

Production and partial purification of extracellular lipase produced by a novel yeast strain *Candida odintsovae* TY42

[Yeni bir maya suşu olan *Candida odintsovae* TY42 tarafından üretilen hücre dışı lipazın üretimi ve kısmi saflaştırılması]

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ABSTRACT

Objective: Nowadays, in most of industrial technology, especially biotechnological processes, use of enzymes is increasingly widespread. With respect to discovery of new enzymes, one of the most successful methods is isolation of microorganisms from natural habitats. Therefore, in this research, we aimed to isolate and characterize lipase producing yeasts and purify the extracellular lipase produced by a novel yeast strain *Candida odintsovae* TY42.

Methods: The yeasts used in the study were isolated from olive oils and identified based on sequence analyses of ITS1-5.8S rRNA-ITS2 and D1/D2 domain of 26S rRNA. The optimal nutritional and physicochemical conditions were determined by investigating the effects of initial pH and temperature and different carbon sources on enzyme production in basal medium. The lipase was purified by ammonium sulfate and acetone precipitation and anion exchange chromatography.

Results: Three lipase producing yeast isolates were obtained from olive oils produced in Aegean region. The sequencing of rRNA regions revealed that novel strains, TY17, TY42 and TY54 identified as *Candida odintsovae*. *C. odintsovae* TY42 showed higher lipase activity and therefore, the yeast was selected for further investigations. The maximum lipase production was achieved in a medium containing fish oil after 144 h of incubation. The lipase was purified 12.8 fold. The molecular weight of the purified lipase was estimated to be about 39 kDa.

Conclusion: This paper is the first study representing lipase production by *C. odintsovae*.

Key Words: *Candida odintsovae*, enzyme purification, extracellular lipase, lipase production.

Conflict of Interest: The authors declare no conflict of interest.

ÖZET

Amaç: Günümüzde, endüstriyel teknolojilerin çoğunda, özellikle de biyoteknolojik işlemlerde enzimlerin kullanımı giderek yaygınlaşmaktadır. Yeni enzimlerin keşfi ile ilgili olarak, en başarılı metotlardan bir tanesi, doğal habitatlardan mikroorganizmaların izolasyonudur. Bu yüzden bu çalışmada, lipaz üretici mayaların izolasyonu ve karakterizasyonu ve yeni bir maya suşu olan *Candida odintsovae* TY42 tarafından üretilen hücre dışı lipazın saflaştırılması amaçlanmıştır.

Metod: Bu çalışmada kullanılan mayalar, zeytinyağlarından izole edilmiş ve ITS1-5.8S rRNA-ITS2 ve 26S rRNA D1/D2 domaininin dizi analizine göre tanımlanmıştır. Optimal besinsel ve fizikokimyasal koşullar, başlangıç pH, sıcaklık ve farklı karbon kaynaklarının bazal ortamda enzim üretimi üzerine etkileri incelenerek saptanmıştır. Lipaz, amonyum sülfat ve aseton çöktürmesi ve anyon değişim kromatografisi ile saflaştırılmıştır.

Bulgular: Lipaz üretici maya izolatları, Ege bölgesinde üretilen zeytinyağlarından elde edilmiştir. rRNA bölgelerinin dizi analizi, bu yeni suşların, TY17, TY42 ve TY54, *Candida odintsovae*'ye dahil olduğunu ortaya çıkarmıştır. *C. odintsovae* TY42, en yüksek lipaz aktivitesi göstermiş ve bu yüzden, bu maya ileriki çalışmalar için seçilmiştir. Maksimum lipaz üretimi, balık yağı içeren ortamda inkübasyonun 144. saatinden sonra elde edilmiştir. Lipaz, 12.8 kat saflaştırılmıştır. Saflaştırılan lipazın molekül ağırlığı yaklaşık olarak 39 kDa olarak hesaplanmıştır.

Sonuç: Bu makale, *C. odintsovae* tarafından lipaz üretimini gösteren ilk çalışmadır.

Anahtar Kelimeler: *Candida odintsovae*, ekstrasellüler lipaz, lipaz üretimi, enzim saflaştırma.

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Enzymes have been produced in large industrial scale for several decades. The global enzyme market increased steadily from \$1.5 billion in 2000 to \$3.1 billion in 2009, and then to \$3.6 billion in 2010 [1,2]. It is expected by Business Communications Company (BCC) research that the worldwide trade would reach \$6.0 billion by the end of 2016 [2] and at least 75% of the industrial enzyme market was employed by hydrolytic enzymes [3]. Lipases are the most extensively used class of enzymes in biotechnology [4].

