Immobilization of Lipase and Lipase Producing Yeast, *Yarrowia lipolytica* NBRC 1658

Lipaz ve Lipaz Üreten *Yarrowia lipolytica* NBRC 1658 Suşunun İmmobilizasyonu

Research Article

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ABSTRACT

Lipase production was screened out of three *Yarrowia* strains and a novel *Candida tropicalis strain* isolated from soil samples and *Yarrowia lipolytica* NBRC 1658 showed the highest lipolytic activity was selected. Lipase produced by *Yarrowia lipolytica* NBRC 1658 was partially purified by ammonium sulfate precipitation and dialysis. Crude lipase, partially purified lipase and the yeast *Yarrowia lipolytica* NBRC 1658 was used for immobilization. Immobilization was carried out on carriers such as Sodium-alginate, k-Carrageenan and Agar-Agar. The highest lipase activity was detected in unimmobilized purified lipase sample as 11.17 U/ml.

Key Words
Lipase, immobilization, *Yarrowia lipolytica*.

ÖZET

Üç farklı Yarrowia suşu ve toprak örneklerinden yeni izole edilmiş bir *Candida tropicalis* suşundan lipaz üretimi araştırılmış ve en yüksek lipolitik aktiviteye sahip suş olan *Yarrowia lipolytica* NBRC 1658 suşu seçilmiştir. Yarrowia lipolytica NBRC 1658 suşundan üretilen lipaz amonyum sülfat çöktürmesi ve diyaliz ile kısmi olarak saflaştırılmıştır. Kaba enzim, kısmi olarak saflaştırılmış lipaz ve *Yarrowia lipolytica* NBRC 1658 suşu ayrı ayrı sodyum-aljinat, k-Karragenan ve Agar-Agar'a tutuklanmıştır. En yüksek lipaz aktivitesi ise immobilize edilmeyen kısmi saflaştırılmış suşta 11.17 U/ml olarak saptanmıştır.

Anahtar Kelimeler
Lipaz, immobilizasyon, *Yarrowia lipolytica*

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INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes [1]. They hydrolyze esters preferentially at the interface between lipid and water in heterogeneous systems [2]. Lipases find immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are by and large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures [3]. Lipases are also defined as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Lipases catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides [4]. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agro-chemicals, and flavour compounds [5].

Enzymes, in addition to their excellent catalytic properties, also have some characteristics that are not very suitable for industrial applications: they are soluble catalysts, they are usually very unstable, they may be strongly inhibited by substrates and products, and they only work well on natural substrates and under physiological conditions [6]. Therefore various strategies have been employed to enhance enzyme stability including genetic engineering, immobilization, and operating in non-aqueous media [7]. The main goal of enzyme immobilization is the industrial re-use of enzymes for many reaction cycles [6]. The advantages of immobilized over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media, leading to significant savings in enzyme consumption [8,9].

This work was undertaken to immobilize lipase enzyme. *Yarrowia lipolytica* NBRC 1658 is selected as lipase source between 3 various *Yarrowia* species and a novel *Candida tropicalis* strain. Under the results of a previous study *Yarrowia lipolytica* NBRC 1658 strain showed the highest protease activity and selected for immobilization. Crude lipase, partially purified lipase and the yeast, *Yarrowia lipolytica* NBRC 1658 was immobilized on three carriers such as Sodium-alginate, k-Carrageenan and Agar-Agar.

MATERIALS AND METHODS

Microorganism

Yarrowia lipolytica NBRC 1658, IFO 1195, and Local Strain was obtained from Hacettepe University, Department of Food Engineering. The novel yeast, *Candida tropicalis* was isolated from soil sample which was collected from an olive oil mill at Tarsus/Mersin regio. The origin of *Yarrowia lipolytica* NBRC 1658, IFO 1195 strains were National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan. And the origin of *Yarrowia lipolytica* Local Strain was Ankara University, Department of Food Engineering culture collection [10]. Lipase production was screened out of three *Yarrowia* strains and a novel *Candida tropicalis strain* isolated from soil samples and *Yarrowia lipolytica* NBRC 1658 showed the highest lipolytic activity was selected for immobilization.

