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Screening for industrially important enzymes from thermophilic bacteria; selection of lipase-producing microorganisms and optimization of culture conditions

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Abstract
In this study, 201 thermophilic bacteria that were isolated from natural hot springs in Aydin/Turkey and registered in Adnan Menderes University Department of Biology culture stocks were used. It was determined that 43 of these bacteria exhibited lipolytic activity and 22 of them exhibited lipase activity. These 22 lipase positive isolates were grown in LB broth medium and the quantitative lipase activities were determined. HBB 134 was chosen as the best lipase producing isolate with the activity of 19.925 U/mL. According to 16S rRNA sequences, it was found that the isolate showed maximum similarity (% 99) with *Anoxybacillus flavithermus*. The best enzyme production from HBB 134 was determined in medium including 0.5% olive oil as carbon source and 0.5% pepton as nitrogen source, pH 6.50 and 45 °C. When the isolate HBB 134 was grown in optimum culture conditions it was determined that production of the lipase started at the beginning of the logarithmic growth phase and it reached maximum level in the middle (12 hour) of the logarithmic phase. It was determined that most of the enzyme activity was intracellular.

Keywords: Screening, Lipase production, *Anoxybacillus flavithermus*, thermophilic, culture conditions.

Introduction

Microbes and their enzymes are used in a wide range of biotechnological applications such as, detergent, fine chemicals, pharmaceutical, bioremediation, food, leather, paper and textile industry. The most important enzymes for industry are lipases, carboxylesterases, cellulases, xylanases, pectinases, amylases and proteases. Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are enzymes that catalyze the hydrolysis of mono-, di- and triacylglycerides to glycerol and free fatty acids at an oil-water interface. Lipases due to their application in a wide range of industrial applications have emerged as key enzymes in rapidly growing biotechnology. Lipases are widely used in biocatalysis due to their ability to catalyze not only the hydrolysis of triacylglycerides in aqueous solutions, but also regio-, enantio- and stereoselective reactions in organic media [1]. Lipases are produced by plants, animals and microorganisms. However, microbial lipases including bacteria, fungi and yeast are also more stable than their corresponding plant and animal sourced enzymes and their production is more suitable, economical and safe [2]. Lipase-producing microorganisms are mostly found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, decaying food, compost heaps and hot springs [3]. Lipases produced from different microbial sources have different properties such as pH and temperature stabilities and substrate specificity. Enzymes from thermophilic organisms are more stable than similar enzymes from mesophilic organisms therefore they are more useful for biotechnological applications [4]. Thermostable enzymes are important to have higher reaction rate at higher operation temperature. Substrate solubility can increase at higher temperatures and thereby avoid environmental contamination [5]. In this case, screening of new thermophilic microbial lipases has special importance in finding novel lipases that have suitable properties for different industrial aplications. Lipase production is influenced by the type and the concentration of the carbon and the nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration [6]. Furthermore, the presence of activators, stimulators, inhibitors and the surfactants, in addition to the amount and the source of inoculum can also influence the production of lipases.

There are many reports about the optimum culture conditions and the nutritional requirements for lipase production. [7, 8, 9, 10, 11, 12]. However, none of the reports have been regarding *Anoxybacillus*. The thermophilic bacterium *Anoxybacillus flavithermus* HBB 134 used in this study was isolated from a hot spring in Alangüllü/Aydın (50°C). Various culture growth

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conditions for the HBB 134 strain affecting lipase production were studied using batch cultures in defined medium.

2. Materials and Methods

2.1. Materials

Analytical reagent grade chemicals were purchased from commercial sources at the highest purity.

2.2. Microorganism

The two hundred one thermophilic bacteria strains have been previously isolated from hot springs in Aydın/Turkey by Dr. Gamze Başbülbül. The strains were grown on Luria-Bertani (LB) medium and stored at -80 °C in 20% skim milk solution.

