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ISOLATION AND IDENTIFICATION OF LIPOLYTIC BACTERIA AND PRODUCTION OF LIPASE

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ABSTRACT

Microbial lipases have highest potentiality due to their reaction selectivity and mild conditions. Accordingly, this work was suggested to isolate and identify some microorganisms capable of lipase production and to optimize the culture conditions for these microorganisms to produce lipase. The organisms were isolated from clayey soil, and identified as *Pseudomonas aeruginosa*, *P. fluorescens* and *Bacillus cereus*. Incubation periods for lipase production from, *P. fluorescens* *P. aeruginosa* and *B. cereus* were one day, 2 days and 3 days, respectively.

The maximal yield of lipase production from *P. aeruginosa*, *P. fluorescens* and *B. cereus* was observed at 30°C. The optimal pH value was 6.0. The maximum enzyme yield was obtained when the production medium was supplied with 1% glucose as a carbon source in the presence of olive oil. The highest growth and lipase production were observed with olive oil as inducer at 1% concentration. Three organic and inorganic nitrogen sources were added separately to the fermentation media, peptone plus urea gave maximum production of lipase (10.9 µmol FFA / ml / min) at nitrogen (N) concentration of 0.92% for *P. aeruginosa*, while *P. fluorescens* recorded 8.50 µmol FFA/ ml/ min and *B. cereus* 4.98 µmol FFA / ml / min.

Keywords:Lipase production, lipolytic bacteria, *P. aeruginosa*,*P. fluorescens* and *B. cereus*.

INTRODUCTION

Many of bacteria were isolated from soil, and they were screened for lipases production (Kokusho *et al.*, 1982). Also, Pabai *et al.* (1996) were isolated lipase-producing bacteria and identified them as: *Pantoea agglomerans* and *Pseudomonas fluorescens*; the presence of butter fat in the growth medium induced more lipase production. Many species of bacteria are known as lipase producers. These bacterial species belong to the genera *Bacillus*, *Pseudomonas*, *Micrococcus*, *Achromobacter*, *Streptococcus*, *Staphylococcus* and *Propionibacterium* (Iwai *et al.*, 1980 and Sugiura, 1984). *P. aeruginosa* (Fernandez et al., 1989 and Bloquel, 1991) and *Bacillus subtilis* (Kennedy and Lennarz, 1979). *Bacillus cereus* and a number of the fluorescent species of Pseudomonas also produce extracellular phospholipase C (Khan *et al.*, 1967; Owens, 1978 and Griffiths, 1983).

Factors affecting microbial extracellular lipase production have been widely investigated in bacteria (Makhzoum *et al.*, 1995 and Ammar & McDaniel, 1984) and can be introduced that incubation period for 10 hours was optimum lipase synthesis by *Pseudomonas fluorescens* 2D (Makhzoum *et al.*, 1995) and the optimal temperature for lipase production by *Pseudomonas fluorescens* 2D was 20°C. Intermediate range of pH 5-8 was optimal for production by *Pseudomonas fragi* (Pabai *et al.*, 1995); pH 6 for *Pseudomonas fluorescens* (Kosugi and Suzuki, 1992).

Accordingly, glucose supported growth (Makhzoum *et al.*, 1995), addition of olive oil to the *Bacillus coagulans* growth medium enhanced the

lipase production (El-Shafei and Rezkallah, 1997). When the media were supplied with serine, arginine and leucine the maximal yield of lipase were produced by *A. anthiceus* and *B. circulans* (Elwan *et al.*, 1985 and Mohamed *et al.*, 1985).

In this work it was suggested to isolate and identify many microorganisms capable for producing microbial lipases and to optimize the culture conditions for these microorganisms to produce lipase.

MATERIALS AND METHODS

Isolation of micro-organisms:

Ten grams of clayey soil samples collected from the top 10 cm were suspended in sterile distilled water (1: 10 dilution) and shaken for 5 min on a rotary shaker (120 rpm) and 1 ml suspension was plated on nutrient agar containing olive oil as a sole source of carbon and energy. Lipolytic bacteria which formed colonies with wide shallow pits and/or surrounded by a large and clear halo and appeared green blue after adding a saturated 20% $CuSO_4$, indicating that lipids were decomposed. Colonies were picked, and the purity of each culture was checked first by streaking several successive times on nutrient agar until purity, after that, the colony was picked and maintained in stock on nutrient agar slopes. Twenty five bacterial isolates were obtained. Morphological, cultural, biochemical and physiological characteristics of these isolates were studied in duplicate series for the purpose of characterization.