Medium and Incubation

1 ml of *Yarrowia lipolytica* NBRC 1658 strain was inoculated in lipase production medium having the composition (g/l): 12 NaH_2PO_4, $2KH_2PO_4$, 0.3 $MgSO_4.7H_2O$, 0.25 $CaCl_2$, 0.005 $FeSO_4.7H_2O$, 0.015 $MnSO_4.7H_2O$, 0.03 $ZnSO_4.7H_2O$, 1 peptone and 1% olive oil v/v, and pH adjusted to 4.5 [11]. And incubated at 30°C, 150 rpm for 72 hours in a rotary incubator.

Preparation of Crude Lipase

After incubation the culture media filtered by Whatman No:1 filter paper and then centrifuged at 7200 rpm for 10 minutes to obtain the cell free supernatant (CFS). The lipase activity was carried out from the CFS. The biomass of *Yarrowia lipolytica* NBRC 1658 was dried in incubator at 30°C for 48 hours and results were computed.

Ammonium Sulfate Precipitation

The method described by Rifaat et al., 2010, was mofidied and used for ammonium sulfate precipitation [12]. Ammonium sulfate was added to the 100 ml of cell free supernatant with stirring to bring the saturation to 40% and after standing

it for 24 hours at 4°C, the precipitate was removed by centrifugation. Lipase activity both in the precipitate and supernatant was determined. Additional ammonium sulfate was added to the supernatant to bring the saturation to 60% and the mixture was left overnight at 4°C. The supernatant was further subjected to saturation 80%. The precipitates were collected, dissolved in distilled water. The enzymatic fractions were subjected to protein and lipase activity determination [13,14]. After ammonium sulfate precipitation the solution was dialyzed against water for 48 hours using dialysis bag.

Lipase Assay

CFS was used as enzyme source for lipase assay. 1 ml of olive oil, 1 ml of enzyme source, 4.5 ml of 50mM asetate buffer (pH 5.6), 0.5 ml of 0.1M $CaCl_2$ were strirred gently and incubated at 30°C, 200 rpm for 30 minutes. The reaction was stopped by adding 20 ml of ethyl alcohol. Lipase activity was determined by titration of the released fatty acids with 50 mM potassium hydroxide (up to final pH=10.5) [14,15]. Total protein amount of CFS was measured by Lowry Method [13]. One unit of lipase activity was defined as the the amount of enzyme that catalysed the release of 1 μmol of fatty acids per minute at 30°C under assay conditions.

Immobilization

Yarrowia lipolytica NBRC 1658 biomass, crude lipase and partially purified lipase was used for immobilization in Sodium-Alginate, k-Carrageenan and Agar-Agar beads. The immobilized samples, crude lipase and partially purified lipase were subjected to lipase activity determination.

RESULTS

The results on optimization of lipase production by *Yarrowia lipolytica* NBRC 1658, IFO 1195, Local Strain and a novel *Candida tropicalis* showed that the highest lipase production was detected by *Yarrowia lipolytica* NBRC 1658. The addition of ammonium sulfate to the production medium increased the lipase production up to 16 U/ml for *Yarrowia lipolytica* NBRC 1658, 11.67 U/ml for *Yarrowia lipolytica* IFO 1195, 10.67 U/ml for *Yarrowia lipolytica* Domestic Strain, and 10.33 U/ml for the novel *Candida tropicalis* whereas before optimization all strains lipase production were ~5 U/ml.