2.3. Screening of industrially important enzymes

The screening of amylolytic thermophilic bacterial strains was carried onto Caso agar plate supplemented with 1% soluble starch. The bacterial strains were streaked as a line on the starch agar plate. After the incubation at 65 ºC for 24 hour, the amylolytic activity was assessed by flooding the plates with 0.3% I_2-0.6% KI solution. Amylase positive strains were determined by the presence of a clear zone of starch hydrolysis around the colony on the starch plates.

Proteolytic production of the thermophilic bacterial strains was screened on Caso agar plates supplemented with 1% skim milk. The bacterial strains were streaked as a line on the skim milk plate. After the incubation at 65 ºC for 24 hour, the plates were observed for clear zone at the end of the incubation period. Clear zone around each isolate indicated a positive result while no clear zone signified a negative result.

The thermophilic bacterial isolates were investigated for their lipolytic activity as reported in the Tween 80 agar method [13]. LB agar medium was supplemented with 0.01% $CaCl_2 \cdot H_2O$ and Tween 80, which were separately autoclaved (Hirayama, HA-40MIV, Japan) for 15 min at 120 °C. Tween 80 was added to the molten agar medium at 45 °C to provide a final concentration of 1%. The medium was agitated until Tween 80 had dissolved completely and then the solution was poured onto Petri dishes. As an indication of the positive outcome for the test, an opaque halo occurred around the colonies.

The isolates that were positive for lipolytic activity were also screened for their lipase activity using Rhodamine B-olive oil agar plate method [14]. LB agar medium was supplemented with arabic gum (to yield a final concentration of 1%) and olive oil, which were autoclaved at 121°C for 15 min. The medium was cooled to about 60 °C and 10 ml Rhodamine B solution (1 mg/ml) and olive oil (to yield a final concentration of 2.5%) were added, and the medium was stirred vigorously and emulsified by mixing for 1 min in a homogenizer (IKA T25B, Germany). Aliquots of 20 ml were poured onto each petri dish and they were left to solidify. Stock cultures were transferred to and incubated in LB agar plates at 65 °C for 24 hours. Each culture was streaked onto the Rhodamine B-olive oil agar plate and was incubated at 65 °C for 24-48 hours. The lipase-producing bacteria were indicated by the presence of orange fluorescent halos around the colonies when the plates were irradiated with 350 nm UV light.

2.4. Selection of best microorganism for lipase production

The lipase producing bacterial strains were cultivated in Erlenmeyer flasks containing 20 mL LB Broth in triplicates. Following incubation at 65 °C and 150 rpm for 24 hours in an orbital shaker (Newbrunswick Scientific Innova 43, USA), the absorbance of the cultures was measured at 600 nm and the lipase activities were determined spectrophotometrically (Shimadzu UV-1700, Japan).

2.5. Identification of HBB 134

One bacterial strain designated as HBB 134 was selected and identified via 16S rRNA gene sequencing. Genomic DNA of the isolate was extracted by using the partially modified phenol-chloroform method as described by Ronimus *et al.* [15]. Then 16S ribosomal DNA was amplified via direct PCR using the following universal primers: 341F (5′-CCT ACG GGA GGC AGC AG3′)-985R (5´ GTA AGG TTC TTC GCG TT 3´) and 20F (5'- AGA GTT TGA TCC TGG CTC AG-3')-1390R (5'- GAC GGG CGG TGT GTA CAA-3') (1300 bp). The PCR products were then sequenced directly in an ABI Prism 3730 XL Genetic analyzer by a professional company (Macrogen Inc., Korea) upon request. The 16S rDNA sequences were analyzed using the GenBank database (www.ncbi.nlm.nih.gov) and the identification was made on the basis of 16S rDNA sequence homology using nucleotide-nucleotide BLAST tool.