Characterization of the bacterial isolates:

The schemata used for identifying these representative bacterial isolates were combination of those described by Krieg and Holt (1984). These bacterial isolates were selected and tested for: morphological, biochemical and physiological characteristics according to methods and techniques referred to by El-Shafey (1999).

Preparation of inoculum:

Test medium was inoculated according to Sugiura *et al.* (1977): Agar slants were inoculated with organisms, each in duplicate and after 48 hours of incubation at 30°C, fairly good growth was obtained. The growth on agar slants was scraped using 5 ml of sterile tap water and dispensed in a flask containing 50 ml of the medium. These cell suspensions were considered as suitable inocula.

Production of lipase:

Bacteria were grown on the appropriate media. After an incubation time, depending on testing factor examined, the contents of the flasks were centrifuged at 4000 rpm for 15 min. culture supernatant was used as a source of crude enzyme.

Culture procedure:

Fifty ml portions of the fermentation medium, i.e., basal mineral salt solution of Sugiura *et al.* (1977), were dispensed in 250 ml conical flasks,

sterilized at 121°C for 15 min. Five ml inoculum were added to each flask. After incubation at 30°C for 3 days, the flasks were centrifuged. The lipase activity was estimated in culture fluids in three replicates.

Estimation of lipase activity:

Lipase activity was estimated as described by Oi *et al.* (1969) with some modification as follows:

The reaction mixture contained 5.0 ml of 5.0% olive oil emulsion in 7% gum acacia, 5.0 ml of 0.2 M Tris-HCl buffer (pH 7.4), 2.0 ml 0.2 M $CaCl_2$, 1.0 ml enzyme solution and 2.0 ml glass distilled water. After the incubation period under the assay conditions, the total amount of liberated fatty acids was titrated against 0.01 mol/L NaOH. The blank was an assay mixture containing boiled enzyme. Lipase calculated according to Xia *et al.* (1996).

Enzyme unit is defined as the amount of enzyme that liberates 1 μmol free fatty acids (FFA) from olive oil in 1 min under the analytical conditions.

Factors affecting lipase production:

1.Effect of environmental conditions:

Incubation period:

This experiment was designed to investigate the influence of fermentation time course. Basal medium was used for bacterial lipase production. Fermentation flasks were incubated at 30°C, and pH 6. Crude enzyme preparations were taken after 12, 18, 24, 48, 72, 96, 120, 144 and 168 hours of incubation for assaying lipase activity.

Incubation temperature:

The production of lipase was tested at different temperatures i.e. 25; 30; 35 and 40°C. Cultures were incubated at these temperatures to the period resulted from experiment (1) "Incubation period"

Medium initial pH:

The basal medium was adjusted to pH ranging from 4 to 9 by using NaOH and HCl to study the effect of initial pH on the growth and lipase production.

2.Effect of nutritional factors:

Carbon sources:

Eight carbon sources were added separately to the basal medium at a concentration of 1% (w/v) to study the effect of each carbon source on production of lipase. The experiment was carried out at 30°C for different times according to selected strain.

Lipid materials:

Six lipid materials were added separately at concentration of 1% to the medium, which contained glucose at optimal concentration and against control without sugar source in the medium.

Lipid materials concentrations:

Different concentrations of olive oil ranged from 0.5-4.0% were added to the production medium to choose the optimal concentration for lipase activity.

Nitrogen sources:

Organic and inorganic nitrogen compounds were added to the production medium to study their effect on lipase production.

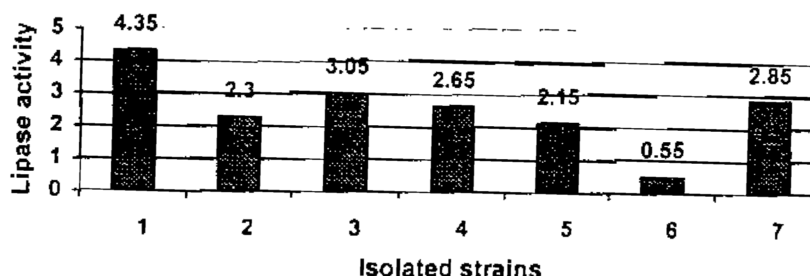
Nitrogen sources concentrations:

Bacterial strains were grown in the production medium provided with different concentrations of peptone and urea ranging from 1.5-5.5% (w/v) to select the optimal concentration.

