Isolation and optimization of thermophilic lipase producing bacteria from soil contaminated with used engines oil

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عزلة وتحديد الظروف المثلى للبكتريا المحبة للحرارة والمنتجة لانزيم اللايبيز من الترب الملوثة بزيوت المحركات المستخدمة

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المستخلص

عزلت اثنان وثلاثون عزلة بكتيرية محبة للحرارة ومنتجة لانزيم اللايبيز بطريقة انتقائية من نماذج التربة الملوثة بزيوت المحركات المستهلكة باستخدام وسط Tributyrin الصلب، اجري الاختبار النوعي لتحديد فعالية انزيم اللايبيز في العزلات وباستخدام اطباق Tributyrin الصلبة وعن طريق قياس المنطقة الشفافة، اظهرت العزلات 6T و 14T و29T القابلية الأعلى على تكوين المنطقة الشفافة وبالتالي انتاج الانزيم مقارنتا بالعزلات الأخرى.

اجريت بعدها غربلة ثانوية للعزلات الثلاثة الانشط على انتاج انزيم اللايبيز وباستخدام الوسط الأساسي السائل الحاوي على 1% زيت الزيتون وذ رقم هيدروجيني 7، اظهرت النتائج ان العزلات الثلاثة أعطت إنتاجية انزيمية مقدارها 4 و10 و 7 وحدة \ مل على التوالي، لذا فقد تم اختيار العزلة المحلية 14T ،حيث شخصت بالاعتماد على الفحوصات المظهرية والفسيولوجية على انها *Bacillus licheniformis*.

درس تاثير كل من فترة الحضن ودرجة الحرارة والرقم الهيدروجيني على قابلية العزلة المحلية *Bacillus licheniformis*14T على انتاج انزيم اللايبيز، بينت النتائج ان اعلى انتاجية انزيمية مقدارها 11 وحدة \ مل حصل عليها بعد 3 يوم من الحضانة في الوسط الزرعي السائل ذو الرقم الهيدروجيني 7 والمحضن بدرجة حرارة 50 م°.

الكلمات المفتاحية : انزيم اللايبيز، البكتريا المحبة للحرارة، *Bacillus licheniformis*

Abstract

Thirty two of thermophilic bacterial isolates producing lipase were selectively isolated from soil samples contaminated with used engines oil using Tributyrin agar medium. The qualitative assay of lipase activity was done on Tributyrin agar plates by measuring diameter of the clear zone. The isolates 6T, 14T and 29T of the isolated strains exhibited a greater zone of clearance than the others, indicating higher lipase activity.

Secondary screening for lipase production was done using liquid basal medium with 1% (w/v) olive oil and pH 7. Results from quantitative assay showed that 6T, 14T and 29T isolates appeared highest ability to produce lipase enzyme 4 U/ml, 10 U/ml and 7 U/mlrespectively. Therefore, the 14T isolate was selected and identified based on their morphological and physiological characteristics which was subsequently identified and designated as *Bacillus licheniformis*.

The effect of incubation time, temperature and pH influencing lipase production from *Bacillus licheniformis*14T isolate was determine. Results showed that the maximal lipase production was 11 U\ml after 3 day of incubation at medium pH7 and temperature 50 ^{0}C.

Key words: lipase, thermophilic bacteria and *Bacillus licheniformis*

Introduction

Many attempts have been made to isolate lipase producing microorganisms since this enzyme is used in numerous biotechnological processes including food, leather, cosmetic, detergents and pharmaceutical industries and industrial wastes management (1).Lipases are produced by many microorganisms such as bacteria, yeast, fungi and actinomyces are known to secret lipases. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies and soil contaminated with oil.The oily environment may provide a good environment for isolation of lipase producing microorganisms(2). Microbial lipases are mostly extracellular and their production is greatly influenced by nutritional and physico- chemical factors such as temperature, pH, nitrogen, carbon sources, inorganic salts, agitation and dissolved oxygen concentration. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol(3).Different classes of extracellular lipolytic enzymes been isolated from many different bacterial species, including *Bacillus* and *Pseudomonas* with different properties and specificities. Many microorganisms are capable of producing lipase and *Bacillus* sp. is the most widely studied group. *Bacillus* species lipases have been purified and biochemically characterized from many *Bacillus species such as Bacillus subtilis, Bacillus pumilus, Bacillus stearothermoleovorans* and their genes have been cloned and sequenced(4).

