than purified lipase (Yılmaz and Sayar, 2015) reported. However, de Almeida et al., (2016) reported that crude and purified lipase enzyme from *Candida viswanathii* have similar optimal temperature values as 50°C and 45°C, respectively. The difference between purified and crude enzyme could be explained the effect of the presence of olive oil in crude enzyme solution.

3.4.3. Effect of pH on lipase activity

Generally, optimal pH range of yeast lipases are variable between 4.0 – 8.0, however there are few example having optimal pH value over 9.0 (Vakhlu et al., 2006). The optimal pH of D44 lipases (Lip1-1, Lip1-2 and Lip4-1) were between pH 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 3.13**).

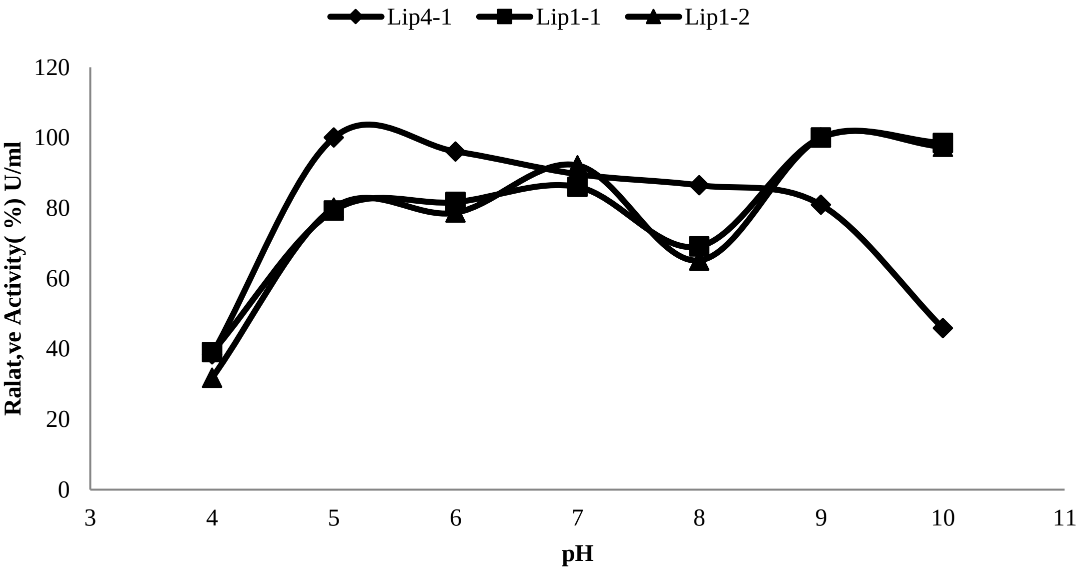

Figure 3.13. Effect of pH on Lip1-1, Lip1-2 and Lip4-1.

In the literature, different optimal pH values of lipases from yeast strains were reported. Rade et al., (2020) reported a novel lipase from the thermophilic fungus *R. emersonii* having higher enzyme activity at acidic conditions as pH 3.5 whereas, five different isolates of *A. niger* from oil seed having optimal enzyme activity at pH 7.5 were introduced. As the most important part in the study of Yılmaz and Sayar, (2015), the crude lipase enzyme from D44 has optimal pH of 9.0 which is similar to that obtained for Lip1-1 and Lip1-2 possess similar optimal pH. Additionally, Syihab et al., (2017) studied the effect of pH on two different lipases from *Pseudoxanthomonas* sp. and they found *variable* optimal pH value between these enzymes as pH of 10 and pH of 8.0 for Lip1 and Lip2, respectively.

3.4.4. Effect of temperature on lipase stability

The effect of temperature on stability of Lip1-1, Lip1-2 and Lip4-1 are shown in **Figure 3.14, 3.15 and 3.16.** It can be seen from **Figure 3.14**, at 50ºC, 20% decrease in activity of Lip1-1 was observed during the first 30 minutes. Then Lip1-1 kept its activity for 4 hours. However, at 60 ºC and 70 ºC, Lip1-1 lost 60% of its activity at 60 ºC after 2 hours of incubation and 90% of its activity during 30 minutes. Therefore, Lip1-1 is more stable at 50°C and the stability was reduced with the increasing temperature. Similar to Lip1-1, Lip1-2 and Lip4-1 showed the similar stability profile that they are stable at 50°C and their stability was decreased with the increasing temperature.

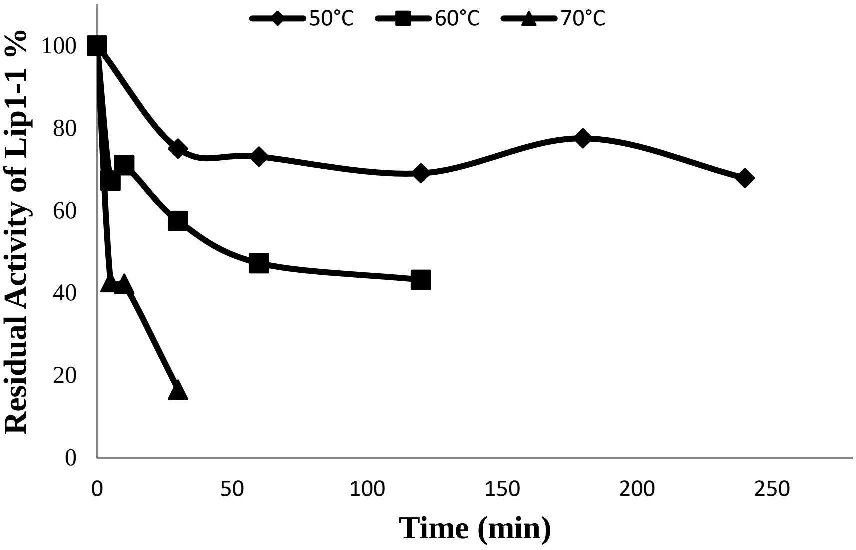

Figure 3.14. Effect of temperature on Lip1-1 stability.