RESULTS AND DISCUSSION

Isolation, screening and identification of the lipolytic bacteria:

Diluted soil samples with sterile water were plated on several different media containing olive oil. Seventy five isolates were streaked and grown at different temperature degrees after streaking on indicator nutrient agar plates and incubated to the suitable incubation period The colonies, proven to produce large hallow zones around their central growth were selected and purified by re-streaking on the same medium. After several plating times on the previous medium using dilution method as well as several successive streakings. Cultures were considered pure when they showed uniform morphological features and as microscopical examinations revealed no contaminating organisms. Seven bacterial isolates were tested and selected according to their lipase activity Fig1.



Lipase activity = μmol FFA / ml / min

Fig. 1 :Lipase activity of isolated strains.

The morphological; physiological and biochemical characteristics of the seven isolates are given in Tables 1-4. Relying upon the standard identification keys previously mentioned, three strains, viz., 1, 2 & 3 are belonging to genus *Pseudomonas* and falling within *Pseudomonas aeruginosa;Pseudomonas* sp. and *Pseudomonas fluorescens* respectively. While the other three strains, viz., 4, 5 & 6 are belonging to genus *Micrococcus* and the last strain, *viz*., 7 is closely related to genus Bacillus and could be identified as *Bacillus cereus (*Krieg and Holt, 1984).

Table 1: Morphological properties of the isolated strains.

Isolate No.	Criteria			
	Cell morphology (shape)	Cell dimensions (µm)	Endospore	Motility
1	Short rod	1.5 x 0.7	-	+
2	Short rod	1.2 x 0.5	-	+
3	Short rod	2.2 x 0.8	-	+
4	Coccoid	0.7 x 0.7	-	-
5	Coccoid	1.3 x 1.3	-	-
6	Coccoid	1.0 x 1.0	-	-
7	Rod	1.6 x 0.8	+	+

Table 2a: Biochemical characteristics of the isolated strains.

Isolate No.	Criteria								
	Nitrate reduc.	Catalase Test	MR test	Indole produc.	Starch hydrol.	Cellulose decomp.	V.P. test pH 7, 5.5, 8	Citrate utiliz.	Litmus milk reaction
1	+++	+	-	-	-	+	---	+	Peptonization
2	++	+	-	-	-	-	---	+	Peptonization
3	+++	+	-	-	-	+	---	+	Peptonization
4	-	+	-	-	-	-	---	-	No change
5	+	+	+	-	-	-	---	-	No change
6	-	+	-	-	-	-	---	-	No change
7	+	+	-	-	+++	+	---	+	Peptonization

Table 2b: Biochemical characteristics of the isolated strains.

Isolate No.	Criteria									
	Gram react-ion	Casein hydrol.	Gelatin Lique-fication	Temperature °C			Growth in NaCl (%)			
				4	30	40	2	5	7	10
1	-	+	+	+	+	-	+	+	+	-
2	-	+	-	-	+	+	+	+	+	-
3	-	+	+	-	+	+	+	+	+	-
4	+	-	-	-	+	+	-	-	-	-
5	+	+	-	+	+	+	+	+	+	-
6	+	+	-	+	+	+	-	-	-	-
7	+	+	+	-	+	-	+	+	+	-

It is also worth pointing out that (Iwai *et al.*, 1980 and Sugiura, 1984) have drawn the attention to the role of many genera such Bacillus, *Pseudomonas*, *Micrococcus*, *Acromobacter*, as lipase producers. Furthermore, (Khan *et al.*, 1967; Owens, 1978 and Griffiths, 1983) pointed out that typical of lipolytic bacteria are species of *Bacillus* (*B. cereus*) and *Pseudomonas* (*P. fluorescens*), but did not refer to genus *Micrococcus*.

Table 3: Acid formation from fermentation of carbohydrates by isolated strains.

Criteria	Monosaccharides										Disaccharides		Polysaccharides				Alcohols			
	Galactose		Glucose		Arabinose		Sorbose		Raffinose		Lactose		Dextren		Xylose		Mannitol		Glycerol	
Isolate No.	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
1	-	-	++++	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-
2	++++	-	++++	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-
3	++++	-	++++	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-
4	-	-	++++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	+++	-	++++	-	-	-	-	-	-	-	++++	-	±	-	-	-	-	-	++++	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, Acid produced; -, Acid not produced; ±, Slightly acid.