A large number of beneficial thermophiles microorganisms which produced lipases with good thermal stabilities have been found in diverse habitats. Thermophilic bacteria are an important source of thermostable enzymes with properties that are often associated with stability in solvents and detergents, giving these enzymes considerable potential for many biotechnological and industrial applications(5). One of these enzymes is a thermostable lipase enzyme that has been applied to the synthesis of biopolymers and biodiesel and used for the production of agrochemicals, cosmetics, and flavors (6).

According to the fact that extracellular thermophilic lipases are quite stable under extreme temperature and often show more resistance to chemical denaturation,this makes them ideal tools in industrial and chemical processesand generally considered as most important group of biocatalyst for biotechnological applications. A small number of thermophilic lipase producing bacteria have been described in the last decades; a few thermostable lipases have

been isolated from thermophiles and hyperthermophiles sites. The knowledge of thermostablelipolytic enzymes in industrial applications is increasing at a rapid and exciting rate (7). High global demand for lipases makes it third largest group of enzymes based on total sales volume after protease and carbohydrase has resulted in increased number of research to identify, isolate and introduce new lipase-producing microorganisms(8). The present paper focused on screening and isolation of microorganisms and optimization of different parameters for maximal enzyme activity.

Materials and methods

Samples collection

Four soil samples were collected from sites contaminated with used engines oil from Baghdad city. The soil sampleswere taken and transported in sterile plastic bags to laboratory and stored at 4ºC when not used immediately.

Isolation of lipolytic bacterial strains

One gm of each soil sample was agitated in a 50ml of conical flask contained25ml of sterile distilled water for 30 min on a rotatory shaker at 50^0C. This sample was serially diluted up to 10^{-6} using saline(0.9%). 0.1 ml of the last dilution was spread on solid tributyrin agar plates by spread plate technique. The plates were incubated at 50^0C for 48 hours to isolate thermophilic bacterial strains. The tributyrin agar plates contain 1% emulsified tributyrin (v/v), 0.3% yeast extract (w/v) 0.5% tryptone(w/v), 2% agar, and pH was adjusted to 7. After incubation period, the presence of clear zone around the colonies indicated lipase production, each colony showing clear zones around growth were picked, and maintained on LB-agar plates for subsequent analysis(9).

Screening for lipase production

Lipolytic isolates were screened by qualitative plate assay, and all the previous isolated bacteria were inoculated on solid tributyrin agar medium with loopfull from each bacterial isolates in the middle of the agar plate.All plates were incubated at 50˚C for 2 days. The diameter of clear zone was measured for each colonyand graded as strong (+++), moderate (++) and weak (+). (9)

Production of lipase in liquid medium

The isolates showing maximum zone of clearance was selected for further analysis. The composition of production medium (basal medium) used in this study was: (% w/v) yeast extract 0.1; NaCl 0.25; $MgSO_4.7H_2O$ 0.05; $CaCl_2.2H_2O$ 0.01; $K_2HPO_4$0.07; KH_2PO_4 0.03; olive oil 1.0 (%v/v); pH 7.0. Overnight cultures were suspended in 5ml of sterile deionized water was used as the inoculum for pre culture to obtain an initial cell density to adjust the turbidity of 0.5 McFarland standards. Submerged microbial cultures were incubated in 100 ml Erlenmeyer flasks containing 25 ml of sterilized liquid medium on a rotary shaker (150 rpm) and incubated at 50 °C. After 2 days of incubation, the

culture was centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the titrimetric method (10).