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are notably versatile because that they can catalyze large number of reactions. Numerous microorganisms including bacteria, fungi, and yeasts produce lipases [5]. Especially, lipases from fungi are important in industrial applications. Due to its large potential in industrial uses as additive agents in foods, fine chemicals, detergents, waste water treatment, cosmetics, pharmaceuticals, leather and medical, the attention in microbial lipase production has increased recently [5-8]. Therefore, screening of microorganisms with lipolytic properties could enable the discovery of novel lipases.

To identify yeast species, the dissimilarities in the internal transcribed spacer (ITS) have been widely used [9]. The region is fairly short and can be easily amplified by universal primers complementary to preserved regions in the rRNA genes [10]. Besides the ITS region, Kurtzman & Robnett [11] determined the differences in the variable D1/D2 domain of 26S rDNA in about 500 species of ascomycetous yeasts. The result showed that nucleotide substitutions in the domain do not exceed 1% among conspecific strains. Therefore, this method is effective for the identification and classification of yeasts [11,12].

The species *Candida odintsovae* (formerly, *Candida odintsovi*) was first isolated from a fermenting birch exudate and assigned to the genus *Candida* by Babeva et al. [13]. Strains of the species are related to ascosporous yeasts belonging to the *Pichia* genus in morphology, physiology, characteristics of coenzyme Q, GC content (%) of DNA and vitamin requirements. *C. odintsovae* is closest to species *Pichia rabaulensis* but differs from it in the characteristics of DNA and in the variety of carbohydrates being fermented.

The aims of this research were to characterize yeasts isolated from olive oils produced in Aegean region of Turkey by sequencing of ITS1-5.8S-rRNA-ITS2 and D1/D2 domain of 26S rRNA regions, and to purify the produced extracellular lipase.

Materials and Methods

Culture isolation

The yeasts used were isolated from olive oils produced in Aegean region of Turkey. Samples were directly streaked

onto Tributyrin Agar media (g/L; yeast extract, 3; peptone, 5; tributyrin, 10 and agar, 15, pH 6.0) in petri plates and were incubated at 27°C [14]. Resultant yeast colonies were isolated and further purified by streaking on Yeast Malt (YM) agar slants (g/L; yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10 and agar, 15) [15].

Sequencing of ribosomal RNA regions

DNA isolation was performed according to the method of Liu et al. [16]. PCR amplification reactions were performed with Corbett Cool Gradient Palm Cycler CGI-96, under the conditions as described elsewhere [15]. The following primers were used for amplifications of ITS1-5.8S rRNA-ITS2 and D1/D2 domain of 26S rRNA regions, respectively: ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC CC GCT TAT TGA TAT GC-3') and NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3').

Both strands of the DNAs were sequenced on an ABI 3130XL automated sequencer (Applied Biosystems, USA) following the manufacturer's instructions. The sequences were compared pairwise using a BLASTN search and were aligned with the sequences of related species obtained from GenBank using CLUSTAL W version 2.0 software. A phylogenetic tree was constructed using the Tamura-Nei neighbor joining method by MEGA software version 5.0 [17]. Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) [18].

Lipase production in submerged fermentation

For lipase production, the composition of the basal medium with an initial pH of 6.0 consisted of yeast extract 0.1% (w/v), peptone 3% (w/v), MgSO₄·7H₂O 0.05% (w/v), KH₂PO₄ 0.1% (w/v) and NaNO₃ 0.3% (w/v) supplied with tributyrin at a concentration of 1% (v/v) as a carbon source. The medium was heat sterilized (121°C for 15 min). After cooling, the oil, previously sterilized by dry heat (180°C for 60 min), was added to the culture medium [19]. The yeast (10⁶ cells/ml) was cultivated in 250 ml Erlenmeyer flasks containing 50 ml of the basal medium and the flasks were incubated in an orbital shaker operating at 150 rpm at 27°C. Samples of 5 ml were taken at equal time intervals (day by day), the cells were separated by centrifugation at 10000 g at 4°C for 15 minutes from culture medium and then lipase activity was determined as below.

Lipase activity determination

Lipase activities were determined by the method of Winkler & Stuckman [20] with some modifications as described below. 45 mg of p-nitrophenol palmitate (p-NPP) was dissolved in 15 ml of 2-propanol and mixed with 135 ml of 25 mM Tris-HCl buffer (pH 7.0) containing Triton-X-100 (0.2%). Liberated p-nitrophenol was determined by measurement of the absorbance at 410 nm following

the termination of the reaction by addition of 0.15 ml of 0.1 M Na₂CO₃ after incubation at 37°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of p-nitrophenol (p-NP) per minute under the standard assay conditions.