Among these bacterial isolates seven tested organisms were selected with considered highly producing lipase and identified; three of them (1, 3 and 7) having highest lipolytic activity and they were used in the following experiments. These seven isolates were submitted to morphological, cultural, biochemical and physiological characteristics and these organisms were examined in duplicate and obtained data are given in Tables 1 to 4. All the isolates are aerobes, catalase positive, they are not indole or acetyl methyl carbinol (a.m.c.) producers and they can be arranged into three major groups.

Table 4 : Assimilation of carbohydrates as sole carbon source by the isolated strains.

Isolate No.	Criteria							
	Monosaccharides				Disaccharides	Polysaccharides	Alcohols	
	Galact-ose	Sorbose	Glucose	Raffin-ose	Lactose	Dextren	Mannit-ol	Glycerol
1	+++	+++	+++	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	+++	+++	+++	++\	+++
5	+++	++	+++	+	+++	+++	+++	+++
6	+++	+++	+++	+++	+++	+++	+++	+++
7	++	+++	++	++	++	+++	+	+

+++, Good growth; ++, Medium growth; +, Growth

The first group includes isolates Nos. (1, 3). Isolate No. 1 and isolate No. 3 have highly lipase activity 4.35, 3.05 μmol FFA/ ml / min, respectively. Whilst lipase activity of isolate No. 2 was 2.30 μmol FFA/ ml /min.

The third group includes one isolate No. (7). It has lower production of lipase activity than isolate 1 and isolate 3, and its quantity was 2.85 μmol FFA/ml/min.

The selected seven bacterial strains and their lipase activity are shown in Table 5.

Table 5: Lipase activity of selected bacterial strains.

Strain No.	Scientific name of organisms	Lipase activity (μmol FFA/ml/min)
1	*Pseudomonas aeruginosa*	4.35
2	*Pseudomonas* sp.	2.30
3	*Pseudomonas fluorescens*	3.05
4	*Micrococcus* sp. (I)	2.65
5	*Micrococcus* sp. (II)	2.15
6	*Micrococcus* sp. (III)	0.55
7	*Bacillus cereus*	2.85

Culture conditions: Incubation period, 3 days; temperature, 30°C; initial pH, 6.

Lipase production:

1- Effect of environmental conditions:

Incubation period:

Production of lipase by bacterial strains is affected significantly by incubation period. Many workers showed that lipase activity increased steeply with time (Pabai *et al.*, 1995). Table 6 show the maximum production of lipase was found to be 2.5 µmol FFA/ml/min by *B. cereus* after 3 days of incubation. While in *P. aeruginosa* the maximum enzyme production was reached after 2 days (3.5 µmol FFA/ml/min). The observed decrease of enzyme activity may be due to the attack of enzyme by proteinases.

Table 6: Effect of incubation period on lipase production by lipolytic bacteria.

Incubation period (h)	micro organism								
	Bacillus cereus			*Pseudomonas fluorescens*			*Pseudomonas aeruginosa*		
	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
12	0.960	0.55	6.21	1.000	0.75	6.30	1.224	1.0	6.70
18	1.130	0.83	6.50	1.500	1.50	6.40	1.424	1.8	6.95
24	2.054	1.20	6.82	1.949	3.40	7.90	1.897	2.0	7.20
48	2.076	1.50	6.85	1.962	3.00	8.00	2.008	3.5	7.45
72	1.988	2.50	7.05	2.119	2.75	8.20	2.723	3.0	7.80
96	2.311	2.20	7.40	3.518	2.00	8.70	3.191	2.5	8.30
120	4.464	1.20	7.50	3.928	1.75	8.90	3.417	2.4	8.70
144	4.793	0.70	7.80	3.718	0.80	9.00	3.632	2.0	8.93
168	4.806	0.50	7.87	2.922	0.50	9.35	3.019	1.2	9.20

Culture conditions = Temperature, 30°C; initial pH, 6.
Lipase activity = µmol FFA/ml/min,
Growth = O.D. 600 nm,
Final pH = Arbitrary units.