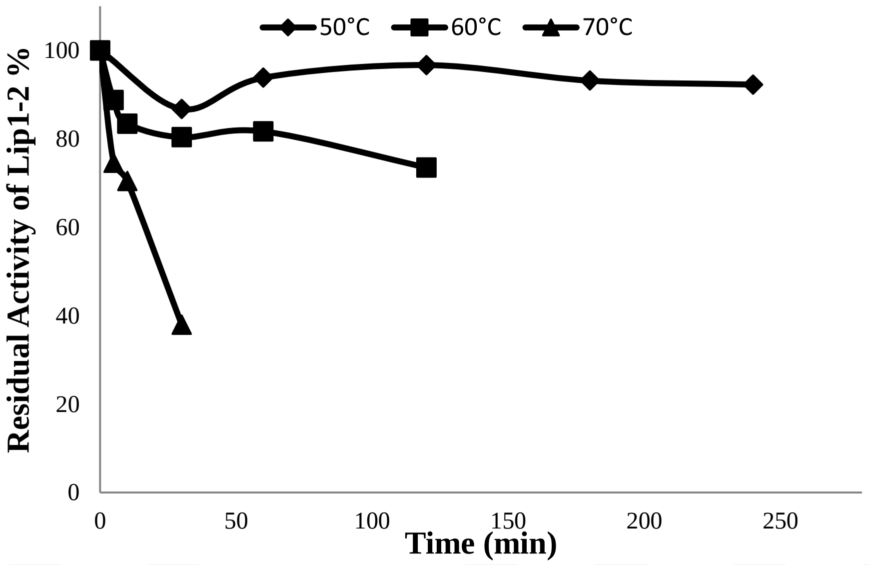

Figure 3.15. Effect of temperature on Lip1-2 stability.

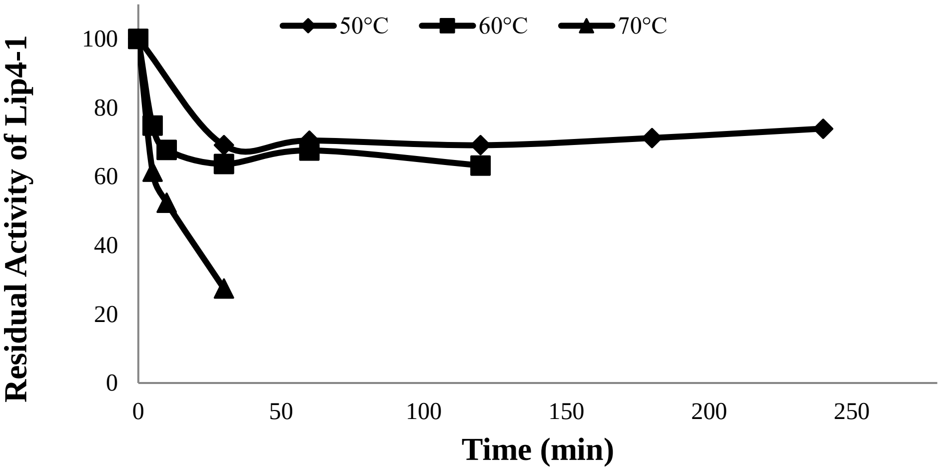

Figure 3.16. Effect of temperature on Lip4-1 stability.

In the study of Yılmaz and Sayar, (2015), crude lipase from D44 was stable at 30°C and kept 20% of its activity during 3 hours of incubation. While purified enzymes started to lose their activity at 60°C after 100 min incubation, crude lipase lost its activity after 5 minutes incubation. In another study (Almeida et al., 2016), half-life of crude and purified lipase from *C. viswanathii* at 50°C were found 23.5 h and 6.7 h at 40°C respectively. As a result, this study exhibited an opposite result when compared to the literature since purified D44 lipases are more thermo-stable compared to crude D44 lipase.

3.4.5. Activity of purified lipase in the presence of ethanol and methanol

Ethanol and methanol are organic solvents that commonly used in biofuel synthesis. Since the crude D44 lipase was used to produce biodiesel (Kula and Sayar, 2019), the effect of ethanol and methanol on purified D44 lipase were investigated.

Table 3.8 represents the organic solvent tolerance of Lip1-1 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 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 higher relative activity than control. It could be said that Lip1-1 is more resistant to methanol than ethanol.

In Table 3.9, effect of ethanol and methanol on activity of Lip1-2 was shown. As seen from Table 3.9 that Lip1-2 is stable in the presence of 10% ethanol and methanol. However, increasing ethanol at concentration from 10% to 20% caused to lose of whole Lip1-2 activity, while 27% of activity lost was observed by increasing methanol concentration from 10% to 20%.

Table 3.8. Effect of organic solvent on Lip1-1.

Organic Solvent	Relative Activity %
Control	100
10% Ethanol	83
20% Ethanol	0
10% Methanol	130
20% Methanol	115

Table 3.9. Effect of organic solvent on Lip1-2.

Organic Solvent	Relative Activity %
Control	100
10% Ethanol	119
20% Ethanol	0
10% Methanol	147
20% Methanol	73

Effect of ethanol and methanol on Lip4-1 activity was also investigated and results are shown in Table 3.10. Based on the results from that table, Lip4-1 lost 30% of their activity at 10% concentration of ethanol and 61% at 20% concentration. However, the presence of methanol did not affect as low as ethanol affected. 10% methanol gave 146% relative activity while 20% methanol was resulted 138% relative activity.

Table 3.10. Effect of organic solvent on Lip4-1.

Organic Solvent	Relative Activity %
Control	100
10% Ethanol	70
20% Ethanol	29
10% Methanol	146
20% Methanol	138

To compare all results, D44 lipases were found to have more tolerance to methanol especially Lip4-1 but, they are not resistance to ethanol at high degree except Lip1-2 at 10% concentration of ethanol. Generally organic solvents have inhibitory effects on enzyme activity by denaturation of amino acid sequences and this was proved by Hernandez-Rodriguez et al., (2009) that the activities of lipases from *Rhizopus* sp. were decreased in the presence of hydrophilic solvents as ethanol and isopropanol.

According to the study of Yılmaz and Sayar, (2015), crude *C. diffluens* D44 lipase was found as organic solvent stable and enzyme has 145.56% and 100.18% residual activity in the presence of 10% and 20% of methanol while 172.86% and 150.67% residual activity in the presence of 10% and 20% of ethanol. As seen in this study, ethanol has less negative effect on crude lipase from *C. diffluens* D44 than methanol. However, purified lipases made an impression on more methanol resistant. When compared purified lipases and crude lipase, Lip1-1 and Lip4-1 among the purified enzymes have more similar relative activities as crude lipase in the presence of methanol and Lip1-2 gave parallel residual activity with crude lipase at 10% ethanol.

3.5. Fatty Acid Sugar Ester Synthesis Using Crude Lipase

Fatty acid esters have been used in wide variety of applications such as anti-microbial agent, bio-lubricant, biosurfactant, emulsifier, biosensor, etc. Furthermore, lipase-catalyzed esters are biodegradable and eco-friendly compared to the traditionally synthesized ones (Siebenhaller et al., 2016).