2.6. Lipase activity assay

Lipase activities were determined by a spectrophotometric assay using *p*-nitrophenil-laurate (pNPL) as substrate [16]. The reaction mixture consisted of 0.1 ml enzyme solution, 0.8 ml 50 mM Tris-HCl buffer (pH 8.0) and 0.1 ml 10 mM pNPL dissolved in ethanol. The hydrolytic reaction was carried out at 65 °C for 30 min. Following the incubation, 0.25 ml of 0.1 M Na_2CO_3 was added to stop the reaction. The mixture was centrifuged (10.000 × *g*, 15 min) (Heraeus-Biofuge pico, Germany) and the absorbance at 410 nm was determined. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol of *p*-nitrophenol (molar absorption coefficient 17.34 mM^{-1} cm^{-1}) from pNPL in one min under the stated experimental conditions.

2.7. Determination of protein concentration

Protein concentration was determined according to the method by Bradford [17]. Bovine serum albumin was used as the standard.

2.8. Biomass determination

Cells were harvested by centrifugation (15 min, 2500 × *g*) (Heraeus-Labofuge 200, Germany) and washed twice with ultra-pure water. The pellet was dried for 24 h at 80 °C and its dry weight was determined.

2.9. Effects of cultivation conditions

The effect of carbon source on lipase production of the strain HBB-134 was studied using LB containing different carbon sources (olive oil, cottonseed oil, corn oil, soybean oil, almond oil, linseed oil, triolein, glycerol, olive mill wastewater, Tween 20, 40, 60 and 80, glucose, fructose, sucrose and lactose) at concentrations of 0.1, 0.5 and 1%. In the case of oils being used as the carbon source, 0.5% gum arabic was added to the LB medium.

The effect of nitrogen source on lipase production of the strain HBB-134 was studied using the medium containing 0.5% olive oil, 0.5% gum arabic, 1% NaCl and different nitrogen sources (yeast extract, tryptone, peptone, casein

hydrolysate, urea, gelatin, meat extract, ammonium sulfate and ammonium nitrate) at concentration of 0.5%.
The effect of pH on lipase production was investigated with the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic at different pH values (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) and 65°C.
The effect of temperature on lipase production was also investigated with the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C) and pH 6.5.
The 250 mL flasks containing 50 mL of the respective media were inoculated (1%, v/v) with overnight grown culture whose absorbance was adjusted to give 0.1 at 600 nm. The cultures were incubated for 24 hours in an orbital shaker (150 rpm). Following incubation, the lipase activity was determined. Three trials were conducted at each assay and the average values were considered.

2.10. Growth kinetics and cellular localization of lipase
The strain HBB-134 was cultivated for 24 h in 50 mL of the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic at 150 rpm on an orbital shaker at 45 °C and pH 6.5. Culture samples were harvested at specific time intervals to determine the pH, biomass and lipase activity. For the detection of the cellular localization of the enzyme, the culture medium was centrifuged (15 min, 20.000 × *g*, 4 °C) (Sigma-3K30, Germany) and the supernatant (S1) was stored for extracellular enzyme analysis. The cells (P1) were washed twice with ultra-pure water and were re-suspended in equal volumes of Tris-HCl buffer (20 mM, pH 8.0, 4°C). A small volume of this fraction was reserved for the determination of the activity of the intact cells. The cell suspension was sonicated for 10 min, at 40% of the maximum power (BANDELIN SONOPULS-HD2200, Germany). The procedure was carried out in an ice bath. Then, the mixture was centrifuged for 15 min at 4°C and 20.000 × *g*, and the supernatant (S2) was kept for the measurement of the intracellular lipolytic activity.

2.11. Statistical analysis
The experiments were performed in triplicates and the statistical analysis was done with the aid of STATISTICA 7.0. One-Way ANOVA was used for testing the differences between the groups. Paired-samples t test was used to compare the means of the control group and the assay group.