The highest lipase producing yeast between the strains was *Yarrowia lipolytica* NBRC 1658 and it was selected for ammonium sulfate precipitation and immobilization. 1 ml of *Yarrowia lipolytica* NBRC 1658 strain was inoculated in lipase production medium (pH adjusted to 4.5) and incubated at 30°C, 150 rpm for 72 hours in a rotary incubator. After incubation the culture media filtered by Whatman No:1 filter paper and then centrifuged at 7200 rpm for 10 minutes to obtain the cell free supernatant (CFS). The lipase activity studies were carried out on the CFS. 100 ml of CFS was used for ammonium sulfate precipitation and the highest purification obtained from crude enzyme was at 80% saturation as 3.85 fold (Table 1). After ammonium sulfate precipitation it was dialysed overnight at +4°C and the enzymatic fractions were subjected to protein and lipase activity determination. At the end of purification, lipase was partially purified 5.63 fold of the crude lipase (Table 2).

The crude lipase, partially purified lipase and the biomass of *Yarrowia lipolytica* NBRC 1658 was used one by one for immobilization in Sodium-Alginate, k-Carrageenan and Agar-Agar beads. All of them entrapped to the carriers and stored at +4°C and afterwards lipase activity measured (Figure 1).

The results of immobilization showed un-entrapped samples had the highest lipase activities. Although partially purified lipase was 3-fold diluted, it showed the highest lipase activity with 11.17 U/ml. Yarrowia lipolytica NBRC 1658 also showed high lipase activity because it is known that Yarrowia species have membrane-bound lipases.

DISCUSSION

The ascomycetous yeast *Yarrowia lipolytica* (formerly *Candida*, *Endomycopsis*, or *Saccharomyces lipolytica*) is one of the more intensively studied 'non-conventional' yeast species [16]. Several lipases have been detected in *Y. lipolytica*, including intracellular, membrane-

Table 1. Ammonium sulfate precipitate.

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	0.201	20.10	5.17	517.0	25.72	100	1
Saturation	40%	10	0.570	5.70	14.33	143.3	25.14	27.71	0.98
	60%	10	0.415	4.15	24.83	248.3	59.83	48.03	2.32
	80%	10	0.448	4.48	44.33	443.3	98.95	85.74	3.85

Table 2. 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	0.201	20.10	5.17	517	25.72	100	1
Ammonium sulfate Precipitation 80%	10	0.448	4.48	44.33	443.3	98.95	85.74	3.85
Dialysis	6	0.544	3.26	28.33	472.17	144.84	91.33	5.63

bound, and extracellular enzymes [17]. There are many studies on lipase production by *Y. lipolytica* strains [1,2,18]. Babu and Rao, 2007, studied on lipase production by *Yarrowia lipolytica* NCIM 3589 strain. Similarly Corzo and Revah, 1999, studied on lipase production by *Yarrowia lipolytica* 681 strain. Lanciotti et al., 2005, screened 62 strains of *Yarrowia* for lipase production on the treatment of olive mill wastewater. Although there are many studies on *Yarrowia* strains, there are not much found on production of lipase by *Yarrowia lipolytica* NBRC 1658 strain. Lipases obtained from different sources are usually subjected to certain prepurification steps before they are purified further. Typically, this is a one step procedure involving precipitation by saturation with an ammonium sulfate [$(NH_4)_2SO_4$] solution [19]. Rifaat et al., 2010, studied on partial purification of lipase from *Fusarium oxysporum* and found 4.3 purification factor on 60% saturation of ammonium sulfate [12]. The method described by Rifaat et al., 2010, was mofidied and used in this study and found 3.85 purification factor on 80% saturation of ammonium sulfate. And after dialysis 5.63 purification factor. It is important that after prepurification steps, lipase enzyme should be purified more with other techniques such as ultrafiltration and/or chromatography.

Lipolytic reaction liberates an acid which can be titrimetrically assayed. The pH-stat method is generally used as a reference lipase assay. This is not so expensive and convenient technique for characterising lipase activity and specificity [20].

Various researchers assayed lipase activity titrimetrically in their studies [15,21,22]. In this study we also assayed lipase activity by titration with 50 mM KOH and lipase activity was calculated.