Enzyme assay

Lipase activity was measured by titrating free fatty acids released by hydrolysis of olive oil using the titration method (11). Olive oil substrate emulsion contained 10% (w/v) olive oil, 10% (w/v) Arabic gum, 0.5 M sodium chloride and 20 mM calcium chloride was blended for 2 min at the maximum speed in a blender. 20 ml substrate was mixed with 2 ml of the lipase enzyme(cell free supernatant) and incubated in a shaker water bath at 125 rpm for 30 min at 30°C. The lipase solution for the positive control was boiled in a water bath for 10 min before addition of the reaction mixture. The reaction was terminated by adding 10 ml ethanol: acetone (1:1) and titrated with 0.02 N sodium hydroxide until the end point was reached with phenolphthalein (0.1%) as an indicator. One unit of lipase activity (U) was defined as the release of 1 µmol of fatty acid per min under the conditions above (10).

Identification of lipase producing isolate

The isolate that showed a higher lipase activity was subjected to identification. It was performed based on their morphological, physiological and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology (11).

Optimization of lipase production:

1- Effect of time

The effect of incubation period on lipase production was tested by using 25 ml of liquid basal medium in 100 ml Erlenmeyer flasks, pH adjusted at 7.0 and supplemented with 10% olive oil as a substrate. Flasks were sterilized by autoclave. After sterilization the media was inoculated with bacterial inoculums in each flask and incubated at 50˚C for different time period (1, 2,3, 4, 5 and 6)days at 150 rpm . After incubation period, the cultures were centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the titrimetric method (12, 13).

2- Effect of temperature

Sterilized basal medium, pH7.0 was used to study the effect of temperature on lipase production. The flasks were inoculated with bacterial inoculums in each flask and incubated at several temperatures (40, 45, 50**,** 55and 60 ˚C**)** for 3 days at 150 rpm. After incubation period the cell free supernatant was used to determine the lipase activity (12, 13).

3- Effect of pH

The effect of pH was determined by preparation of sterilized basal liquid medium with different pHs value from 5.0 - 9.0 (5.0,6.0,7.0, 8.0 and 9.0) respectively using 1N HCl and 1N NaOH solutions for adjusting. The flasks were inoculated with bacterial inoculums in each flask and incubated at 50 ˚C for 3 days. After incubation period, the cell free supernatant

was used to determine the lipase activity (12, 13).

Results and discussion

Isolation and screening of lipase producing bacteria

Thermophilic microorganisms can be isolated from natural environment such as compost and soil, also thelipase producing microbes have been found in diverse habitats such as industrial wastes and soil contaminated with oil (14).Thirty two different isolates of lipolytic thermophilic bacteria were isolated from four soil samples contaminated with used engines oil using solid tributyrin agar plates by spread plate technique. Table (1),also results in table(1) confirmed that all isolates were potent to produce lipase and also indicate that lipolytic bacteria are widespread in the oil-contaminated environments.

Table(1): Thermophilic bacterial isolates isolated from soil samples obtained from four sites contaminated with used engines oil using solid tributyrin medium with 1% of olive oil as a substrate. Plates were incubated for 3 days at 50˚C.

No.	Contaminated soil samples	Number of bacterial isolates that we obtained
1	first	5
2	second	10
3	third	8
4	fourth	9
Σ		32

Then the isolated strains were screened for extracellular lipase on solid tributyrin agar medium and observed for the presence of clear zone around the colonies. Table(2) shows that three of them 6T, 14T and 29T were produced a big clear zone than the others, and indicating higher lipase activity.

Table (2):Primary screening for thermophilic bacteria to produce lipase enzyme on solid tributyrin medium with 1% of olive oil as a substrate. Plates were incubated for 3 days at 50˚C.

Symbol of isolates	Lipase activity (mm)	Symbol of isolate	Lipase activity (mm)	Symbol of isolate	Lipase activity (mm)
1T	11	12T	8	23T	11
2T	14	13T	9	24T	12
3T	9	14T	27	25T	13
4T	11	15T	12	26T	7
5T	10	16T	14	27T	11
6T	17	17T	13	28T	6
7T	12	18T	10	29T	20
8T	7	19T	11	30T	12
9T	5	20T	8	31T	9
10T	12	21T	6	32T	13
11T	6	22T	9		

The selected isolates 6T, 14T and 29T were then screened based on their ability to produce lipase enzyme in liquid basal medium with 1% of olive oil after 2 days of incubation.A result shows that 14T isolate exhibited a maximum lipase production, which produced 10 U/mlwas selected for further research, while other two isolates 6T and 29T showed 4 U/ml, and 7 U/ml respectively, Table (3). Therefore, the bacterial isolate which showed maximum lipase production was further characterized and identified by morphological, biochemical characteristics, results

suggested this isolate as *Bacillus licheniformis* according to Bergey's Manual of Determinative Bacteriology.

