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Immobilization of Candida tropicalis Lipase and Cells Isolated from Olive Pulp

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Research Article

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ABSTRACT

Yeast isolated from waste olive pulp obtained from a local olive oil mill was identified and determined to be a *Candida tropicalis* strain. Lipase production optimization of *C. tropicalis* strain has been completed. The lipase produced according to the optimization parameters was partially purified by ammonium sulfate precipitation and dialysis. Crude enzyme, partially purified lipase, and cells of the *C. torpicalis* strain were separately immobilized into sodium alginate, k-Carrageenan, and Agar-Agar, respectively, and their lipase activities were investigated. The highest lipase activity was determined as 10.83 U/ml in the partially purified sample that was not immobilized.

Keywords: Lipase, Immobilization, *Candida tropicalis*, Sodium-alginate, k-Carrageenan

Zeytin küspesinden İzole Edilen Candida tropicalis Lipazının ve Hücrelerinin İmmobilizasyonu

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ÖZ

Yerel bir zeytin yağı imalathanesinden elde edilen atık zeytin küspesinden izole edilen maya tanımlanmış ve *Candida tropicalis* suşu olduğu saptanmıştır. *C. tropicalis* suşunun lipaz üretim optimizasyonu tamamlanmıştır. Optimizasyon parametrelerine göre üretilen lipaz amonyum sülfat çöktürmesi ve diyaliz ile kısmi olarak saflaştırılmıştır. Kaba enzim, kısmen saflaştırılmış lipaz ve *C. torpicalis* suşunun hücreleri ayrı ayrı sırasıyla sodyum aljinata, k-Karragenana ve Agar-Agar'a tutuklanarak lipaz aktiviteleri araştırılmıştır. En yüksek lipaz aktivitesi immobilize edilmeyen kısmi saflaştırılmış örnekte 10,83 U/ml olarak saptanmıştır.

Anahtar Kelimeler: Lipaz, İmmobilizasyon, Candida tropicalis, Sodyum-aljinat, k-Karragenan

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Introduction

The olive oil production industry, a traditional and important agricultural industry in Mediterranean countries, accounts for approximately 97% of the world's olive oil production (Rharrabti and Yamani, 2018; Dias et al., 2021). According to 2023-2024 data, one of the top three olive oil producers worldwide is Turkey and it's olive oil production was 210 thousand tons. Three processes such as traditional press system, the three-phase centrifuge system and the two-phase centrifuge system are used to extract olive oil. As a result of these systems, two types of waste/residue are formed: liquid and solid. The seeds, skins and pulp remaining after the olives are pressed constitute the olive pomace (olive pulp). These wastes are of great importance in obtaining new microbial strains for lipase production.

Lipases, triacylglycerol ester hydrolases (E.C. 3.1.1.3), are serine hydrolases that carry out the hydrolysis and synthesis of esters of glycerol and long-chain fatty acids. Industrial lipases are generally esterase enzymes that hydrolyze fats and oils to diglycerides, monoglycerides, and finally glycerol and free fatty acids, respectively (Meghwanshi and Vashishtha, 2018). In an aqueous or nonaqueous system, lipases catalyze many reactions such as hydrolysis, ester synthesis, transesterification, and enantioresolution of esters (Hou, 2002). Lipases from different sources show significant differences in their enzyme specificity, which allows them for different industrial uses. In the energy industry, lipases attract attention as a non-toxic, biodegradable and renewable alternative fuel (such as biodiesel production) and energy source (Matuoog and Yunjun, 2017). Apart from the energy sector, lipases also have applications especially in the food, pharmaceutical and detergent industries. Lipases are used to obtain polyunsaturated fatty acids from solid or liquid oils. For this reason, it is used as a food additive and supplement in the medical sector. Lipases can also be used in the production of various chemicals, including food, pesticides and drugs. Dyeing of wool is enriched with reactive dyes using lipase (Ulbrich-Hofmann, 2012). Another area of use of lipases is bioremediation. For instance, it can be used in the treatment of wastewater from the meat industry. It is applied in the treatment of oily wastes containing solid and liquid oils discharged by the dairy industry, restaurants, slaughterhouses, hospitals or health centers (Basheer et al., 2011). Lipases can synthesize a chiral intermediate for the production of Polixatel, which is applied as an anticancer drug, especially in ovarian cancer (Fukaya et al., 2016). Lipases are also included in biosensor applications (Melani et al., 2019).

