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A Novel Yeast Isolated From Olive Mill Waste Candida tropicalis; Optimization of Medium Composition For Lipase Production

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Abstract: A novel microorganism that was isolated from an olive mill waste sample was screened for lipase production. This novel strain was identified and determined by 18S rDNA analysis and it was detected that the strain was 92% *Candida tropicalis* in ratio. Optimization of lipase production was carried out by the addition of nitrogen and carbohydrate sources into the lipase production medium as well as the effect of pH and temperature parameters were studied to increase the lipase production. Maximum growth conditions for the strain were detected at 4.0 pH medium and 30°C growth temperature. The effect of various nitrogen sources on lipase production showed that ammonium sulfate increased lipase production whereas urea, peptone and casein did not show a distinct effect. In addition presence of various sugars in the lipase production medium did not increase the lipase production efficiently although some oils did. The highest lipase activity was determined as 10.67 U/ml, with the addition of 1% ammonium sulfate and 1% olive oil into the production medium.

Keywords: *Candida tropicalis*, Lipase Production, Optimization

Zeytin Değirmeni Atıklarından Yeni İzole Edilen Bir Maya *Candida tropicalis*; Lipaz Üretimi İçin Besiyeri Kompozisyonun Optimizasyonu

Öz: Bu çalışmada bir zeytin değirmeni atık örneğinden yeni izole edilen bir mikroorganizma lipaz üretimi için taranmıştır. Bu yeni suş 18S rDNA analizi ile tanımlanmış ve suşun %92 oranında *Candida tropicalis* (yuvarlakmaya) olduğu tespit edilmiştir. Lipaz üretim ortamına azot ve karbonhidrat kaynakları ilave edilerek lipaz üretiminin optimizasyonu gerçekleştirilmiş, ayrıca lipaz üretimini artırmak için pH ve sıcaklık parametrelerinin etkisi incelenmiştir. Suş için maksimum büyüme koşulları 4.0 pH ortamında ve 30°C büyüme sıcaklığında tespit edilmiştir. Çeşitli azot kaynaklarının lipaz üretimi üzerindeki etkisi araştırıldığında, amonyum sülfatın lipaz üretimini arttırdığını, üre, pepton ve kazeinin ise belirgin bir etki göstermediğini saptanmıştır. Birtakım yağların artırmasına rağmen lipaz üretim ortamında çeşitli şekerlerin bulunması lipaz üretimini verimli bir şekilde artırmamıştır. En yüksek lipaz aktivitesi, üretim ortamına %1 amonyum sülfat ve %1 zeytinyağı ilavesiyle 10.67 U/ml olarak belirlenmiştir.

Anahtar kelimeler: *Candida tropicalis*, Lipaz üretimi, Optimizasyon

Introduction

According to Markets and Markets report on the industrial enzymes market, published in October 2016, the industrial enzymes market was estimated to be valued at USD 4.61 Billion in 2016, and the global industrial enzymes market is projected to reach USD 6.30 Billion by 2022 in terms of value, at a CAGR of 5.8% from 2017. This huge market size led researchers to show interest in the production of enzymes. Lipases (E.C. 3.1.1.3) are placed only after proteases and carbohydrases in the world enzyme market and share about 5% of it by the year 2006 (Vakhlu and Kour, 2006) and it still keeps it's leading place in 2017. These enzymes can be defined as carboxylesterases that catalyze the hydrolysis of long-

chain acylglycerols to glycerol, free fatty acids, and mono- and diglycerides (Aehle, 2007). Therefore they also can be named triacylglycerol acyl hydrolases. The hydrolysis of fats occurs at the water/lipid interface in aqueous media, while in non-aqueous media, the biochemical reactions driven by lipases include hydrolysis, interesterification, alcoholysis, acidolysis and esterification (Yu et al, 2016). Lipases also catalyze aminolysis in addition to the hydrolytic activity on triglycerides (Joseph et al, 2008). They hydrolyze esters preferentially at the interface between lipid and water in heterogeneous systems (Corzo and Revah, 1999). Furthermore, lipases have chemo-, region- and stereo-selective properties, which make lipases one of the most desirable enzymes for many industries (Haki and Rakshit, 2003). One of the most important classes of industrial enzymes are lipases (Babu and Rao, 2007) and they find immense applications in food, dairy, detergent and pharmaceutical industries. 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 (Jaeger and Eggert, 2002)).