Table (3): Secondary screening for thermophilic bacteria to produce lipase enzyme in liquid basal medium with 1% of olive oil as a substrate. Flasks were incubated for 2 days at 50˚C.

No.	Bacterial isolates	Lipase activity(U/ml)
1	6T	4
2	14T	10
3	29T	7

Production of extracellular lipase

Most extracellular bacterial lipases are influenced by nutritional and physiological factors and these factors such as pH value, incubation period, temperature, agitation and carbon sources are demonstrated to obtain the best optimum conditions for highly growth and lipolytic activity(15).The best thermophilic lipase-producing bacteria isolate *Bacillus licheniformis* was selected to various characterizations.

1- Effect of time

The effect of different incubation periods on lipase production by the *Bacillus licheniformis* was evaluated from 1 to 6 day. The isolate was inoculated in liquid basal medium and was harvested at one day interval. The maximum enzyme activity (13 U/ml) was observed after 3 days of incubation, Figure (1). Furthermore, the enzyme activity was gradually decreased after 3 days.

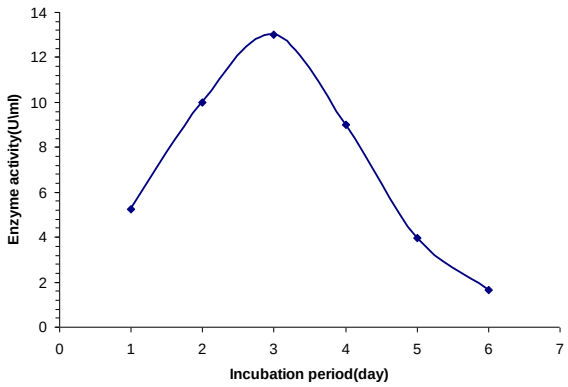

Figure (1): Effect of different incubation period on lipase production (IU/ml) by the selected bacterium *Bacillus licheniformis*.

2- Effect of temperature

Different temperatures were tested for lipase production by culturing *Bacillus licheniformis* at temperatures from 40 to 60°C in liquid basal medium, Figure (2), the highest enzyme production by isolate was obtained at 50 °C, while the enzyme production has slightly decreased after 50°C, which indicates that these isolate is able to grow and produce lipase at higher temperatures.

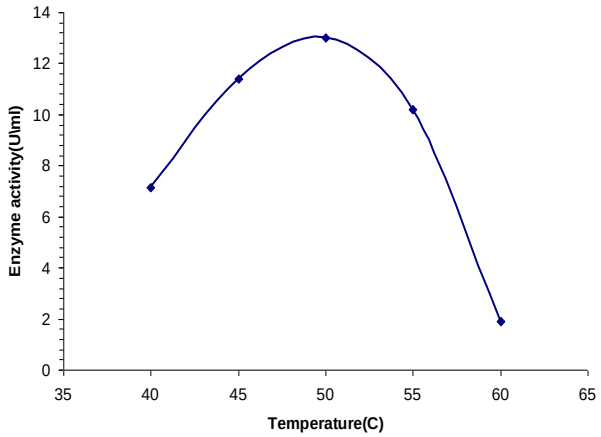

Figure (2):Effect of different temperature on lipase production (IU/ml) by the selected bacterium *Bacillus licheniformis*.

3-Effect of pHs

The *Bacillus licheniformis* was inoculated in liquid basal media with 1% of olive oil. The pH of the medium adjusted to different pHs and incubated at 50 °C. The samples were withdrawn after 3 days and the lipase activity obtained was determined. The maximum activity of 13 U/ml was found to be expressed at pH 7, Figure (3), and whilethe enzyme activity decreased rapidly at alkaline pHs.