Lipase sources can be plant, animal or microbial (Gupta et al., 2004). Microbial lipases are generally more stable than animal or plant lipases (Hou, 2002). Extracellular lipase production is higher than intracellular lipase production, and extracellular lipases are produced by bacteria, yeasts, and molds. Yeasts have some advantages over bacteria and molds. For instance, they are more resistant to high substrate concentrations. They also have greater tolerance to metal ions. For these reasons, they allow for ease of growth and processing and the use of less refined and cheaper substrates such as agricultural industrial by-products (Rane and Sims, 1993; Rehman et al., 2014). Many yeasts have been studied in the production of lipase. Among these, *Candida* species are the most prominent (Sakpuntoon et al., 2020). Candida species that produce lipase include C. curvata, C. tropicalis, C. valida, C. rugosa, C. utilis, C. pellioculosa, C. antarctica, C. cylindraceae, C. deformans, and C. parapsilosis (Ghosh et al, 1996; Tokak et al, 2019; Başkan and Açıkel, 2023). The yeast most commonly used in commercial lipase production is Candida rugosa (Sakpuntoon et al, 2020). However, other species that produce lipase should not be ignored. Among the lipase-producing species other than *Candida*, *Yarrowia lipolytica* (formerly *Candida lipolytica*) stands out. Additionally, *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Geotrichum* species (such as *G. candidum*, *G. asteroids*, *Geotrichum* sp. FO401B), *Kluyveromyces lactis*, *Trichosporon* species (such as *T. asteroides*, *T. cutaneum*, *T. fermentans*) are also taken place in the literature as lipase producing species (Vakhlu and Kour, 2006).

Enzyme immobilization refers to an enzyme that is physically or chemically confined or localized to a specific area or region and retains abundant catalytic activity over several subsequent cycles (Kumar et al., 2023). Free enzymes have many disadvantages as well as advantages. The most important of these is that free enzymes are easily broken down and inactivated. Another is that free enzymes cannot be recovered and reused (Matuoog and Yunjun, 2017). Therefore, immobilization of enzymes is required, especially in industrial application areas. Immobilization increases the recyclability of enzymes and also increases enzyme stability and activity (Mehta et al., 2017). There are various methods or techniques for immobilization of enzymes using different supports, such as physical adsorption, covalent binding, membrane confinement, encapsulation, entrapment and cross-linking (Thangaraj and Solomon, 2019[a]). While physical adsorption is reversible, the others are irreversible. The carriers used in lipase immobilization are very important because their interactions with enzyme molecules can affect the activity and stability of immobilized lipases (Thangaraj and Solomon, 2019[b]). These carriers can be a synthetic organic polymer, a biopolymer, or an inorganic polymer. There are various enzyme immobilization carrier materials, including glass beads, macroporous resin, mesoporous silica, sol-gel material, magnetic particles and carbon nanotube (Matuoog and Yunjun, 2017).

In this study, a yeast strain obtained from olive pulp taken from a local olive oil mill was identified as *Candida tropicalis* and after lipase enzyme optimization, it was partially purified by ammonium sulfate precipitation and

dialysis and both yeast and crude enzyme extract were immobilized on sodium-alginate, k-Carrageenan and agar-agar carriers and enzyme stability was investigated.

Material and Methods

Isolation and Identification of The Microorganism

Waste olive pulp obtained from a local olive oil mill located in the Tarsus/Mersin region was taken under sterile conditions. 1 g of olive pulp was taken and transferred to a tube containing 10 ml of 0.9% NaCl, and homogenization was ensured. 0.1 ml of this solution was taken and inoculated into Yeast Medium Agar (YMA) prepared in a petri plate and incubated at 30°C for 72 hours. The yeast strain with lipolytic activity obtained after incubation was purified by streak-plate technique. The purified strain was identified by 18S rRNA (Kebabcı and Cihangir, 2022).