Much differences in data concerning the time elapsed to maximal production of lipases are reported by Gomaa and El-Shawaf (1998) who found that the maximum of relative activity of *Micrococcus* sp. was reached after 4 days at 30°C. While El-Shafei and Rezkallah (1997) reported that the lipases of *B. cereus* and *B. coagulus* reached a maximum level after incubation for two days with continuous agitation. Also, Songgang *et al.* (1997) showed that maximal lipase production by *P. pseudoalcaligenes* was after 34 hours of incubation at 24°C similar to 26°C. Pabai *et al.* (1995) reported that *P. fragi* CRDA 323 lipase maximum was after 4 days and lipase activity/weight ratio of dry cells per milliliter of culture did not substantially drop after that. Qiaoqin *et al.* (1992) show that a bacterial strain F-1903 was isolated from Fujian province soil with maximal lipase production after 28 hours. In the present work the strain *P. aeruginosa* attained maximum production within one day.

Incubation temperature:

The results of incubation temperature are given in Table 7. The results of final pH showed slightly acidic trend in case of *B. cereus* from pH

6.70 to 6.10 within 72 hours, nearly neutral 7.40, 6.78 in case of *P. fluorescens* and 7.80, 7.2 in case of *P. aeruginosa* at 25, 40°C but alkaline 8.50, 8.91 in case of *P. fluorescens* and 8.50, 9.00 with *P. aeruginosa* at 30, 35°C.

Similar results were reported by Gomaa and El-Shawaf (1998) with *Pseudomonas* sp.; Korish (1994) with *Rhizopus arrhizus*; Shady (1993) with *P. aeruginosa* and *B. subtilis* and Okeke and Okolo (1990) with *Acremonium strictum*, who reported that maximum enzyme production was attained at 30°C. Also, Motawee (1995) indicated that the optimum production and high amount of lipase enzyme were at 20-30°C for *P. fluorescens* after 4 days.

Table 7: Effect of incubation temperature on the biosynthesis of lipase production by lipolytic bacteria.

Temperature °C	Microorganism								
	Bacillus cereus[1]			*Pseudomonas fluorescens*[2]			*Pseudomonas aeruginosa*[3]		
	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
25	2.563	0.40	6.70	2.655	1.025	7.40	2.324	0.124	7.80
30	2.727	2.50	6.50	2.400	3.400	8.50	2.478	4.450	8.50
35	0.323	1.40	6.22	3.526	2.150	8.91	3.448	1.100	9.00
40	0.202	0.05	6.10	0.261	0.200	6.78	1.261	0.80	7.20

Culture conditions= [1]**incubation period 3 days; initial pH, 6.**
[2] **Incubation period 1 day; initial pH, 6.**
[3]**Incubation period 2 days; Initial pH, 6..**

Lipase activity = **µmol FFA/ ml/min,**
Growth = **O.D. 600 nm,**
Final pH = **Arbitrary units.**

Culture pH:

In this experiment, the influence of pH on the production of lipase was investigated. Table 8 show growth and lipase production by different microorganisms. It was shown that the pH value highly affected lipase production in culture filtrates of all tested strains. From the obtained results, it could be noticed that *B. cereus* attained optimal lipase activity at pH 6 and its final pH was acidic 6.32, but this was slightly alkaline in case of *P. fluorescens* and *P. aeruginosa* (7.40 to 7.5, respectively).

Table 8: Effect of initial pH-value on lipase production by lipolytic bacteria.

Initial pH	Microorganism								
	Bacillus cereus[1]			*Pseudomonas fluorescens*[2]			*Pseudomonas aeruginosa*[3]		
	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
4	0.419	1.0	4.85	0.233	0.40	5.60	0 41	0.075	5.4
5	0.538	1.05	6.01	0.354	0.90	6.85	0.443	0.600	6.3
6	2.727	2.65	6.32	2.400	3.40	7.40	2.278	5.000	7.5
7	2.826	2.00	7.20	2 420	3.00	8.20	2.400	3.050	8 4
8	3.472	1.85	7.75	2.400	2.80	8.50	2.308	2.600	8.7
9	2 539	0.50	7.90	2.3900	2.10	8.84	2.202	1.500	9.2

Culture conditions= [1]**incubation period, 3 days; temperature, 30°C.**
[2] **Incubation period 1 day; temp., 30°C.**
[3]**Incubation period 2 days; initial pH, 6..**

Lipase activity = **µmol FFA/ml/min,**
Growth = **O.D. 600 nm,**
Final pH = **Arbitrary units.**

These findings are in agreement with those obtained by many workers who found that the optimum pH was 6.0 for lipase production by different species of bacteria and fungi such as *P. aeruginosa* and *B. subtilis* (Shady, 1993). *Aspergillus fumigatus* (Mohamed *et al.*, 1988) and *B. stearothermophilus* (Ammar and McDaniel, 1984). They are on the other hand in contrast with those reported by Gomaa and El-Shawaf (1998) with *Pseudomonas* sp. and *Micrococcus* sp. who recorded that lipases production was with maximum at pH 8 and gradual decreased thereafter. In addition low or high pH can cause inactivation due to denaturation of the enzyme protein.