Improvement of lipase production

Effects of different carbon sources. Glucose, fish oil, sunflower oil, olive oil, Tween-20 and Tween-80 with the concentrate of 1% were used to study the effects of carbon sources on lipase production.

Effect of initial pH. To investigate the effect of initial pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) on the production of lipase, the media are buffered with acetate buffer (for pH 5-6) and Na-phosphate buffer (for pH 6-8).

Effect of temperature. In the submerged fermentation, lipase production experiments were performed at different temperatures (15, 20, 25, 30, 35 and 40°C) to determine temperature of maximum lipase production.

Purification of extracellular lipase

C. odintsovae TY42 was grown in basal medium supplied with fish oil at a concentration of 1% (v/v) in five 1000 ml Erlenmeyer flasks with a 250 ml working volume on a rotary shaker (150 rpm) at 30°C. After 144 hours of incubation, the culture broth was separated from cells by centrifugation at 10000 g at 4°C for 15 minutes. The extracellular lipase was concentrated by gradient ammonium sulfate precipitation (25-75%) from supernatant. The precipitate collected by centrifugation was dissolved in 0.05 M of Tris-HCl buffer (pH 7.0) and dialyzed overnight in the same buffer. Dialyzed sample was concentrated with addition of 1:1 volume of chilled acetone and allowed for precipitation during 1 hour at 4°C. Precipitates were collected by centrifugation at 10000 g at 4°C for 15 minutes and dissolved in 0.05 M of Tris-HCl buffer (pH 7.0).

Concentrated sample was applied to Dowex 1X4-200 column that had been pre-equilibrated with 0.05 M of Tris-HCl buffer (pH 7.0) for anion exchange chromatography. The bound protein was eluted by increasing concentration of NaCl in 0.05 M of Tris-HCl buffer (pH 7.0) from 50 mM to 200 mM with stepwise gradient. Fractions that exhibited lipase activity were pooled and concentrated.

Protein determination

Protein concentrations were determined by Bradford

assay by using bovine serum albumin as standard [21]. Protein concentrations in fractions were monitored by measuring the absorbance at 280 nm during ion exchange chromatography.

SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was performed according to method of Laemmli [22] on a 4% polyacrylamide stacking gel and 12% polyacrylamide resolving gel. For determination of relative molecular weight, a mixture of reference proteins including phosphorylase b (94 kDa); albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa) was run along with the sample that partially purified. Protein bands were visualized by Coomassie brilliant blue G 250 staining.

Results and Discussion

Amplification and sequencing of ITS1-5.8S-ITS2 and 26S rRNA regions

Lipase-producing microorganisms have been isolated in different locations such as industrial wastes, oil processing factories, dairies, soils polluted with oils [23]. Although researches of the inherently resultant microorganisms in olive oils have been limited and do not give a complete picture for olive oils produced in all of the olive-producing parts of the world, latest research has revealed the occurrence of a rich microflora in fresh olive oil [24]. The environments containing oils could provide good habitats for isolation of lipase-producing organisms.

In the present research, three different yeast colonies were obtained from Tributyrin Agar petri dishes and their DNA was isolated. PCR amplification of the ITS1-5.8S-ITS2 and 26S rRNA regions of the yeasts isolates rendered products ranging from 450-497 and 562-566 bp, respectively. The sequences obtained from sequencing of rRNA regions were compared with GenBank using the BLASTN tool and accession numbers were taken (Table 1). Results revealed that the isolates belong to *Candida odintsovae*. In Fig. 1, phylogenetic analysis of the 26S rRNA sequences of the *C. odintsovae* strains with 26S rDNA sequences with over 93% similarity belonging to eight different species from NCBI database is presented.