In this study, sugar esters or glycolipids synthesis were achieved by crude lipase enzymes from *C. albidus* D24 and *C. diffluens* D44 based on the study of Siebenhaller et al., (2018). However, they were used commercial purified Novozyme 435 lipase as a catalyst for the production of sugar esters in honey and agave syrup as a vegan alternative. Depends on that study, 2.5 ml of concentrated crude lipases were incubated with 1.66 gr fructose, which is found the highest amount sugar content in honey and 200 µl of vinyl decanoate and vinyl octanoate in a 100 ml erlenmeyer flask at 50 rpm and 50ºC for 48 hours. After reaction, organic phase in each erlenmeyer flask was collected (**Figure 3.17**) to be subjected for analysis with TLC as shown in **Figure 3.18**.

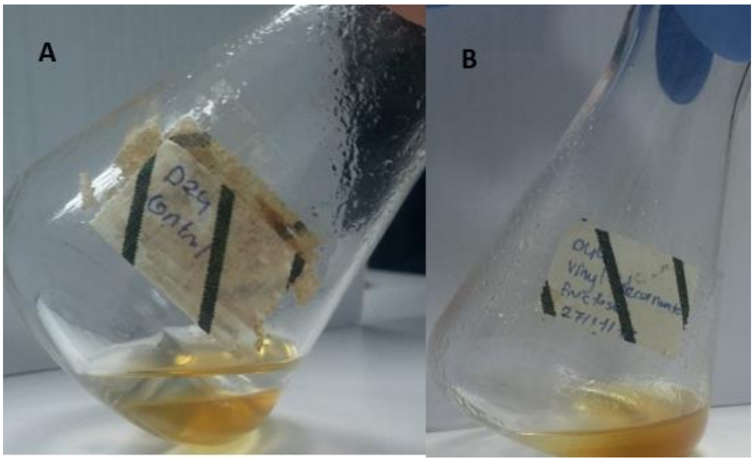

Figure 3.17. Sugar ester synthesis A) control without vinyl ester B) synthesis reaction medium containing vinyl decanoate and fructose.

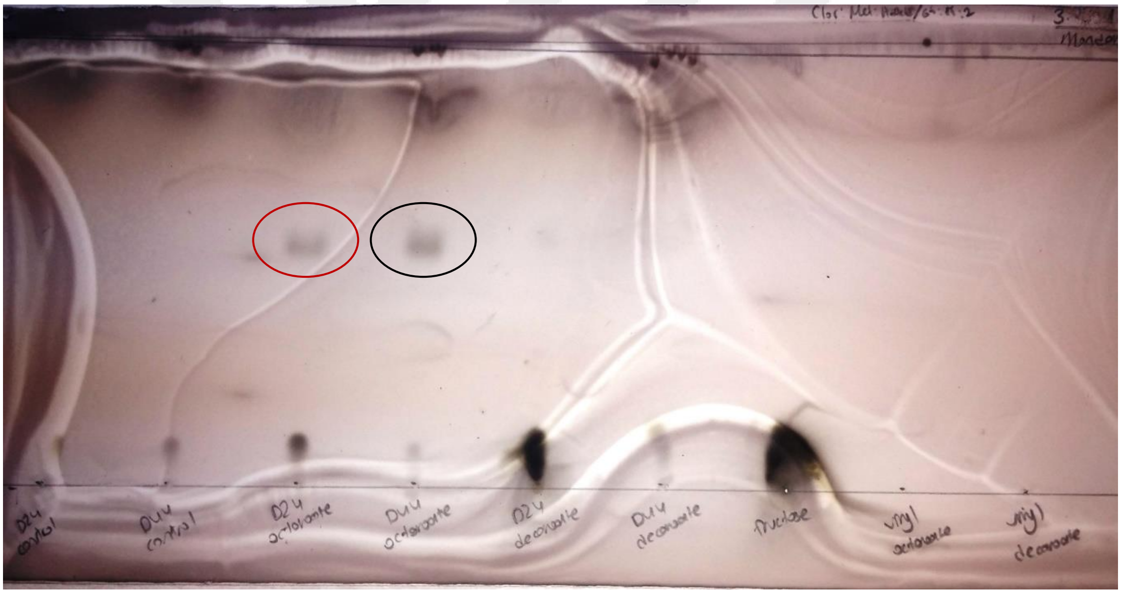

Figure 3.18. Sugar ester synthesis analysis with TLC.

After qualitative analysis of sugar esters by TLC method, two different bands were visualized in TLC plate that belong to D24 & vinyl octanoate ester reaction (red circle) and D44 & vinyl octanoate ester reaction (black circle), respectively (**Figure 3.18)**. The migration (R_f) of visualized bands on the plate were calculated divided the length of bands by the length of mobile phase in order to compare to the study of Siebenhaller et al., (2018). R_f values were found as 0.56 for both of D24 and D44 sugar esters. When they were compared to reference study (Siebenhaller et al., 2018), it revealed that they have same R_f values. Therefore, it could be said that these bands belong to fructose mono octanoate.

Briefly, using crude lipase for sugar ester synthesis was achieved successfully and it proved that using crude enzymes, that are not purified by expensive and difficult purification methods, is an environmentally friendly and more affordable way.

4. CONCLUSIONS

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 usage of lipases have opened remarkable opportunities on biotechnological applications for both research and industry. Based on that, it is of great importance to identify microorganisms in which lipases are produced, to reveal their biochemical reaction functions, to examine their mechanisms of action and their kinetic properties in detail. In order to understand the structural and conformational integrity of lipases, they are purified.

In this thesis, it was presented that the purification and characterization of lipase from *C. albidus* D24 and *C. diffluens* D44 isolated from petroleum sludge. Since lipase from *C. albidus* D24 affected by ammonium sulfate and acetone, it was not purified efficiently in the preliminary step of purification. However, lipase from *C. diffluens* D44 was purified by acetone precipitation, DEAE sepharose anion exchange and Sephadex G-100 gel filtration chromatography, respectively. It was resulted in partially purified three types of lipases from *C. diffluens* D44 as Lip1-1, Lip1-2 and Lip4-1. Their purification folds were found as 1.0, 0.8 and 1.2 together with low enzyme recovery of 2.4%, 7.2% and 4.5%, respectively. These lipases were characterized in terms of optimum temperature, optimum pH, thermo stability and organic solvent tolerance. Consequently, while Lip1-1 had the highest activity at 60ºC, optimum temperature of Lip1-2 and Lip4-1 was found at 65ºC. Optimal pH was determined for three lipases as 9.0 for Lip1-1 and Lip1-2 and 5.0 for Lip4-1. They were strongly resistant to high temperature between 50ºC and 60ºC. Therefore, 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. The synthesis of fructose mono octanoate from crude lipase of *C. albidus* D24 and *C. diffluens* D44 was also achieved based on TLC analysis in this study.