Extreme alkaline optimal pH ranges were found by Songgang *et al.* (1997) at pH 9.4 or 9.5 by *P. pseudoalcaligenes*. pH 8.5 for *P. fragi* and other *Pseudomonas lipases* (Gilbert *et al.*, 1991) and different strains of *P. fragi* at pH 9.0 (Nishio *et al.*, 1987) and pH 9.5 (Watanabe *et al.*, 1977) as produced by *P. fragi* CRDA 323. However, the lipases from fungi such as *R. delemar* (Iwai and Tsujisaka, 1974) were reported to be more active in an acid region. The range of production in this work fall between 5.6-8.5.

2- Effect of nutritional factors:

Carbon source:

The present study was carried out to select the proper carbon source for growth and lipase production by test strains. On the bases of the suggestion of Iwai *et al.* (1973) that lipase produced might be derived from small quantities of carbohydrates present in the medium with lipid materials as inducer; the basal medium of Sugiura *et al.* (1977) was amended with 1% from different sugars and 1% olive oil as inducer. After intermittent sterilization of medium, the pH was adjusted at 6.0, then inoculated with isolated strains at the optimum conditions stated experimentally for each strain.

The results in Table 9 show that strain *B. cereus* reacted positively with sugars in the presence of olive oil. the highest amount of enzyme produced was detected when glucose was applied with olive oil, where its activity was 2.850 µmol FFA/ ml/min for growth 2.727 O.D., and final pH was acidic (6.550).

Table 10 with *P. fluorescens* show that the best amount of lipase was obtained discendengly in the presence of olive oil with glucose, sucrose, arabinose and inulin, respectively. The highest amount of enzyme was 5.05 µmol FFA/ml/min in the presence of glucose with olive oil, O.D. referring the amount of growth which was 2.40.

Table 11 show also that the presence of olive oil with different sugars produced the maximal amount of lipase by *P. aeruginosa* compared to its absence. The best sugars which gave the highest amount of lipase were glucose (5.50), followed by xylose (4.95) and inulin (4.65 µmol FFA/ ml/ min).

These results are in agreement with those obtained by Korish (1994) who reported that the highest yield of lipase was obtained with glucose after four days followed by maltose, starch, galactose and fructose in descending order.

Table 9: Effect of different sugars on lipase production by *Bacillus cereus* in presence and absence of olive oil.

Culture condition		Glucose	Galactose	Lactose	Maltose	Sucrose	Inulin	Arabinose	Xylose	without sugar
(A) Sugar with olive oil	Lipase activity	2.850	2.000	0.600	0.350	2.000	1 600	0.650	2.000	-
	Growth	2.727	2.008	2.450	2.504	2.033	2 494	1.900	1.809	-
	Final pH	6.550	5.830	6.680	5.800	9 200	7.280	5.250	5.260	-
(B) Sugar without olive oil	Lipase activity	0.000	0.000	0.000	0.100	0.500	1.800	1.650	0.000	1.000
	Growth	0.170	0.281	0.128	2.991	0.160	0.243	0.240	0.445	0.643
	Final pH	6.220	6.03	6.260	8.500	6.210	6 230	5.710	7.330	6.450
Difference A-B	Lipase activity	2 850	2.000	0 600	0.250	1.500	-0.200	-1.000	2.000	-
	Growth	2.557	1.727	2.322	-0.487	1.873	2.251	1.660	1.264	-

* Negative values in lipase activity and growth means sugar inhibition.

Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm.

Table 10: Effect of different sugars on lipase production by *Pseudomonas fluorescens* in presence and absence of olive oil.