3. Results and Discussion
3.1. Screening of industrially important enzymes
The bacterial strains collected from hot springs were qualitatively screened for their amylolytic, proteolytic and lipolytic activity. Fifty-eight of the two hundred one bacteria strains were observed amylolytic activity on starch agar plates while in that of proteolytic activity fifty two of them were positive on caso agar. In the Tween 80 agar method, forty-three of the bacteria strains were observed lipolytic activity. Of the 43 bacterial strains subjected to Rhodamine B-olive oil agar plate method, only 22 strains showed lipase activity (Fig. 1). In order to select the best lipase producing isolate, all the lipase positive isolates were assayed for lipolytic activity quantitatively. HBB 134 has been selected as the isolate displaying the most enzymatic activity up to 19.925 U/ml and the further studies have been conducted using this isolate.

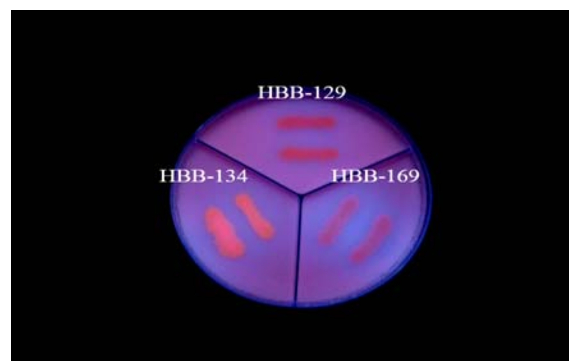

Fig 1: Detection of lipase activity on Rhodamine-B plates. HBB 129 and 134 are positive, HBB 169 is negative cultures.

3.2. Identification of the HBB 134 Strain
The 16S rDNA sequence of the HBB 134 strain showed highest similarity to *Anoxybacillus flavithermus* (99%) according to BLAST results. The HBB 134 strain was registered in the GenBank database system, from which obtained the accession number GQ342689. The phylogenetic tree was represented in Figure 2.

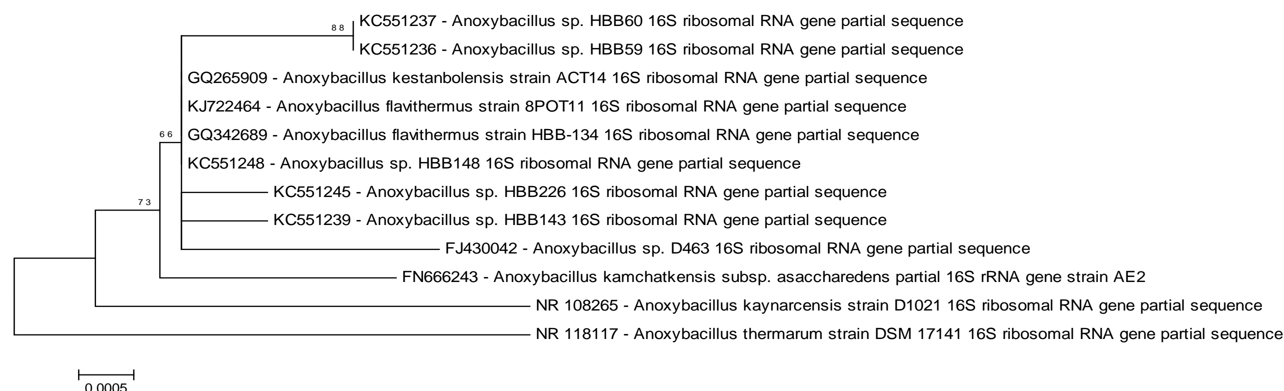

Fig 2: The phylogenetic tree resulting from analysis of the 16S rDNA sequences of *Anoxybacillus flavithermus* HBB 134.