To evaluate the purification results, relatively lower purity and yield were obtained by these methods when compared to literature. Therefore, in further studies, lower salt concentration of equilibration and binding buffers used in anion exchange chromatography or different ion exchange chromatography having more ion strength in order to capture lipases on column than DEAE sepharose. Besides that, new purification strategies such as aqueous two phase partitioning, three phase partitioning, or reverse micellar system could be used to purify lipases in high purification fold and high yield with less purification steps. To identify the partially purified lipases from *C. diffluens* D44, the related bands on SDS-PAGE gel can be cut and treated with trypsin enzyme therefore, their peptide fragments can be observed with MALDI-MS analysis. By comparing with the information in the protein tools, structural information about the enzyme can be obtained. Furthermore, synthesized sugar esters can be purified using flash chromatography and characterized by Q-ToF mass spectrometry.

REFERENCES

Adrio, J. L., Demain, A. L. (2014) Microbial enzymes: tools for biotechnological processes. Biomolecules 4:117–139.

Afsar, A., Cetinkaya, F. (2008) A research on increasing the effectiveness of degreasing process by using enzymes. Tekst Konfeksiyon 18:278–283.

Ahmed, E. H., Raghavendra, T., Madamwar, D. (2010) An alkaline lipase from organic solvent tolerant *Acinetobacter* sp. EH28: application for ethyl caprylate synthesis. Bioresource Technology, 101:3628–3634.

Ai, L., Huang, Y., Wang, C. (2018) Purification and characterization of halophilic lipase of *Chromohalobacter* sp. from ancient salt well. Journal of Basic Microbiology, 58(8), 647–657.

Akoh C. C., Min, D. B. (2002) Food Lipids: Chemistry, Nutrition, and Biotechnology, 2nd Edition, 269-270.

Al-Zuhair, S. (2011). Biochemical catalytic production of biodiesel. In Handbook of Biofuels Production: Processes and Technologies (pp. 134–159). Elsevier Inc.

Anbu, P. (2013) Characterization of an Extracellular Lipase by *Pseudomonas koreensis* BK-L07 Isolated from Soil. Preparative Biochemistry and Biotechnology 44(3): 266-280.

Anggriani, L., Budiarti, S. R. I., Mubarik, N. R. (2020) Lipase-producing ability of bacteria from inasua (Fish fermented product) from Central Moluccas, Indonesia. Biodiversitas, 21(2), 622–628.

Aqeel, Sultan. M., Javaid, Asad, M., Tahir, Mahmood, R., Mahmood, M., Nazir, S., Basir, Shah, M. (2020) Purification and characterization of lipase produced by indigenously isolated bacteria. Pure and Applied Biology, 9(4), 2521–2528.

Arai, T., Yusa, S., Kirimura, K., Usami, S. (2006) Erratum to: "Cloning and sequencing of the cDNA encoding lipase I from *Trichosporon fermentans* WU-C12." FEMS Microbiology Letters.

Arora, N. K., Mishra, J., Mishra, V. (2020) Microbial Enzymes: Roles and Applications in Industries. Microbial Enzymes : Roles and Applications in Industries (Vol. 11). Springer Singapore: Singapore.

Ayaz, B., Ugur, A., Boran, R. (2015) Purification and characterization of organic solvent-tolerant lipase from *Streptomyces* sp. OC119-7 for biodiesel production. Biocatalysis and Agricultural Biotechnology, 4 (1): 103–108.

Bailey, J. E., Ollis, D. F. (1986) Applied enzyme catalysis. In:Biochemical engineering fundamentals, 2nd Edition. McGraw Hill, New York, NY, pp 157-227.

Barbe, S., Lafaquière, V., Guieysse, D., Monsan, P., Remaud-Siméon, M., André, I. (2009) Insights into lid movements of *Burkholderia cepacia* lipase inferred from molecular dynamics simulations. Proteins: Structure, Function, and Bioinformatics, 77(3), 509–523.

Barros, M., Fleuri, L. F., MacEdo, G. A. (2010) Seed lipases: Sources, applications and properties - A review. Brazilian Journal of Chemical Engineering. Assoc. Brasiliera de Eng. Quimica / Braz. Soc. Chem. Eng.

Benjamin, S., Pandey, A. (2001) Isolation and characterization of three distinct forms of lipases from *Candida rugosa* produced in solid state fermentation. Brazilian Archives of Biology and Technology.

Bharathi, D., Rajalakshmi, G. (2019) Microbial lipases: An overview of screening, production and purification. In Biocatalysis and Agricultural Biotechnology (Vol. 22, p. 101368). Elsevier Ltd.

Bindiya, P. A., Ramana, T. (2014) Optimization of lipase production from an indigenously isolated marine *Aspergillus sydowii* of Bay of Bengal. Journal of Biochemical Technology 3(5): 203-211.

Boran, R., Ugur, A., Sarac, N., Ceylan, Ö. (2019) Characterization of *Streptomyces violascens* OC125-8 lipase for oily wastewater treatment. 3 Biotech, 9, 5.

Böer, E., Mock, H. P., Bode, R., Gellissen, G., Kunze, G. (2005) An extracellular lipase from the dimorphic yeast *Arxula adeninivorans*: Molecular cloning of the ALIP1 gene and characterization of the purified recombinant enzyme.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1–2), 248–254.

Cao, X., Liao, L., Feng, F. (2020) Purification and characterization of an extracellular lipase from *Trichosporon* sp. and its application in enrichment of omega-3 polyunsaturated fatty acids. LWT, 118, 108692.

Carvalho, A., Fonseca, T., Mattos, M., Oliveira, M., Lemos, T., Molinari, F., Serra, I. (2015) Recent Advances in Lipase-Mediated Preparation of Pharmaceuticals and Their Intermediates. International Journal of Molecular Sciences, 16(12), 29682–29716.

Chandra, P., Enespa, Singh, R., Arora, P.K. (2020) Microbial lipases and their industrial applications: a comprehensive review. Microb Cell Fact. 19:169.

Charcosset, C. (2011) Comprehensive Biotechnology. In Membrane Systems and Technology, 2nd edition; Moo-Young, M., ed.; Academic Press: Burlington, MA, USA. 603–618.

Dalmau, E., Montesinos, J. L., Lotti, M., Casas, C. (2000) Effect of different carbon sources on lipase production by *Candida rugosa*. In Enzyme and Microbial Technology (Vol. 26, pp. 657–663). Elsevier Science Inc.

Darvishi, F., & Hosseini, B. (2015) Biological Journal of Microorganism (Vol. 4). University of Isfahan.