Lipase production in submerged fermentation

Yeasts having lipase production in petri dishes were

Table 1. Molecular characterization of *C. odintsovae* strains used in the study

Isolate no.	ITS1-5.8S-ITS2 rRNA region			D1/D2 domain of 26S rRNA region		
	Size of PCR product (bp)	Homology (%)	Accession number	Size of PCR product (bp)	Homology (%)	Accession number
TY17	495	99	KC148190.1	566	100	KC148198.1
TY42	450	99	KC148194.1	565	99	KC148202.1
TY54	497	100	KC148195.1	562	99	KC148203.1

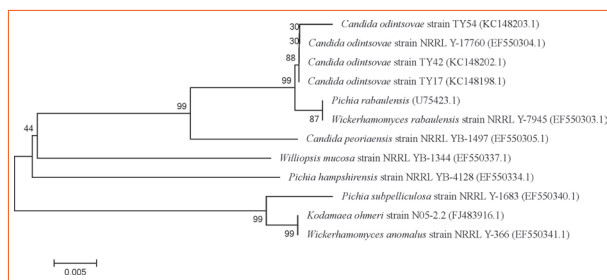


Figure 1. Phylogenetic tree representing the placement of *C. odintsovae* strains. The sequences of the related species were obtained from the GenBank, NCBI.

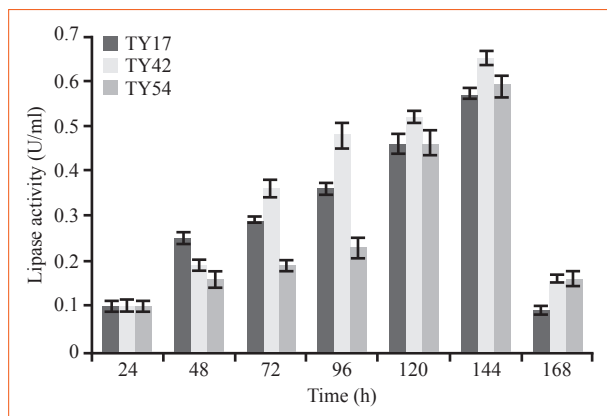


Figure 2. Effect of incubation period on lipase production by *C. odintsovae* strains. Submerged fermentation process using basal medium supplemented with tributyrin at pH 6.0 and temperature 30°C.

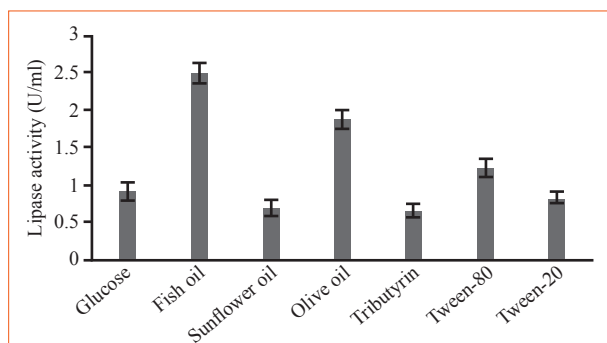


Figure 3. Effects of different carbon sources on lipase production by *C. odintsovae* TY42. Submerged fermentation was performed for 144 h at pH 6.0 and temperature 30°C.

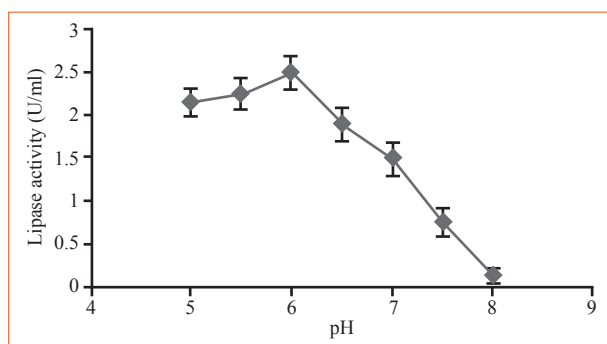


Figure 4. Effect of initial pH on lipase production by *C. odintsovae* TY42. Fermentation medium was supplemented with fish oil at 30°C for 144 h.

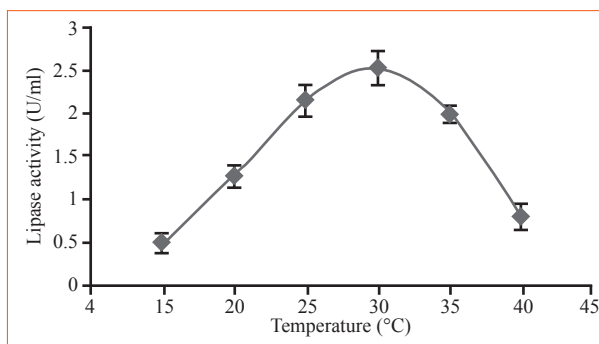


Figure 5. Effect of incubation temperature on lipase production by *C. odintsovae* TY42. Submerged fermentation was performed at pH 6.0 for 144 h and fish oil was used as carbon source.