3.3. Effect of carbon and nitrogen sources on lipase production
Lipases are generally produced using carbon sources possessing lipid structures such as triacylglycerols, fatty acids, glycerol or tweens. In addition, other complex sources including sugars, sugar alcohols, polysaccharides and whey

are also used to increase the production of lipases [18].
Sugars, nitrogen compounds, volatile acids, polyalcohols, pectins, oil, polyphenols and tannins providing the color for the olive mill wastewater constitute the main components of the olive mill wastewater [19]. Olive mill wastewater has not been reported to have significant effects on cellular development. Enzyme production was slightly lower in 0.1% concentration than in the control culture and it was gradually increasing up to 1% concentration (Table 1). This increase in activity could be attributed to the oil that the olive mill wastewater contains. Glycerol has been determined to increase enzyme production in increasing amounts from 0.1% concentration up to 1% (Table 1).
While Tween 20 completely inhibits bacterial growth, lipase production has not been determined in media containing Tween 40, 60 and 80 since bacterial growth was very poor (data not shown). Glucose, fructose and sucrose have all caused a decrease in bacterial growth at all concentrations. The highest activity among the sugars, whose effect has been investigated, has been determined upon addition of lactose into the medium. As lactose concentration was increased in the medium, cellular growth was determined to increase, but not the enzyme production, to the same extent. For this reason, the highest activity was determined at a lactose concentration of 0.1% (Table 1). Tan *et al.* [20] have reported that lactose and mannitol have been the carbohydrates that have increased lipase production the most in *Penicillium camembertii Thom* PG-3 among the candidates.

Table 1: Effect of carbon sources on lipase production

Carbon sources	Specific activity (U/mg dry wt cell)		
	0.1 %	0.5 %	1 %
Control 1 (LB medium)	13.44	13.44	13.44
Control 2 (LB medium + gum arabic)*	16.73	16.73	16.73
Olive mill wastewater	12.68	14.36	18.56
Glycerol	14.76	16.72	21.22
Glucose	16.27	14.58	10.68
Fructose	11.72	14.41	3.03
Sucrose	5.80	11.14	9.10
Lactose	19.01	16.09	16.11
Olive oil	17.67	40.38	20.03
Cottonseed oil	12.26	16.55	17.89
Corn oil	16.88	26.79	22.46
Soybean oil	18.82	15.80	14.83
Almond oil	27.03	22.09	21.95
Triolein	32.44	28.83	15.50

Anoxybacillus flavithermus HBB 134 was grown at 65°C for 24 h on LB medium with the addition of the indicated carbon sources. Activity was assayed in the cellular fraction of the cultures. Values are means of three different cultures. *Control 2 used for oils

The effect of lipids added into the medium to induce enzyme production varies from organism to organism. However, lipids are generally known to increase the production of lipolytic enzymes. The sampled oils were observed to increase enzyme production. The highest amount of lipase production was 40.38 U/mg (2.4 fold of the control) in the medium containing 0.5% olive oil (Table 1). Similarly, during the production of lipase by *Aspergillus* sp. [21], *Penicillium restrictum* [22] and *Bacillus* sp. LBN 4 [23], the highest lipolytic activity has been attained in media containing olive oil.
Inorganic nitrogen sources (urea, ammonium sulfate, ammonium nitrate) have increased bacterial growth significantly, but not the amount of enzyme production. The determined activity values were much below that of the control value. The organic nitrogen sources with a richer content have yielded similar results to that of the control medium containing tryptone and yeast extract. Peptone has been determined as the highest enzyme activity yielding nitrogen source with 22.57 U/mg activity. This value is higher than that of LB control medium containing 1% tryptone and 0.5% yeast extract (Table 2). Obtaining higher enzyme efficiency using nitrogen source at a lower concentration (0.5% peptone) than the case for the control experiment indicated more enzyme production from less chemical consumption providing an economical advantage. Organic nitrogen sources were usually preferred in studies reported in the literature [10, 21, 24, 25].

Table 2: Effect of nitrogen sources on lipase production

Nitrogen sources	Specific activity (U/mg dry wt cell)
Control (LB)	20.71
Peptone	22.57
Tryptone	22.42
Yeast extract	19.18
Beef extract	20.80
Casein hydrolysate	17.61
Gelatin	0.78
Urea	0.55
Ammonium sulphate	0.43
Ammonium nitrate	0.35

Anoxybacillus flavithermus HBB-134 was grown at 65°C for 24 h on the medium containing 0.5% olive oil, 0.5% gum arabic, 1% NaCl with the addition of the indicated nitrogen source at a concentration of 0.5%. Activity was assayed in the cellular fraction of the cultures. Values are means of three different cultures.