There had been many researches on lipase production from various yeast and bacteria as well as pancreatic lipases from animals. Industrially used lipases generally are produced from bacteria and/or yeast cultures. However lipases used in industrial applications are often immobilized on various carriers. This process gives advantage to the lipase enzyme; a better stability, multiple usage, recovery etc. Though immobilization of enzymes has several advantages, the selection of support material and the method of immobilization are prominent factors influencing the enzymatic reactions [23]. The immobilization of microbial cells (or enzymes) by entrapment in gels such as calcium alginate, *K*-carrageenan, and chitosan is a well-known technique [24]. In this study we immobilized lipase enzyme and ***Yarrowia lipolytica*** NBRC 1658 cells in sodium alginate, *K*-carrageenan and agar-agar by entrapment. Athough the results showed that immobilized lipase and microbial cells had activity loss, the stability and recovage of immobilized samples takes advantage to the non-immobilized

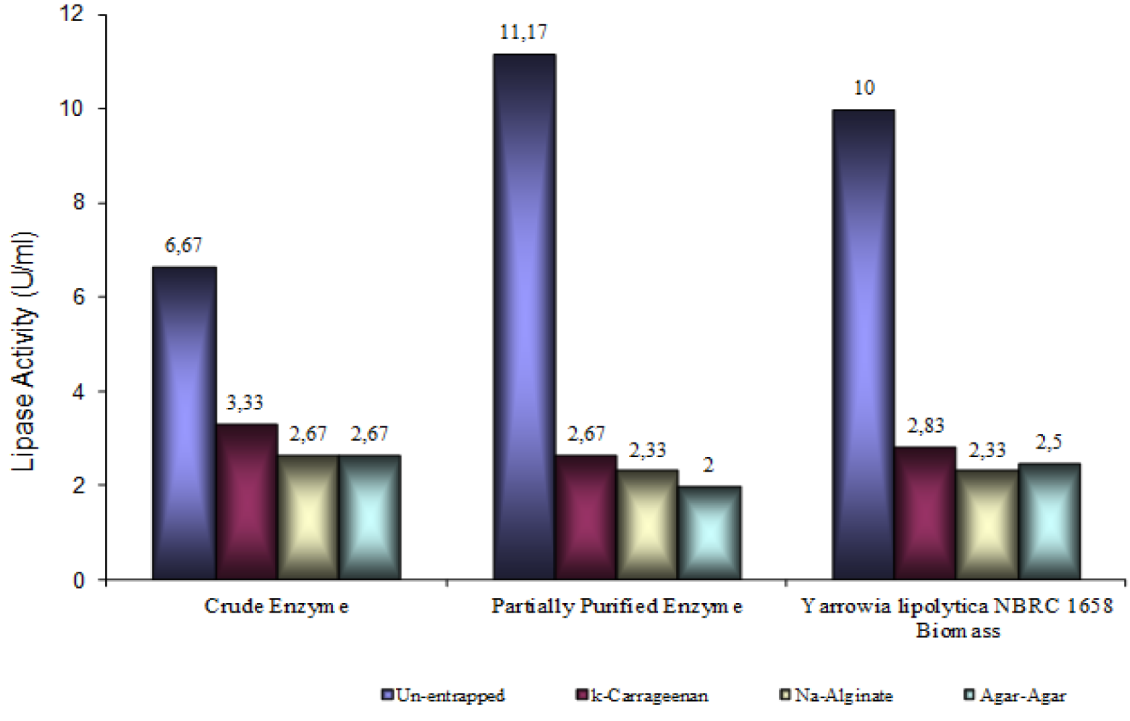

Figure 1. Entrapment of crude lipase, partially purified lipase and Yarrowia lipolytica NBRC 1658 biomass in Sodium-Alginate, k-Carrageenan and Agar-Agar.

samples. The stability, pH and temperature optimization of the immobilized samples should be studied.

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