Lipases are by and large produced from microbes (Gupta et al, 2004) According to a report by Business Communications Company, Inc. in 2007, lipases are forecasted as the fastest growing class of enzyme (Gupta et al, 2015). Since lipases are physiologically necessary for living organisms, they are ubiquitous and can be found in diverse sources, such as plants, animals and microorganisms (Rahman et al, 2006). More abundantly, however, they are found in bacteria, fungi and yeasts (Haki and Rakshit, 2003). The major sources include microbial lipases; among these yeast and fungal lipases are of special interest because they can carry out various stereoselective reactions. These lipases are highly diverse and are categorized into three classes based on oxyanion hole: GX, GGGX and Y. The detailed phylogenetic analysis showed that the GX family is more diverse than GGGX and Y families (Gupta et al, 2015). Microbial lipases have a great potential for commercial applications due to their stability, selectivity and broad substrate specificity because many unnatural acids, alcohols or amines can be used as substrates. There are also a certain number of lipases produced by yeasts, most of them belonging to the *Candida* genus, that have been used for the biotechnological purpose (Cardenas et al, 2001).

This work was undertaken to optimize lipase production by a novel yeast, *Candida tropicalis* (Sesli et al, 2020). For this purpose soil samples were collected from olive mill wastes and various microorganisms were isolated. A yeast which was then identified as *Candida tropicalis* by 18S rDNA analysis, showed the highest lipase activity and was selected for the optimization of lipase production. The pH optima of lipase production medium as well as optimum temperature and stirring speed, were studied. However effect of different carbon and nitrogen sources was also detected to increase lipase production

Material and Method

Isolation and Identification: Soil samples were collected into sterilized plastic bags, from an olive mill in Tarsus/Mersin regio. Under sterilized conditions 1 g of soil sample was washed with 10 ml of 0.9% NaCl solution. 1 ml of the mixed solution inoculated on modified Yeast Medium Agar plates and incubated at 30°C for 72 hours. Novel yeasts were isolated by streak-plate technique and then assayed for lipolytic activity. A strain showed the highest activity was selected. Besides biochemical and morphological tests, the novel strain then was identified by 18S rDNA phylogenetic analysis. Catalase, amilase and urease activities were examined. Also nitrate reduction of the strain were tested. Colony morphology and microscopy studies were studied.

Medium and Incubation: 1 ml of novel *Candida tropicalis* liquid sample was inoculated in lipase production medium (Hatzinikolaou et al, 1996). The lipase medium composition was (g/l): 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 $ZnSO_4.7H_2O$, 1 peptone “as initial nitrogen source and displaced by other nitrogen sources during optimization”. Medium pH was adjusted to 4.5 and after sterilization 1% olive oil (v/v) was added. Incubation was carried out at 30ºC, 100 rpm for 72 hours in a rotary incubator.

Optimization of Lipase Production: Lipase production medium, as described above, was prepared for the optimization. Primarily initial pH was adjusted between 3-9 and optimum pH range was detected. Afterwards optimum temperature range was detected between 10-40ºC. Also stirring speed of the incubator between 100-250 rpm with 50 rpm intervals was carried out.

Carbon and nitrogen sources affecting growth and lipase production in *Candida tropicalis* were studied.

Various carbohydrates (with and without 1% olive oil) were added to the production medium individually. As carbon sources, also 1% olive oil, extra virgin olive oil, sunflower oil, corn oil, soybean oil and canola oil were added to the medium separately. For detection of nitrogen sources; 1% protease peptone, peptone, yeast extract, casein, urea, ammonium oxalate, ammonium nitrate, ammonium carbonate and ammonium sulfate were added with and without 1% olive oil. The results of the experiments were determined and computed.

Preparation of Crude Lipase: After incubation the culture media was 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. Biomass was determined by dry weight at 30°C for 48 hours and was expressed as g of cell dry weight per 100ml.

Lipase Assay: CFS was used as an 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" (Kamzolova et al. 2005; Sugihara et al. 1991). One unit of lipase activity was defined as the amount of enzyme that catalysed the release of 1 µmol of fatty acids per minute at 30°C under assay conditions.