Lipase Production Medium and Determination of Lipase Activity

Lipase production medium was prepared as grams per liter; 1 peptone, 12 NaH_2PO_4, 2 KH_2PO_4, 0.3 $MgSO_4.7H_2O$, 0.25 $CaCl_2$, 0.005 $FeSO_4.7H_2O$, 0.015 $MnSO_4.7H_2O$, 0.03 $ZnSO_4.7H_2O$ (Hatzinikolaou et al., 1996). The pH of the medium was adjusted to 4.5 and sterilized at 121°C under 1 atm pressure for 15 minutes. After sterilization, 1% olive oil was added when the ambient temperature dropped below 65°C. When the medium reached room temperature, 1 ml of the stock culture was taken and inoculated into the medium and left for incubation at 30°C and 100 rpm for 72 hours. After incubation, the medium was first filtered through Whatman No:1 filter paper and then centrifuged at 7200 rpm for 10 minutes to obtain the supernatant (CFS). The supernatant obtained was used for the measurement of the lipase activity.

The supernatant obtained after incubation in lipase production medium was used in the determination of lipase activity. 1 ml of supernatant (enzyme source), 1 ml of olive oil, 4.5 ml of 50 mM pH 5.6 acetate buffer and 0.5 ml of 0.1 M CaCl2 were mixed and incubated at 200 rpm for 30 minutes. After incubation, 20 ml of 97% ethyl alcohol solution was added to stop the reaction. Lipase activity was determined by titrating the released fatty acids with 50 mM potassium hydroxide up to pH 10.5 (Sugihara et al., 1991; Kamzolova et al., 2005). One unit of lipase activity was defined as the activity that released 1 µmol of fatty acid under the conditions given above.

Ammonium Sulfate Precipitation and Dialysis

A modified method was used for ammonium sulfate precipitation (Kebabci and Cihangir, 2011). Ammonium sulfate was added to 100 ml of supernatant to bring it to 40% saturation and the process was carried out for 24 hours at +4°C with continuous stirring. At the end of the period, the precipitate was collected by centrifugation. Lipase activities in the precipitate and supernatant were determined. Then, the same procedure was applied for 60% and 80% ammonium sulfate saturation. At each stage, precipitates were collected and lipase activity and total protein were determined. After ammonium sulfate precipitation, the sediment was placed in a dialysis bag for further purification and dialyzed against water for 48 hours, after which lipase activity and total protein amounts were determined by the Lowry method (Lowry et al., 1951). The standard protein curve prepared with bovine serum albumin was used to calculate the protein amount.

Immobilization

Sodium-alginate, k-carrageenan and agar-agar were used as carrier materials for immobilization. These carrier materials were formed into beads to immobilize *Candida tropicalis* biomass, crude lipase and partially purified lipase and investigated for lipase activity.

Results

As a result of optimization of the lipase production medium of the newly isolated and identified *Candida tropicalis* strain, the enzyme activity was increased from ~5 U/ml to 10.33 U/ml. In the optimization, parameters such as pH of the production environment, temperature, carbon source, nitrogen source were investigated. It was determined that 1% ammonium sulphate added to the lipase production medium as a nitrogen source increased enzyme production. Addition of 1% ammonium sulphate plus 1% olive oil increased lipase production from 10.33 U/ml to 10.67 U/ml in the same medium (Kebabcı and Cihangir, 2022).

Lipase production was carried out by inoculating 1 ml of *Candida tropicalis* from the stock culture into modified lipase production medium to which 1% ammonium sulfate and 1% olive oil were added, at 30°C and 100 rpm for 72 hours. At the end of incubation, the medium was filtered through Whatman No:1 filter paper. The resulting blend was centrifuged at 7200 rpm for 10 minutes to obtain cell-free medium (CFS). Ammonium sulfate precipitation and dialysis processes were carried out using this cell-free medium. 100 ml CFS was used for ammonium sulfate precipitation and the highest purification obtained from the crude enzyme was 4.72-fold at 80% saturation (Table 1). The medium obtained after ammonium sulfate precipitation was subjected to dialysis at +4°C overnight. Protein and lipase activity were determined in enzymatic fractions for all processes. At the end of the purification, 7.25-fold partial purification was achieved (Table 2).