Culture condition		Glucose	Galactose	Lactose	Maltose	Sucrose	Inulin	Arabinose	Xylose	without sugar
(A) Sugar with olive oil	Lipase activity	5.05	0.500	0.000	0 000	3.200	2.350	3.000	1.500	-
	Growth	2.40	3.460	2.530	3.788	2.300	2.485	2.583	2.644	-
	Final pH	8.30	8.280	8.470	8.500	8.830	9.080	8.640	9.080	-
(B) Sugar without olive oil	Lipase activity	2 00	1.550	1.450	2.400	1.250	3.650	2.200	1.500	2.00
	Growth	3.12	3.044	3.216	3.808	3.648	3.472	2.781	2.340	2.23
	Final pH	8.75	7.000	9.010	8.740	9.000	8.640	6.680	5.600	7.89
Difference A-B	Lipase activity	3.05	-1.050	-1.450	-2.400	1.950	-1.300	0.800	0.000	-
	Growth	-3.72	0.416	-0.686	-0.020	-1.348	-0.987	1.802	2.304	-

* Negative values in lipase activity and growth means sugar inhibition. Lipase activity = µmol FFA / ml / min,Growth = O.D. 600 nm.

Nadkarni (1971) reported that addition of glucose to a synthetic medium increased both growth and lipase production by *P. aeruginosa*. Alford and Pierce (1963) reported that *P. fragi* produced good levels of lipase activity in the presence of glucose in synthetic medium containing a variety of amino acids. In respect of xylose addition Okeke and Okolo (1990) reported that xylose at a concentration of 2% (w/v) was the optimum for lipase production from *Acremonium strictum*, while in the present experiment xylose was unfavourable for lipase production.

Also, Ammar and McDaniel (1984) reported that sucrose 0.5% was the best sugar to achieve maximum lipase production by *B. stearothermophilus*, which is a thermophilic Bacillus. In this work sucrose was unfavourable for lipase production with the mesophilic short rod organism *P. aeruginosa*, but *P. pseudoalcoligenes* lipase was favoured by soybean flcur as found by Songgang *et al.* (1997). The presence of glucose (1%) in bouillon broth markedly decreased the lipoprotein lipase production by *Pseudomonas* sp. and *S. aureus* as observed by (Narasaki *et al.*, 1968, Mates & Sudakevitz, 1973).

Effect of lipids:

As previously shown in obtained results, olive oil have promoting effect on lipase activity. Various lipid materials were added to the media to determine whether they also promote lipase production or not. This was done using the previous basal medium of Sugiura *et al.* (1977).

Data in Table 12 show that the presence of glucose which was shown to be the best sugar in the previous experiment with lipid materials gave the highest amount of growth and lipase production by *B. cereus*. In Table 13 with *P. flourescenes* showed that olive oil with glucose gave the highest amount of lipase. While with *P. aeruginosa* showed in Table (14) that all lipid materials with glucose gave the best amount of lipase except palm oil and maize oil.

These findings are in agreement with those obtained by El-Shafei and Rezkallah (1997) who found that the presence of either olive oil or tributyrin and with lesser extent castor oil were promotive to lipase enzyme but it was drastically reduced in the presence of animal fat, cotton seed oil, margarine or glycerol. Pabai *et al.* (1996) reported that butter fat induced more lipase production when present in the growth medium. Handelsman and Shoham (1994) showed that optimal lipase production by *Bacillus* sp. was observed in medium containing 1% Tween 80. Korish (1994) indicated that maize oil, olive oil and palm oil enhanced, significantly, the biomass growth and lipase activity. Lipase added to the medium inhibited the enzyme synthesis, as was found to be the case with *P. fragii* (Smith & Alford, 1966).

Lipid concentration:

From the abovementioned results in Table 15 it was found that olive oil with glucose was the best inducer for lipase production. Then olive oil was added to the fermentation medium in different concentrations varying from 0.5 to 4%.

Table 11: Effect of different sugars on lipase production by *Pseudomonas aeruginosa* in presence and absence of olive oil.

Culture condition		Glucose	Galactose	Lactose	Maltose	Sucrose	Inulin	Arabinose	Xylose	without sugar
(A) Sugar	Lipase activity	5.50	1.650	2.230	0.200	0.050	4.650	3.400	4.950	-
with	Growth	2.508	4.002	3.000	3.022	2.114	1.805	2.083	2.067	-
olive oil	Final pH	8.100	9.000	9.050	8.950	9.160	9.160	8.960	9.190	-
(B) Sugar	Lipase activity	2.150	0.800	1.200	1.000	1.500	1.750	1.400	1.900	2.150
without	Growth	2.350	3.324	2.924	2.580	3.176	2.242	3.860	2.344	2.575
olive oil	Final pH	9.250	8.290	9.190	9.340	9.220	9.220	8.710	7.830	9.040
Difference	Lipase activity	4.200	0.850	1.030	-0.800	-1.450	3.900	2.000	4.050	-
A-B	Growth	-3.416	0.678	0.076	0.442	-1.062	-4.38	-1.777	-0.277	-

* Negative values in lipase activity and growth means sugar inhibition.
Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm.