Das, A., Shivakumar, S., Bhattacharya, S., Shakya, S., Swathi, S. S. (2016) Purification and characterization of a surfactant-compatible lipase from *Aspergillus tamarii* JGIF06 exhibiting energy-efficient removal of oil stains from polycotton fabric. 3 Biotech, 6(2), 131.

Dave, R., Madamwar, D. (2010) Preparations for the use of *Candida rugosa* lipase in non-conventional solvents. Biocatalysis and Biotransformation. Taylor & Francis.

De Almeida, A. F., Dias, K. B., Da Silva, A. C. C., Terrasan, C. R. F., Tauk-Tornisielo, S. M., Carmona, E. C. (2016) Agroindustrial Wastes as Alternative for Lipase Production by *Candida viswanathii* under Solid-State Cultivation: Purification, Biochemical Properties, and Its Potential for Poultry Fat Hydrolysis. Enzyme Research.

de Meneses, A. C., Lerin, L. A, Araújo P. H. H., Sayer, C., de Oliveira, D. (2019) Benzyl propionate synthesis by fed-batch esterification using commercial immobilized and lyophilized Cal B lipase. Bioprocess Biosyst Eng 42(10):1625–1634.

Demuner, B. J., Pereira, J. N., Antunes, A. (2011) Technology prospecting on enzymes for the pulp and paper industry. J Technol Manag Innov 6:148–158.

Dueramae, S., Bovornreungroj, P., Enomoto, T., Kantachote, D. (2017) Purification and characterization of an extracellular lipolytic enzyme from the fermented fish-originated halotolerant bacterium, *Virgibacillus alimentarius* LBU20907. *Chemical Papers*, *71*(10), 1975–1984.

Divakar, S., Manohar, B. (2007) Use of lipases in the industrial production of esters. In Industrial Enzymes: Structure, Function and Applications (pp. 283–300). Dordrecht: Springer Netherlands.

Enzymes Market by Product Type (Industrial Enzymes and Specialty Enzymes), Source (Microorganism, Plant, and Animal), Type, Industrial Enzyme Application, Specialty Enzymes Application, and Region – Global Forecast to 2025. (Retrieved August 12, 2020, from https://www.researchandmarkets.com/reports/5009185/enzymes-market-by-product-type-industrial?utm_source=dynamic&utm_medium=GNOM&utm_code=lb9cnl&utm_campaign=1375386+-+Global+Enzymes+Market+(2019+to+2025)+-+Demand+for+An+Alternative+to+Synthetic+Chemicals+Presents+Opportunities&utm_exec=jamu273gnomd.

Enzymes Market Size, Share | Global Industry Report, 2020-2027. Retrieved August 12, 2020, from https://www.grandviewresearch.com/industry-analysis/enzymes-industry.

Erarslan, A., Kazan D. (1998). Enzimlerin Saflşatırılması ve Nitelendirilmesinde Temel Yöntemler. TÜBİTAK, Marmara Araştırma Merkezi Gen Mühendisliği ve Biyoteknoloji Araştırma Enstitüsü Enzim ve Fermentasyon Teknolojisi Grubu, 37 – 50.

Fukaya, T., Lim, B., & Levine, M. (2016) Enhancer Control of Transcriptional Bursting. *Cell*, *166*(2), 358–368.

Geoffry, K., Achur, R. N. (2018) Screening and production of lipase from fungal organisms. Biocatalysis and Agricultural Biotechnology. Elsevier Ltd.

Gilham, D., Lehner, R. (2005) Techniques to measure lipase and esterase activity in vitro. Methods, 36(2 SPEC. ISS.), 139–147.

Guerrand, D. (2017) Lipases industrial applications: Focus on food and agroindustries. In OCL - Oilseeds and fats, Crops and Lipids (Vol. 24, p. D403). EDP Sciences.

Gumel, A. M., Annuar, M. S. M., Heidelberg, T., Chisti, Y. (2011) Lipase mediated synthesis of sugar fatty acid esters. Process Biochemistry. Elsevier.

Gupta, N., Rathi, P., Gupta, R. (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. Analytical Biochemistry, 311(1), 98–99.

Gururaj, P., Ramalingam, S., Nandhini Devi, G., Gautam, P. (2016) Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from *Acinetobacter* sp. AU07. Braz. J. Microbiol., 47(3): 647–657.

Gutiérrez, A, del Rio, J. C., Martinez, M. J., Martinez, A. T. (2001) The biotechnological control of pitch in paper pulp manufacturing. Trends Biotechnology 19:340–348.

Hasan, F., Shah, A. A., Hameed, A. (2006) Industrial applications of microbial lipases. Enzyme and Microbial Technology, 39(2), 235–251.

Hube, B., Stehr, F., Bossenz, M., Mazur, A., Kretschmar, M., Schäfer, W. (2000) Secreted lipases of *Candida albicans*: Cloning, characterization and expression analysis of a new gene family with at least ten members. Archives of Microbiology, 174(5), 362–374.

Iftikhar, T., Niaz, M., Anwer, M., Qaiser Abbas, S., Saleem, M., & Jabeen, R. (2011) Ecological Screening of Lipolytic Cultures and Process Optimization for Extracellular Lipase Production from Fungal Hyperproducer. Pak. J. Bot (Vol. 43).

Jermsuntiea, W., Aki, T., Toyoura, R., Iwashita, K., Kawamoto, S., Ono, K. (2011) Purification and characterization of intracellular lipase from the polyunsaturated fatty acid-producing fungus *Mortierella alliacea*. New Biotechnol 28:158–164.

Kademi, A., Lee, B., Houde, A. (2003) Production of Heterologous Microbial Lipases by Yeasts. Indian Journal of Biotechnology, Volume 2.

Kakugawa, K., Shobayashi, M., Suzuki, O., Miyakawa, T. (2002) Purification and characterization of a lipase from the glycolipid-producing yeast *Kurtzmanomyces* sp. I-11. Bioscience, Biotechnology and Biochemistry.

Kamini, N. R., Fujii, T., Kurosu, T., Iefuji, H. (2000) Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp. S-2. Process Biochemistry, 36(4), 317–324.

Khan, F. I., Lan, D., Durrani, R., Huan, W., Zhao, Z., Wang, Y. (2017) The lid domain in lipases: Structural and functional determinant of enzymatic properties. Frontiers in Bioengineering and Biotechnology. Frontiers Media S.A.

Kishan, G., Gopalakannan, P., Muthukumaran, C., Thirumalai, K., Muthukumaresan, M., Dharmendira, K., Tamilarasan, K. (2013) Statistical optimization of critical medium components for lipase production from *Yarrowia lipolytica* (MTCC 35). Journal of Genetic Engineering and Biotechnology 11(2): 111-116.