Sodium-alginate, k-Carrageenan and agar-agar beads were prepared as carriers for immobilization. The prepared beads, 3-4 mm, were used in the study. *Candida tropicalis* biomass and crude lipase and partially purified lipase were trapped in carriers and stored at +4°C, and then lipase activity determinations were carried out. Data obtained as a result of immobilization showed that the highest lipase activities were in the non-immobilized samples. Partially purified lipase showed the highest

lipase activity with 10.83 U/ml, despite being diluted 7.25-fold (Figure 1).

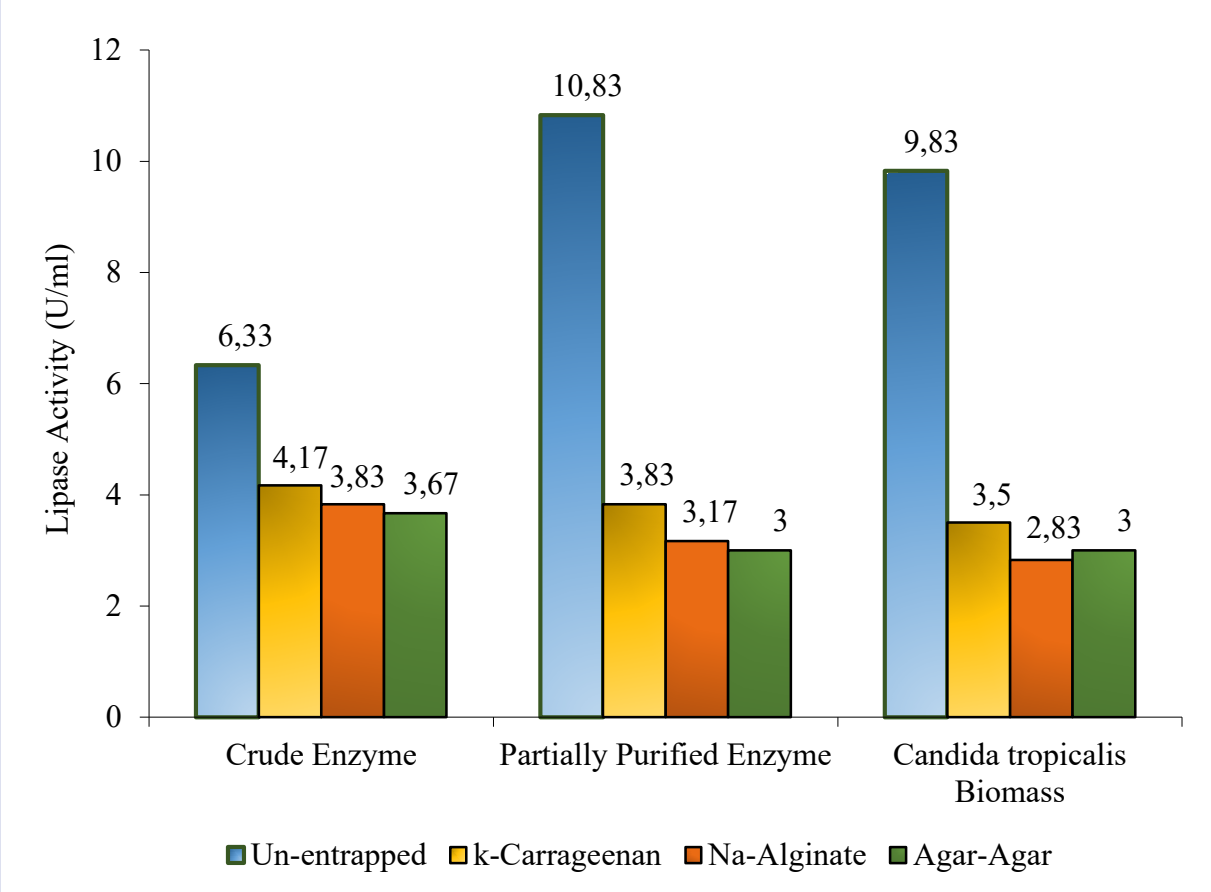

Figure 1. Lipase activities of crude lipase, partially purified lipase and Candida tropicalis biomass trapped in Sodium-Alginate, k-Carrageenan and Agar-Agar.