firstly examined for the extracellular lipase production in submerged fermentation process using basal medium supplied with tributyrin at pH 6.0 and temperature 30°C. During the experiment, lipase productions were evaluated daily until seventh days. After incubation for 144 h, lipase productions by yeast strains in the media were reached maximum value (Fig. 2). Also, noteworthy decreases in the productions were detected for all of the investigated yeasts. The decrease can be related to the hydrolytic action of proteases [25], to the decrease in cell growth, or to adsorption of the enzyme produced at the aqueous-organic interface [26]. On the other hand, while *C. odintsovae* T17 and TY54 strains showed 0.57 and 0.59 U/ml lipase activities respectively in the medium containing tributyrin, *C. odintsovae* T42 strain showed higher lipase activity (0.65 U/ml). Therefore, the strain was selected for further investigation.

Improvement of lipase production

Effects of different carbon sources. In the present research, six different carbon sources were tested to study their effects on lipase production by *C. odintsovae* T42. All the carbon sources improved lipase production over the control (tributyrin, 0.65 U/ml) with especially high levels being obtained with fish oil at pH 6.0 and temperature 30°C (2.49 U/ml) (Fig. 3).

Effect of initial pH. The optimum pH for lipase production by *C. odintsovae* T42 in submerged fermentation supplemented with fish oil was determined by evaluating the activity at 30°C over a range of pH values. The lipase production was found to be the best at pH 6.0 (Fig. 4). Below and above the pH value, lipase production diminished stepwise.

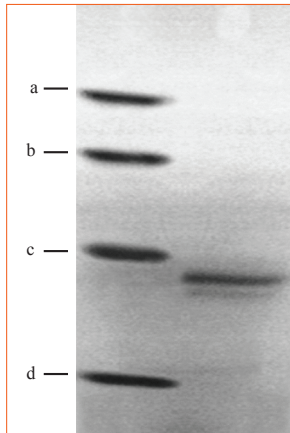
Effect of temperature. The effect of temperature on the lipase production by *C. odintsovae* T42 was evaluated in media incubated at 15, 20, 25, 30, 35 and 40°C. Maximum lipase production was achieved using fish oil as a sole carbon source at pH 6.0 and temperature 30°C. (Fig. 5).

Purification of extracellular lipase

The extracellular lipase produced by *C. odintsovae* TY42

Table 2. Purification of the extracellular lipase of *C. odintsovae* TY42

Purification Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)
Crude extract	227	33.7	0.15	1
(NH ₄) ₂ SO ₄ precipitate	45.8	20.6	0.45	3
Acetoneprecipitate	8.7	4.8	0.55	3.7
Dowex 1x4-200 anion exchanger	0.175	0.33	1.9	12.8

**Figure 6.** SDS-PAGE of purified lipase from *C. odintsovae* TY42 (right) and molecular weight markers (left): a, phosphorylase b (94 kDa); b, albumin (67 kDa); c, ovalbumin (43 kDa); d, carbonic anhydrase (30 kDa). The gel was stained with Coomassie brilliant blue (G-250).

was partially purified from the supernatant prepared from the cell culture by ammonium sulfate-precipitation, dialysis, acetone-precipitation and anion exchange chromatography. A final purification of 12.8-fold was achieved and the lipase had a specific activity of 1.9 U/mg. A summary of the purification steps is presented in Table 2. The purified *C. odintsovae* lipase was homogeneous as determined by SDS-PAGE and the apparent molecular weight of the purified lipase was estimated to be about 39 kDa (Fig. 6).

Conclusion

The industrial requirements for new lipase resources with unique enzymatic features and obtained at low costs has encouraged the isolation of new lipolytic organisms. The introduction of new lipase producer microorganism is important because microbial lipases have gained special industrial attention due to their ability towards extremes of temperature, pH, and organic solvents, and chemo-, regio-, and enantio-selectivity. From the industrial viewpoint, *C. odintsovae* isolated from olive oil has the capacity of being a source for entrepreneurs. No doubt, additional studies such as optimization of the lipase production and characterization of the produced lipase are needed before the use of lipase for the industrial applications.

Acknowledgements

This work was supported by the Scientific Research Unit of Ege University (11-FEN-015). We would like to extend our thanks to the Izmir Institute of Technology, Biotechnology and Bioengineering Central Research Laboratories (BIYOMER) for providing laboratory facilities for

DNA sequencing.

This publication is dedicated to the Yusuf BARKUT.

Conflict of Interest

There are no conflicts of interest among the authors.

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