Results

Soil samples were collected from an olive mill in Tarsus/Mersin regio and besides bacteria four isolated yeasts were assayed for lipolytic activity. A novel yeast strain that showed the highest activity was selected for future experiments. After biochemical and morphological tests carried out, 18S rDNA phylogenetic analysis was applied and the novel strain was identified as *Candida tropicalis* in 92% ratio [Fig. 1].

For detection of the biochemical properties of *Candida tropicalis* catalase activity was determined and was found that it was catalase positive. Also biochemical tests showed that nitrate reduction of the strain was negative, it's amilase activity was negative and urease activity was positive.

Colony morphology of the strain were also determined as S type colony. The strain was dyed by methylene blue. At microscopy examination no pseudohyphae or hyphae were detected. 18 RNA analysis and identification was carried out by a private biotechnology company located in Ankara.

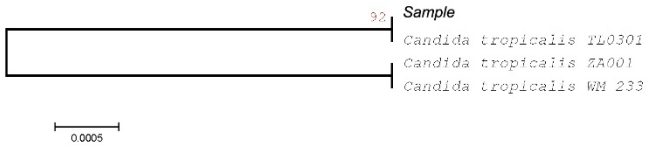

Figure 1. Identification of novel yeast strain by 18S rDNA analysis.

The strain's optimum pH range of growth was 3-7 and maximum was 4. The optimum pH range on lipase production was 3-5 and maximum were at 4 [Fig. 2].

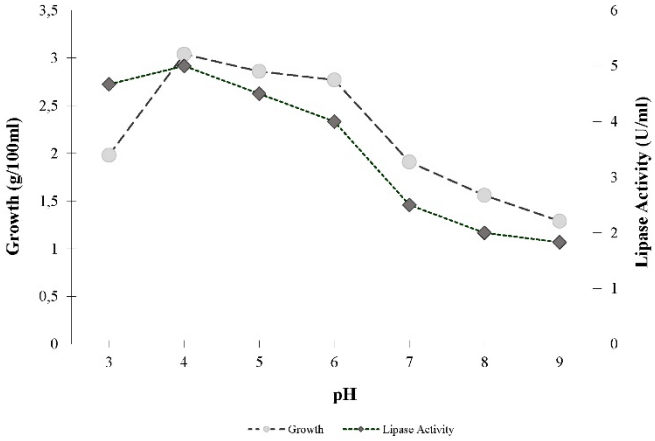

Figure 2. Effect of pH on growth and lipase production by *Candida tropicalis*.

The growth temperature was also detected and maximum growth and lipase production was determined at 30°C [Fig. 3].

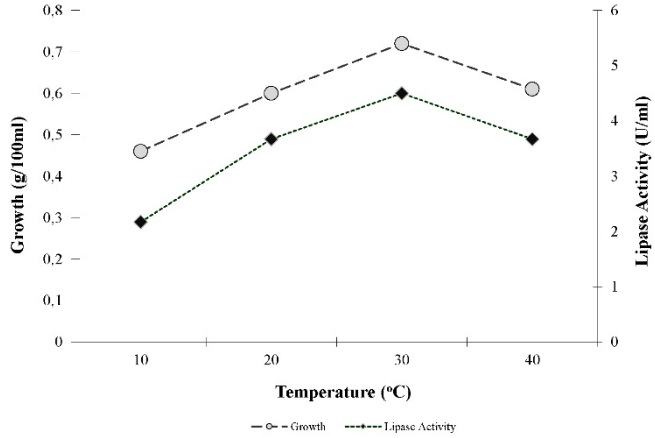

Figure 3. Effect of temperature on growth and lipase production by *Candida tropicalis*.

Stirring speeds between 100-250 rpm were studied for growth and lipase production. It showed that maximum growth and lipase production were detected at 100 rpm stirring speed [Fig. 4].

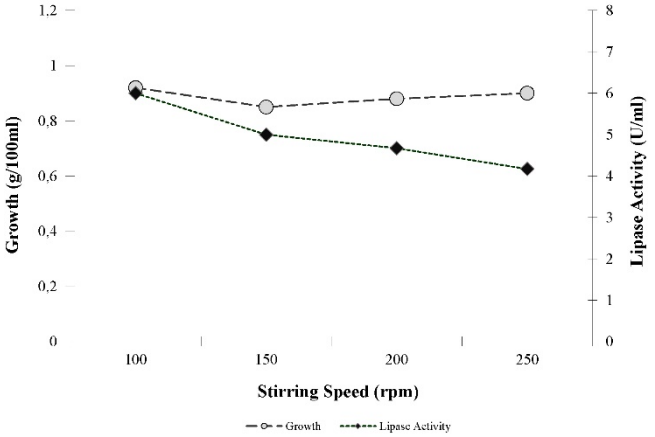

Figure 4. Effect of stirring speed on growth and lipase production by *Candida tropicalis*.