Kula, C., Sayar, N. A. (2019) Multi-objective optimization of a novel crude lipase-catalyzed fatty acid methyl ester (FAME) production using low-order polynomial and Kriging models. International Journal of Green Energy, 16(8), 657–665.

Kumar, A., Dhar, K., Kanwar, S. S., Arora, P. K. (2016) Lipase catalysis in organic solvents: Advantages and applications. Biological Procedures Online. BioMed Central Ltd.

Kumari, A., Gupta, R. (2012) Purification and Biochemical Characterization of a Novel Magnesium Dependent Lipase from *Trichosporon asahii* MSR 54 and its Application in Biodiesel Production. Asian Journal of Biotechnology, 4(2), 70–82.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259), 680–685.

Li, M., Zhang, H., Wang, J. xin, Pan, Y. (2012) A new essential protein discovery method based on the integration of protein-protein interaction and gene expression data. *BMC Systems Biology*, *6*(1), 15.

Massadeh, M., Sabra, F., Dajani, R., Arafat, A. (2012) Purification of Lipase Enzyme Produced by *Bacillus stearothermophilus* HU1. International Conference on Eco-Systems and Biological Sciences.

Melani, N. B., Tambourgi, E. B., Silveira, E. (2020) Lipases: From Production to Applications. Separation and Purification Reviews. Taylor and Francis Inc.

Navarro del Hierro, J., Gutiérrez-Docio, A., Otero, P., Reglero, G., Martin, D. (2020) Characterization, antioxidant activity, and inhibitory effect on pancreatic lipase of extracts from the edible insects Acheta domesticus and Tenebrio molitor. Food Chemistry, 309, 125742.

Neugnot, V., Moulin, G., Dubreucq, E., Bigey, F. (2002) The lipase/acyltransferase from *Candida parapsilosis*. European Journal of Biochemistry, 269(6), 1734–1745.

Nerurkar, M., Joshi, M., Adivarekar, R., (2015) Bioscouring of cotton using lipase from marine bacteria *Bacillus sonorensis*. Appl Biochem Biotechnol 175:253–265.

Nomura, D. K., & Casida, J. E. (2016) Lipases and their inhibitors in health and disease.

Oishi, H., Morimoto, T., Watanabe, Y., Tamat, Y. (1999) Purification and characterization of phospholipase B from *Kluyveromyces lactis*, and cloning of phospholipase B gene. Bioscience, Biotechnology and Biochemistry, 63(1), 83–90.

Ota, Y., Sawamoto, T., Hasuo, M. (2000) Tributyrin specifically induces a lipase with a preference for the sn-2 position of triglyceride in *Geotrichum* sp. FO401B. Bioscience, Biotechnology and Biochemistry, 64(11), 2497–2499.

Pignède, G., Wang, H., Fudalej, F., Gaillardin, C., Seman, M., Nicaud, J. M. (2000) Characterization of all extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. Journal of Bacteriology, 182(10), 2802–2810.

Paiva, A. L., Balcão, V. M., Malcata, F. X. (2000) Kinetics and mechanisms of reactions catalyzed by immobilized lipases.

Patel, R. N. (2008) Synthesis of chiral pharmaceutical intermediates by biocatalysis. Coordination Chemistry Reviews 252(5): 659-701.

Pérez, M. M., Gonçalves, E. C. S., Vici, A. C., Salgado, J. C. S., de Moraes Polizeli, M. de L. T. (2019) Fungal Lipases: Versatile Tools for White Biotechnology (pp. 361–404).

Perfect, J. R., Casadevall, A. (2011) The History of *Cryptococcus* and *Cryptococcosis* . In *Cryptococcus* (pp. 17–26). American Society of Microbiology, 17-18.

Priji, P., K. N. Unni, S. Sajith, P. Binod and S. Benjamin (2015) Production, optimization, and partial purification of lipase from *Pseudomonas* sp. strain BUP6, a novel rumen bacterium characterized from Malabari goat. Biotechnol Appl Biochem 62(1): 71-78.

Rajashekharappa, S. (2017) Reverse Micelles as a Bioseparation Tool for Enzymes. Article in Journal of Proteins and Proteomics.

Rehman., Maqsood UR. (2020) Purification and Characterization of Lipase from Psychrophilic Bacteria *Pseudomonas Mandelii* HTB2 from Batura Glacier, Pakistan.

Ren, K., Lamsal, B. P. (2017) Synthesis of some glucose-fatty acid esters by lipase from *Candida antarctica* and their emulsion functions.

Rotticci-Mulder, J. C., Gustavsson, M., Holmquist, M., Hult, K., Martinelle, M. (2001) Expression in *Pichia pastoris* of *Candida antarctica* lipase B and lipase B fused to a cellulose-binding domain. Protein Expression and Purification, 21(3), 386–392.

Salihu, A. and M. Z. Alam (2012) Production and applications of microbial lipases: a review. Scientific Research and Essays 7(30): 2667-2677.

Salleh, A. B., Abdul Rahman, N. Z. R., Basri, M. (2006) New Lipases and Proteases. Nova Science Pub Inc; 1st Edition.

Sangeetha, R., Arulpandi, I., Geetha, A. (2011) Bacterial lipases as potential industrial biocatalysts: An overview. Research Journal of Microbiology, 6(1), 1–24.

Sandoval, G., Herrera-López, E. J. (2018) Lipase, phospholipase, and esterase biosensors (review). In: Sandoval G (ed) Lipases and phospholipases. Methods in molecular biology, vol 1835. Humana Press, New York, NY.

Sanchez, S., Demain, A. L., (2017) Useful microbial enzymes – an introduction. Biotechnology of microbial enzymes. Elsevier, Amsterdam, pp 1–11.

Saraswat, R., Bhushan, I., Gupta, P., Kumar, V., Verma, V. (2018) Production and purification of an alkaline lipase from *Bacillus* sp. for enantioselective resolution of (±)-Ketoprofen butyl ester. 3 Biotech, 8, 491.

Saxena, R. K., Sheoran, A., Giri, B., Davidson, W. S. (2003) Purification strategies for microbial lipases. Journal of Microbiological Methods. Elsevier.

Satyanarayana, T., Kunze, G. (2009) Yeast biotechnology: diversity and applications, Springer, 1st Edition. Dordrecht, Netherlands.

Sharma, R., Chisti, Y., Chand Banerjee, U. (2001) Production, purification, characterization, and applications of lipases. Biotechnology Advances (19) 627 – 662.