3.4. Effect of initial pH and temperature on lipase production

Small differences in the pH of the environment may effect bacterial growth and enzyme production. HBB 134 does not grow at pH values below 6.0 or above 8.5 however, since growth is very weak at pH 8.50, activity could not be determined. The highest enzymatic activity was determined as 19.51 U/mg in the medium with pH 6.50 (Table 3). HBB 134 could not grow at 30 °C and the highest amount of bacterial growth has been attained at 50 °C and the highest enzymatic production was attained at 45 °C with an activity of 79.69 U/ mg cell (Table 4).

Table 3: Effect of initial pH on lipase production

İnitial pH	Specific activity (U/mg dry wt cell)
5.00	-
5.50	-
6.00	6.86
6.50	19.51
7.00	13.50
7.50	11.03
8.00	8.81
8.50	-
9.00	-

Anoxybacillus flavithermus HBB-134 was grown on the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic at different pH values for 24 h at 65°C. Activity was assayed in the cellular fraction of the culture. Values are means of three different cultures.

The fact that the isolate could not grow at 30 °C indicated that it was a thermophillic bacterium rather than a thermotolerant isolate. Lipase production (45 °C) and cellular growth (50 °C) did not display any parallelism. Similar results were encountered in different studies. The optimum growth temperature was 65 °C for *Bacillus thermoleovorans* ID-1 whereas the optimum lipase production was attained at 50 °C [26]. The optimum growth temperature was 60 °C for *Thermus thermophilus* whereas the optimum lipase production was attained at 70 °C [27].

Table 4: Effect of temperature on lipase production

Temperature (°C)	Specific activity (U/mg dry wt cell)
30	-
35	62.52
40	72.14
45	79.69
50	71.03
55	48.88
60	40.52
65	18.93
70	2.76
75	3.09
80	4.31

Anoxybacillus flavithermus HBB-134 was grown on the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic at different temperatures for 24 h at pH 6.5. Activity was assayed in the cellular fraction of the culture. Values are means of three different cultures.

3.5. Growth kinetics and cellular localization of lipase

When HBB 134 strain was cultured at the optimum conditions, the enzyme production was determined to begin in early logarithmic phase, peaking in mid-exponential phase (12th hour). Most of the enzymatic activity was identified in the intracellular fraction (S2) (Table 5). However, the enzyme activity determined in the intact cellular fraction (P1) was similar to that, which was observed in the intracellular fraction. This situation indicated that the substrate passed through the cellular wall prior to hydrolysis. Previously conducted studies have also yielded similar results [28, 29, 30]. A decrease in activity has been observed at the 15th hour at all fractions. This loss of activity was thought to result from the loss of stability in extracellular environment. Chen *et al.* [7] have reported that extracellular development of lipase production was dependent upon three factors: (i) protease formation, (ii) pH stability of the lipase, (iii) reactivity of the lipase at different pH values.

pH value of the culture environment began to rise starting from the 3rd hour that the growth began steadily, reaching a value of 8.28 at the end of the 24th hour. This situation indicated that the organism utilizes the compounds in the medium to produce alkali products. If the extracellular enzymatic activity of the isolate were sufficient, it would have been expected that the olive oil in the medium would hydrolyze to form fatty acids and therefore decrease the pH value of the medium. However, since a significant portion of the lipase activity was intracellular, the nitrogen source of the medium was mostly used and alkaline products were formed in return.