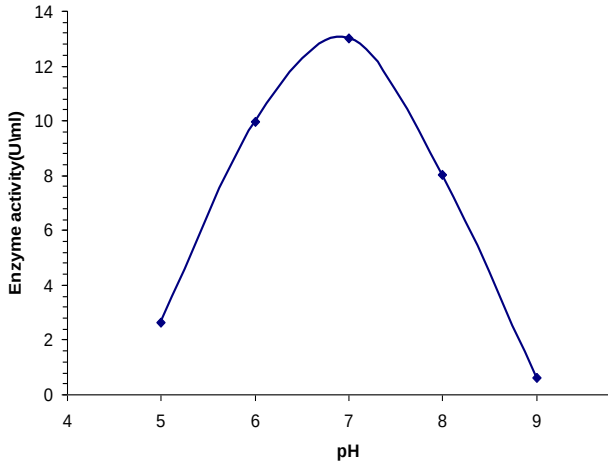

Figure (3):Effect of pH_S on lipase production (IU/ml) by the selected bacterium *Bacillus licheniformis*.

Bacteria were the predominant organisms isolated from the samples. The existence of lipase producing microorganisms in diverse environment such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, compost and hot springs have been studied(15). Many researchers have isolated the lipase producing microbes from the different sources. Highly thermostable extracellular lipase producing *Bacillus* strain isolated from a hot spring(14), several lipase-producing bacteria were isolated from wastewater of an oil processing plant and the strain possessing the highest lipase activity was identified as *Pseudomonas aeruginosa*. Also, the enzyme exhibited maximum lipolytic activity at 45°C, pH 8.0 after 1 day (16).Tributyrin agar plate assay is the most common methods reported for measuring the activity of lipases by the appearance of degradation haloes on culture media supplemented with desired substrates tributyrin. The production of bacterial lipase by tributyrin agar method was done. Among the two hundred isolates screened for lipolytic activity, thirty two isolates exhibited high lipolytic activity (> 50 mm), ten isolate showed moderate activity (25 to below 50 mm), fifty three isolates showed low lipolytic activity (< 25 mm) and one hundred and five isolates have no lipolytic activity(9). Bacterial Lipase producers were isolated from oil spilled soil samples.Among the 200 isolates 20 shows clear zone in the tributyrin medium. The bacterial isolate which showed lipolytic activity was screened for lipase production in the screening medium. In the screening medium the isolate *Pseudomonas gessardii* shows maximum lipase production which produced 12U\ ml, while others showed less than 5U\ ml. Also, the results showed that the lipase production was maximum at pH7, temperature 37^0C and incubation time 2 days (15). Twenty eight lipase producing thermophilic bacterial isolates were isolated. One strain was selected as a best thermophilic lipase producer and identified

as *Bacillus stearothermophilus*. Different factors such as temperature, pH and incubation period were studied for improving lipase production. Maximum lipaseproduction was achieved at 60ºC, after 24 h. of fermentation at pH 10(17). A total of 93 isolates of thermophilic lipase-producing bacteria were isolated from soil samples. Two isolates *Burkholderiapseudomallei* and *Staphylococcus Pasteuri* showed the highest lipase production (14). Five lipases-producing thermophilic bacteria strains were isolated from Hot Spring samples using Rhodamine B-olive oil agar method and identified as *Bacillus halodurans*(18).Six strains of thermophilic extracellular-lipase producing isolates were isolated from oil-contaminated soil samples by rhodamine B plates at 55 °C and the isolates were identified as genus *Bacillus*(19). Totally, twenty two of thermophilic bacterial strains were isolated from water samples using tributyrin agar method.Two bacterial isolates which identified as species of *Bacillus* and *Aeribacillus* showed maximum production of lipase at 50 ^{0}C in liquid medium (20). The lipase enzyme produced from *Bacillus sp* at different range of temperature was from 0.12 U/ml to 1.34U/ml. The optimum temperature for lipase enzyme production was at 45°C (1.34U/ml) and the enzyme production was affected and decreased after increase of temperature above 45°C to 60°C (21).

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