Show, P.-L., Ling, T.-C., C.-W. Lan, J., Tey, B.-T., N. Ramanan, R., Yong, S.-T., Ooi, C.-W. (2015) Review of Microbial Lipase Purification Using Aqueous Two-phase Systems. Current Organic Chemistry, Volume 19.

Siebenhaller, S., Hajek, T., Muhle-Goll, C., Himmelsbach, M., Luy, B., Kirschhöfer, F., Syldatk, C. (2017) Beechwood carbohydrates for enzymatic synthesis of sustainable glycolipids. Bioresources and Bioprocessing, 4(1), 25.

Siebenhaller, S., Gentes, J., Infantes, A., Muhle-Goll, C., Kirschhöfer, F., Brenner-Weiß, G., Syldatk, C. (2018) Lipase-Catalyzed Synthesis of Sugar Esters in Honey and Agave Syrup. Frontiers in Chemistry, 6, 24.

Singh, A. K., Mukhopadhyay, M. (2012) Overview of fungal lipase: a review. Applied biochemistry and biotechnology 166(2): 486-520.

Singh, R., Kumar, M., Mittal, A., Mehta, P. K. (2016) Microbial enzymes: industrial progress in 21st century. 3 Biotech. Springer Verlag.

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., Tripathi, C. (2017) Strategies for Fermentation Medium Optimization: An In-Depth Review. Frontiers in microbiology 7: 2087.

Singh, R. S., Singh, T., Singh, A. K. (2019) Enzymes as diagnostic tools. In Biomass, Biofuels, Biochemicals: Advances in Enzyme Technology (pp. 225–271). Elsevier.

Singh, P., Patel, V., Shah, V., Madamwar, D. (2019) A Solvent-tolerant Alkaline Lipase from *Bacillus* sp. DM9K3 and Its Potential Applications in Esterification and Polymer Degradation. Applied Biochemistry and Microbiology, 55(6), 603–614.

Sivaramakrishnan, R., Incharoensakdi, A. (2016) Purification and characterization of solvent tolerant lipase from *Bacillus* sp. for methyl ester production from algal oil. Journal of Bioscience Bioengineering, 121(5): 517–522.

Somashekar, B. R., Lohith, K., Manohar, B., Divakar, S. (2007) Inhibition of *Rhizomucor miehei* and *Candida rugosa* lipases by d-glucose in esterification between l-alanine and d-glucose. Journal of Bioscience and Bioengineering, 103(2), 122–128.

Starodub, N. F. (2006) Biosensors for the evaluation of lipase activity. J Mol Catal B: Enzym 40(3):155–160.

Staroń, J., Dąbrowski, J. M., Cichoń, E., Guzik, M. (2018) Lactose esters: synthesis and biotechnological applications. Critical Reviews in Biotechnology. Taylor and Francis Ltd.

Sumarsih, S., Hadi, S., Andini, D. G. T., Nafsihana, F. K. (2019) Carbon and Nitrogen Sources for Lipase Production of *Micrococcus* sp. Isolated from Palm Oil Mill Effluent-Contaminated Soil. In IOP Conference Series: Earth and Environmental Science (Vol. 217, p. 12029). Institute of Physics Publishing.

Sun, Y., Shi, Q.H., Zhang, L., Zhao, G.F., Liu, F.F. (2011) Adsorption and Chromatography. In Comprehensive Biotechnology Adsorption and Chromatography, 2nd ed.; Moo-Young, M., ed.; Academic Press: Burlington, MA, USA. 665–679.

Syihab, F. S., Fida, M., Akhmaloka, A., Made, P. W. (2017) Purification and characterization of thermostable and alcohol tolerant lipase from *Pseudoxanthomonas* sp. African Journal of Biotechnology, 16(31), 1670–1677.

Palmer, T., Boner, P. L. (2008) Enzymes Biotechnology Handbook: Enzymes in Biotechnology, Enzymes in NIIR Board of Consultants & Engineers, 2nd Edition., UK.

Reis, P., Holmberg, K., Watzke, H., Leser, M. E., Miller, R. (2009) Lipases at interfaces: A review. Advances in Colloid and Interface Science.

Tan, T., Lu, J., Nie., K., Deng, L., Wang, F. (2010) Biodiesel production with immobilized lipase: A review. Biotechnology Advances.

Trojanowicz, M. (2014) Enantioselective electrochemical sensors and biosensors: a mini-review. Electrochemical Communication, 8:47–52.

Turati, D. F. M., Almeida, A. F., Terrone, C. C., Nascimento, J. M. F., Terrasan, C. R. F., Fernandez-Lorente, G., Pessela, B. C., Guisan, J. M., Carmona, E. C. (2019) Thermotolerant lipase from *Penicillium* sp. section Gracilenta CBMAI 1583: Effect of carbon sources on enzyme production, biochemical properties of crude and purified enzyme and substrate specificity. Biocatalysis and Agricultural Biotechnology, 17, 15–24.

Ünlüer, O. B., Özcan, A., Uzun, L. (2014) Preparation of a novel hydrophobic affinity cryogel for adsorption of lipase and its utilization as a chromatographic adsorbent for fast protein liquid chromatography. Biotechnology Program 30(2): 376–382.

Vakhlu, J., Kour, A. (2006) Yeast lipases: enzyme purification, biochemical properties and gene cloning. Electronic Journal of Biotechnology, 9(1), 717–3458.

Vishnoi, N., Dixit, S., Mishra, J. (2020) Microbial Lipases and Their Versatile Applications (pp. 207–230). Springer, Singapore.

Yalçın, H. T., Çorbacı, C., and Ucar, F. B. (2014) Molecular characterization and lipase profiling of the yeasts isolated from environments contaminated with petroleum. Journal of Basic Microbiology, 54 Supply 1: S85-92.

Yang, F., Weber, T. W., Gainer, J. L., & Carta, G. (1997) Synthesis of lovastatin with immobilized *Candida rugosa* lipase in organic solvents: Effects of reaction conditions on initial rates. Biotechnology and Bioengineering, 56(6), 671–680.

Yılmaz, D. E., Sayar N.A. (2015) rganic solvent stable lipase from *Cryptococcus diffluens* D44 isolated from petroleum sludge. Journal of Molecular Catalysis B: Enzymatic 122: 72-79.

Zehani, N., Dzyadevych, S. V., Kherrat, R., Jaffrezic-Renault, N. J. (2014) Sensitive impedimetric biosensor for direct detection of diazinon based on lipases. Front Chem 2:44.

Zhekova, B., Dobrev, G. (2019) Isolation and purification of lipase produced from *Rhizopus arrhizus* in solid state fermentation by fractional precipitation. Bulgarian Chemical Communications, Volume 51, Special Issue D (pp. 184 – 188).