Table 5: Lipase production from *A. flavithermus* HBB 134 and localization of lipase

Time (h)	Dry weight (mg/mL)	Volume activity (U/mL)				
		Final pH	S1	P1	S2	P2
0	0	6.50	0	0	0	0
3	0.04	6.58	0	0	0	0
6	0.14	6.56	1.173	4.083	4.846	0.481
9	0.30	7.09	1.192	6.545	6.090	1.955
12	0.34	7.57	1.455	7.013	6.545	1.487
15	0.38	8.04	1.186	4.981	5.698	1.051
18	0.48	8.04	1.583	5.307	5.692	1.147
21	0.44	8.27	1.429	5.237	5.654	1.987
24	0.42	8.28	1.423	5.692	5.307	1.872

Anoxybacillus flavithermus HBB-134 was grown on the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum arabic for 24 h at 45 °C and pH 6.5. S1 was culture supernatant; P1 was intact cells; S2 was supernatant of broken cells; P2 was broken cells.

4. Conclusion

Of the 201 bacterial strains screened on Rhodamine B-olive oil agar plate, 22 strains showed lipase activity. Lipase production is generally induced using several oils. In this study the highest amount of lipase production was 40.38 U/mg in the medium containing 0.5% olive oil. This value was 2.4 fold higher than the control. Evaluation of the results obtained from the investigation of the effect of carbon and nitrogen sources indicated that the medium containing 0.5% peptone, 0.5% olive oil, 0.5% gum arabic and 1% NaCl was determined as “the enzyme production medium”. The highest enzymatic activity was determined as 79.69 U/mg in the enzyme production medium with pH 6.50 and 45°C. However, HBB 134 may be used for enzyme production in a range of temperatures between 40°C and 50°C. Most of the enzymatic activity was determined in the intracellular fraction (S2). However, the enzyme activity determined in the intact cellular fraction (P1) was similar to that, which was observed in the intracellular fraction. Enzyme activity could be assayed using intact cells routinely since the results of S2 and P1 fractions were similar.

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References

1. Dheeman DS, Frias JM, Henehan GTM. Influence of cultivation conditions on the production of a thermostable extracellular lipase from *Amycolatopsis mediterranei* DSM 43304. Journal of Industrial Microbiology and Biotechnology, 2010; 37:1-17.
2. Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. Enzyme and Microbial Technology, 2006; 39:235-251.
3. Wang Y, Srivastava KC, Shen GJ, Wang HY. Thermostable alkaline lipase from a newly isolated