The addition of carbon and nitrogen sources into the lipase production medium showed that carbohydrates used as carbon sources did not give a significant effect on lipase production, however, with olive oil growth increased significantly [Fig. 5 and Fig. 6].

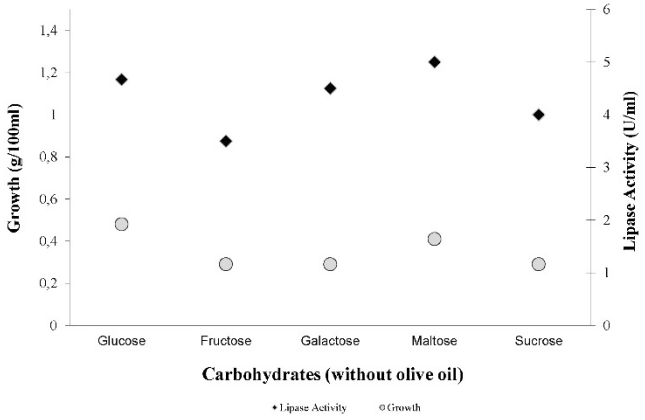

Figure 5. Effect of carbohydrates without olive oil on growth and lipase production by *Candida tropicalis*.

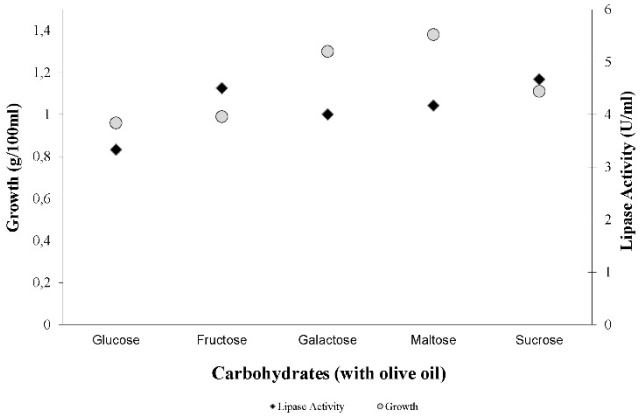

Figure 6. Effect of carbohydrates with olive oil on growth and lipase production by *Candida tropicalis*.

Furthermore effect of oils was determined. It was obvious that oils increased lipase production and growth according to carbohydrate additives. Maximum lipase activity was detected in extra virgin olive oil, corn oil, soybean oil and canola oil as 5.17 U/ml (Fig. 7).

Figure 7. Effect of oils on growth and lipase production by *Candida tropicalis*.

Although carbon sources did not affect lipase production as expected, the addition of nitrogen sources did. Ammonium sulfate increased lipase production more than other nitrogen and carbon sources, with olive oil lipase production was determined as 10.67 U/ml although without olive oil it was 10.33 U/ml [Fig. 8 and Fig. 9]. Ammonium oxalate and ammonium nitrate also showed a distinct effect on lipase production.

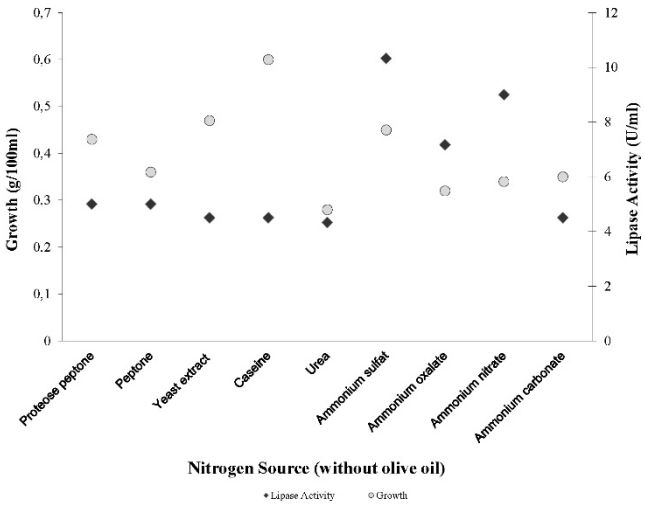

Figure 8. Effect of nitrogen sources with olive oil on growth and lipase production by *Candida tropicalis*.