APPENDICES

Appendix A

Table A.1. Raw data of the calibration curve using as p-NPP substrate

Buffer volume (ml)	Sample Volume (ml)	µg p-NP	µmol p-NP	OD410	OD410	OD410	Mean	µM p-NP(µmol /ml)
0	0	0	0	0	0	0	0.00	0.00
2	0.1	41.733	0.3	0.086	0.089	0.093	0.09	0.13
2	0.1	83.466	0.6	0.181	0.174	0.175	0.18	0.27
2	0.1	125.199	0.9	0.264	0.254	0.264	0.26	0.40
2	0.1	166.932	1.2	0.363	0.362	0.362	0.36	0.53
2	0.1	208.665	1.5	0.462	0.461	0.458	0.46	0.67
2	0.1	250.398	1.8	0.528	0.53	0.529	0.53	0.80
2	0.1	292.131	2.1	0.598	0.6	0.611	0.60	0.93
2	0.1	333.864	2.4	0.708	0.714	0.702	0.71	1.07
2	0.1	375.597	2.7	0.752	0.781	0.787	0.77	1.20
2	0.1	417.33	3	0.88	0.872	0.862	0.87	1.33
2	0.1	459.063	3.3	0.943	0.952	0.969	0.95	1.47
2	0.1	500.796	3.6	1.047	1.04	1.026	1.04	1.60

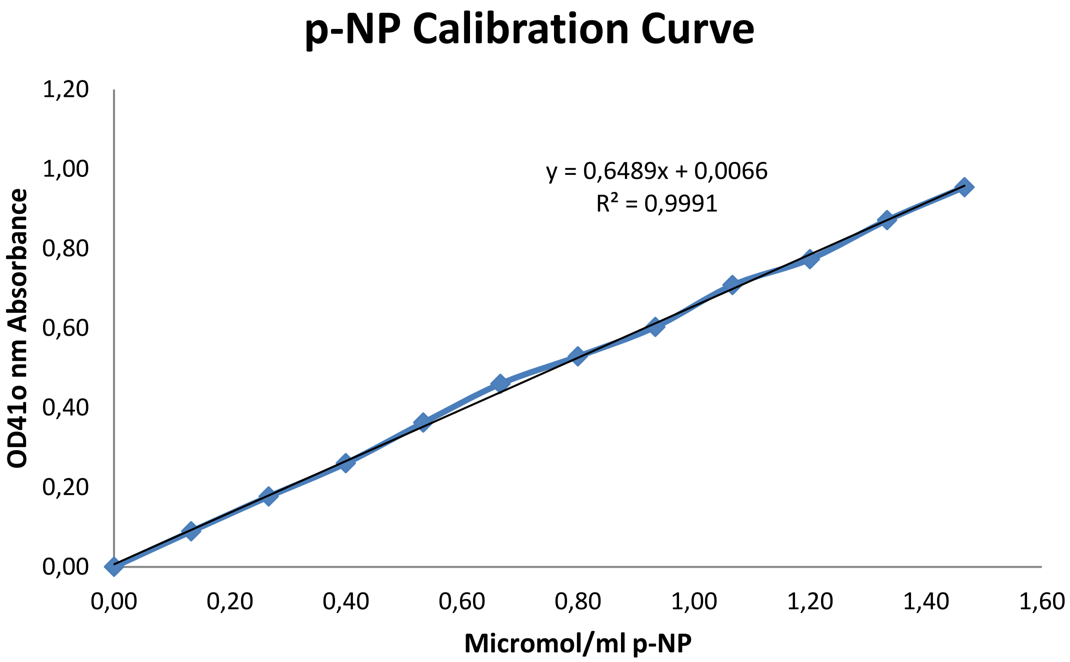

Figure A.1. Calibration curve used for enzyme activity calculation

Appendix B

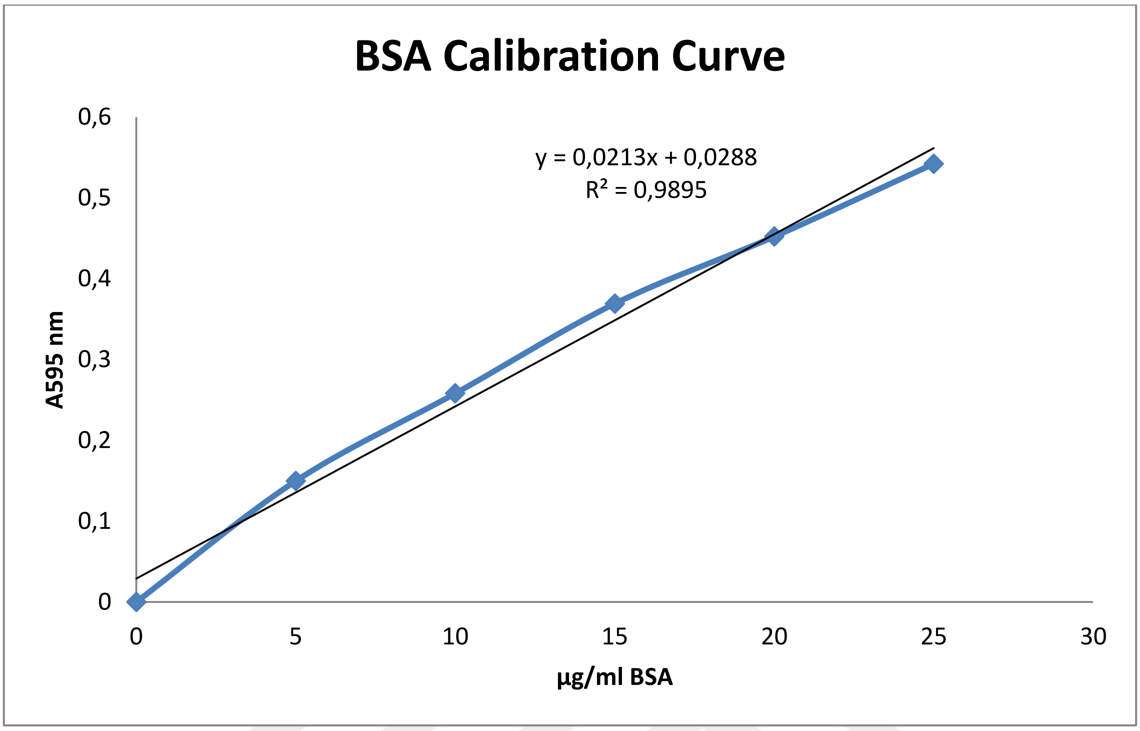

Figure B.1. BSA calibration curve used for Bradford protein assay

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PRESENTATIONS AT INTERNATIONAL CONGRESS/SYMPOSIUMS

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