thermophilic *Bacillus* strain, A30-1 (ATCC 53841). Journal of Fermentation and Bioengineering, 1995; 79: 433-438.
4. Imamura S and Kitaura S. Purification and characterization of a monoacylglycerol lipase from the moderately thermophilic *Bacillus* sp. H-257 Journal of Biochemistry, 2000; 127:419-425.
5. Mozhaev VV. Mechanism-based strategies for protein thermostabilization. TIBTECH, 1993; 11:88-95.
6. Elibol M, Ozer D. Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. Process Biochemistry, 2000; 36:325-329.
7. Chen SJ, Cheng CY, Chen TL. (1998). Production of an alkaline lipase by *Acinetobacter radioresistens.* Journal of Fermentation and Bioengineering, 1998; 86: 308-312.
8. Kademi A, Aït-Abdelkader N, Fakhreddine L, Baratti JC. Characterization of a new thermostable esterase from the moderate thermophilic bacterium *Bacillus circulans.* Journal of Molecular Catalysis B-Enzymatic, 2000; 10: 395-401.
9. Dominguez A, Deive FJ, Sanroman MA, Longo MA. Effect of lipids and surfactants on extracellular lipase production by *Yarrowia lipolytica*. Journal of Chemical Technology and Biotechnology, 2003; 78:1166-1170.
10. Li CY, Cheng CY, Chen TL. Fed-batch production of lipase by *Acinetobacter radioresistens* using Tween 80 as the carbon source. Biochemical Engineering Journal, 2004; 19: 25-31.
11. Torres S, Baigori MD, Castro GR. Effect of hydroxylic solvents on cell growth, sporulation, and esterase production of *Bacillus licheniformis* S-86. Process Biochemistry, 2005; 40:2333-2338.
12. Ertuğrul S, Dönmez G, Takaç S. Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improving its enzyme activity. Journal of Hazardous Materials, 2007; 149:720-72.
13. Smibert RM, Krieg NR. (1994). Methods for general and molecular bacteriology. In: Gerhardt, P., Murray, R.G.E., Wood, W.A., Krieg, N.R. editors, American Society for Microbiology. Washington, D.C. 1994, 622.
14. Kouker G, Jaeger KE. Specific and sensitive plate assay for bacterial lipases. Applied and Environmental Microbiology, 1987; 53: 211-213.
15. Ronimus RS, Parker LE, Morgan HW. The utilization of RAPD-PCR for identifying thermophilic and mesophilic *Bacillus* species. FEMS Microbiology Letters, 1997; 47: 75-79.
16. Sigurgisladottir S, Kanarosdottir M, Jonsson A, Kristjansson JK, Mathiasson E. Lipase activity of thermophilic bacteria from Icelandic hot springs. Biotechnology Letters, 1993; 15: 361–366.
17. 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, 1976; 72:248-254.
18. Gupta R, Gupta N, Rathi P. Bacterial lipases: an overview of production, purification and biochemical properties. Applied Microbiology and Biotechnology, 2004; 64: 763-781.
19. Rozzi A, Malpei F. Treatment and disposal of olive mill effluents. International Biodeterioration and Biodegredation, 1996; 38: 135-144.
20. Tan T, Zhang M, Xu J, Zhang J. Optimization of culture conditions and properties of lipase from *Penicillium camembertii Thom* PG-3. Process Biochemistry, 2004; 39: 1495-1502.
21. Cihangir N, Sarıkaya E. Investigation of lipase production by a new isolate of *Aspergillus* sp. World Journal of Microbiology and Biotechnology, 2004; 20: 193-197.
22. De Azeredo LAI, Gomes PM, Sant'Anna Jr. GL, Castilho LR, Freire DMG. Production and regulation of lipase activity from *Penicillium restrictum* in submerged and solid-state fermentations. Current Microbiology, 2007; 54: 361-365.
23. Bora L, Kalita MC. Production and optimization of thermostable lipase from a thermophilic *Bacillus* sp. LBN 4. The Internet Journal of Microbiology, 2007; Volum 4 Number 1.
24. Gulati R, Sexana RK, Gupta R, Yadav RP, Davidson WS. Parametric optimisation of *Aspergillus terreus* lipase production and its potential in ester synthesis. Process Biochemistry, 1999; 35: 459-464.
25. Kumar S, Kikon K, Upadhyay A, Kanwar SS, Gupta R. Production, purification and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. Protein Expression and Purification, 2005; 41: 38-44.
26. Lee DW, Koh YS, Kim KJ, Kim BC, Choi HJ, Kim DS, Suhartono MT, Pyun YR. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiology Letters, 1999; 179: 393-400.
27. Dominguez A, Pastrana L, Longo MA, Rua ML, Sanroman MA. Lipolytic enzyme production by *Thermus thermophilus* HB27 in a stirred tank bioreactor. Biochemical Engineering Journal, 2005; 26: 95-99.
28. Kademi A, Aït-Abdelkader N, Fakhreddine L. A thermostable esterase activity from newly isolated moderate thermophilic bacterial strains. Enzyme and Microbial Technology, 1999; 24: 332-338.
29. Kakariari E, Georgalaki MD, Kalantzopoulos G, Tsakalidou E. Purification and characterization of an intracellular esterase from *Propionibacterium freudenreichii* spp. *freudenreichii* ITG 14. Lait, 2000; 80: 491-501.
30. Bakır Ateşlier ZB and Metin K. Production and partial characterization of a novel thermostable esterase from a thermophilic *Bacillus* sp. Enzyme and Microbial Technology, 2006; 38:628-635.