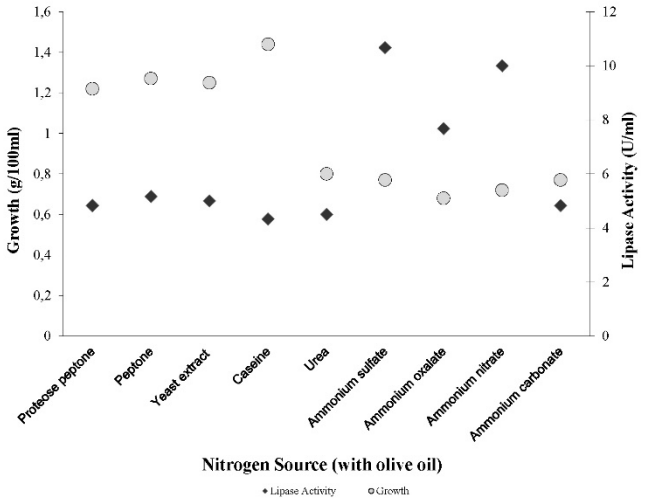

Figure 9. Effect of nitrogen sources without olive oil on growth and lipase production by *Candida tropicalis*.

Discussions

Only about 2% of the world's microorganisms have been tested as enzyme sources (Hasan, 2006). Companies of the enzyme industry continue searching for economical sources of high activity lipases also with simple growth and lipase producing conditions. New lipases from microbial sources have been reported sporadically. The need for novel lipases is obvious, but little effort has been made for conducting a large-scale systematic screening for new lipases (Hou, 1997). *Candida* sp. is the most potential lipase producer from yeasts reported in the literature (Treichel, 2010). According to Vakhlu and Kour (2006), the main terrestrial species of yeasts that were found to produce lipases are: *Candida tropicalis*, *C. rugosa*, *C. antarctica*, *Candida deformans* and *Yarrowia lipolytica*, et al.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors (Treichel, 2010). Therefore many researchers aimed to change the composition of the medium by addition of different carbon and nitrogen sources and changing physicochemical factors such as temperature, pH, and dissolved oxygen (Rajendrean and Thangavelu, 2007; Treichel, 2010). The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer (Sharma et al, 2001; Gupta et al, 2004; Treichel, 2010). However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization (Treichel, 2010).

Different carbon sources affecting growth and lipase production in *Candida rugosa* were studied on a defined medium (Dalmau et al, 2000). And carbohydrates and acids non-related to fats did not induce lipase production. A present study was therefore undertaken to investigate the effect of different growth media (with and without olive oil) supplemented with various nitrogen (yeast extract, tryptone and proteose-peptone) and carbon sources (glucose and fructose) on lipase production by *C. rugosa* (Fadıloğlu and Erkmen, 2002). In this study high yields of the enzyme were obtained with yeast extract and proteose-peptone in the medium with olive oil.

The initial pH of the growth medium and temperature are important for lipase production therefore culture pH and growth temperature should be assayed for lipase production optima. Researchers studied the optimization of lipase production generally adjusted culture pH between 3-11 and growth temperature between 10-70ºC. Stirring speed is as important as carbon and nitrogen sources, the culture pH and growth temperature. Alonso et al, (2005), studied lipase production by a Brazilian wild strain of *Yarrowia lipolytica* (formerly *Candida lipolytica*) at different stirring speeds and air flow rates and maximum lipase activity was detected in the late stationary phase at 200 rpm.

In this study a novel yeast isolated from olive mill soil was identified as *Candida tropicalis* by 18S rDNA

analysis. Optimization of lipase production assayed by carbon and nitrogen sources, culture pH, growth temperature and stirring speed conditions. According to the results obtained, the lipase production medium was modified as (g/l): 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 $ZnSO_4.7H_2O$ and %1 ammonium sulfate, initial pH adjusted to 4.5, and after sterilization 1% olive oil (v/v) added. Maximum lipase production assayed at 30ºC growth temperature, 100 rpm stirring speed at 72 hours incubation.

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