Table 1. Partial purification of lipase enzyme by ammonium sulfate precipitation.

Fractions		Total Volume (ml)	Protein		Enzyme			Yield (%)	Purification Fold
			mg/ml	Total (mg)	U/ml	Total Unit Activity	Total Specific Activity (U/mg protein)		
Crude Enzyme		100	0.252	25.20	5.33	533.0	21.15	100	1
Saturation	%40	10	0.620	6.20	15.17	151.7	24.47	28.46	1.16
	%60	10	0.456	4.56	26.33	263.3	57.74	49.40	2.73
	%80	10	0.483	4.83	48.17	481.7	99.73	90.38	4.72

Table 2. Purification of lipase enzyme after dialysis.

Fractions	Total Volume (ml)	Protein		Enzyme			Yield (%)	Purification Fold
		mg/ml	Total (mg)	U/ml	Total Unit Activity	Total Specific Activity (U/mg protein)		
Crude Enzyme	100	0.252	25.20	5,33	533.0	21.15	100	1
Ammonium sulfate	10	0.483	4.83	48.17	481.7	99.73	90.38	4.72

Precipitation 80%								
Dialysis	5	0.578	3.89	29.83	596.6	153.37	111.93	7.25

Discussions

Enzyme biotechnology, one of the sub-branches of biotechnology, has a significant share in the industrial field. The size of the global enzyme market is estimated to be approximately 6.4 billion USD in 2024. The lipase market is thought to be approximately 760 million US dollars in 2024. While molds have a 60% share in the production of industrial enzymes, bacteria have a 24% share, yeasts have a 4% share and plants and animals have a 10% share (Fasim et al., 2021). Immobilization of lipases and the microorganisms that produce them is gaining importance in the sustainable enzyme market. It is observed that lipases used especially in synthesis and biotransformation are immobilized. Because recycling of expensive lipases is of industrial importance (Sharma et al., 2001). Immobilization can also increase enzyme stability and activity. Lipases are produced by many *Candida* species, but *C. rugosa* is one of the most commonly used for lipase production (Vakhlu and Kour, 2006). *C. tropicalis*, is also being studied for lipase production. There are many studies in the literature on *Candida tropicalis* and the lipase it produces. The majority of these are related to biofuel production. Thangavelu et al. (2020) investigated *Candida tropicalis* ASY2 strain to produce microbial lipids for use as biodiesel feedstock. In a study carried out by Kutty et al. (2012) the degradation of petroleum hydrocarbons by immobilized *Candida tropicalis* (SD 302) strain was studied. There are also studies on phenol degradation from immobilized *Candida tropicalis* strains (Chen et al., 2002; Varma and Gaikwad, 2010; Kumar et al., 2018; Silva et al., 2019). Another degradation study was carried out by Tan et al. (2014) on acid Orange G using *Candida tropicalis* TL-F1 strain. In a study carried out by Pothayi et al. (2022) the production of lipase enzyme in *C. tropicalis* strains using various parameters was investigated. In another study, *C. tropicalis* strain was used in the production of flavor compounds from olive mill waste (Guneser et al., 2017). Scientific studies show that research on *C. tropicalis* and its lipase is especially focused on biofuel and biodegradation. Various carriers are used for the immobilization of *Candida tropicalis* lipase and biomass. Immobilization of microbial cells or their enzymes in gels such as calcium alginate, K-carrageenan and chitosan is a well-known technique (Fadnavis et al., 2003). In our study, lipase and biomass produced by *Candida tropicalis* were immobilized on these three carriers. Although loss of activity is observed after immobilization, it is advantageous when it comes to the stability of immobilized samples. It would be appropriate to use the newly isolated *Candida tropicalis* strain especially in biofuel and biodegradation studies.

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