Table 12: Effect of different oils in presence and absence of glucose on lipase production by *Bacillus cereus*.

Culture condition		Sunflower oil	Cotton oil	Maize oil	Olive oil	Palm oil	Tween 80	Without sugar
(A) Lipid	Lipase activity	2.050	2.400	2.650	2.900	2.500	2.200	-
materials	Growth	2.346	2.136	2.883	2.355	3.246	1.008	-
with glucose	Final pH	4.970	4.960	5.820	4.940	5.140	4.990	-
(B) Lipid	Lipase activity	0.700	2.350	0.900	1.500	1.600	1.900	1.200
materials	Growth	2.490	0.568	0.742	1.026	0.569	0.094	0.970
without glucose	Final pH	7.450	6.150	6.230	6.240	6.150	6.100	6.220
Difference	Lipase activity	1.350	0.050	2.200	1.650	0.900	0.300	-
A-B	Growth	-0.144	1.568	2.141	1.329	2.677	0.914	-

* Negative values in lipase activity and growth means glucose inhibition.
Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm.

Table 13: Effect of different oils on lipase production by *Pseudomonas fluorescens* in presence and absence of glucose.

Culture condition		Sunflower oil	Cotton oil	Maize oil	Olive oil	Palm oil	Tween 80	Without sugar
(A) Lipid	Lipase activity	1.700	1.450	0.900	5.200	2.400	1.400	-
materials	Growth	2.632	2.440	2.65	2.628	2.427	2.871	-
with glucose	Final pH	8.380	7.460	7.670	7.980	5.860	8.310	-
(B) Lipid	Lipase activity	0.000	2.100	2.250	2.000	1.450	0.350	2.00
materials	Growth	2.189	2.202	2.266	3.232	1.992	1.918	6.12
without glucose	Final pH	7.900	8.160	7.840	7.890	7.820	8.800	8.75
Difference	Lipase activity	1.700	-0.650	-1.350	1.200	0..950	1.050	-
A-B	Growth	0.443	0.238	0.379	-0.604	0.435	0.953	-

* Negative values in lipase activity and growth means glucose inhibition.
Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm.

Table 14: Effect of different oils on lipase production by *Pseudomonas aeruginosa* in presence and absence of glucose.

Culture condition		Sunflower oil	Cotton oil	Maize oil	Olive oil	Palm oil	Tween 80	Without sugar
(A) Lipid	Lipase activity	3.400	2.150	1.750	5.550	0.300	4.800	-
materials	Growth	2.125	2.744	2.626	2.372	2.522	2.429	-
with glucose	Final pH	8.290	8.240	7.790	8.370	8.400	9.07	-
(B) Lipid	Lipase activity	2.200	2.550	3.950	3.750	4.550	1.700	2.150
materials	Growth	2.303	2.200	2.410	2.253	2.354	1.919	5.224
without glucose	Final pH	8.620	8.530	8.420	8.660	8.570	9.200	9.25
Difference	Lipase activity	1.200	-0.400	-2.150	1.800	-4.25	3.600	-
A-B	Growth	-0.178	0.544	0.216	0.119	0.168	0.510	-

* Negative values in lipase activity and growth means glucose inhibition.
Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm.

The obtained results show that 1% olive oil concentration gave the highest amount of lipase by the three tested strains. The best organism was *P. aeruginosa* followed by *P. fluorescens* and at the end *B. cereus*.

These results are in agreement with those obtained by Shady (1993) who reported that olive oil 1% concentration gave the highest amount of lipase for *P. aeruginosa*. Also, in line Handelsman and Shoham (1994) and Okeke and Okolo (1990) who stated that optimal lipase production by *Bacillus* sp. and *Acremonium strictum* was on medium containing 1% (v/v) Tween 80.

Table 15: Effect of olive oil concentration on lipase production by lipolytic bacteria.

microorganism	*Bacillus cereus* (1)			*Pseudomonas fluorescens* (2)			*Pseudomonas aeruginosa* (3)		
Lipid concentration %	Growth	Lipase activity	Final PH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
0.5	2.539	0.65	7 95	2.182	2.30	8.28	2.335	2.20	8.55
1.0	2.650	2.85	6.55	2 400	5.30	8 30	2.372	5.50	8 37
2 0	2.100	1.70	7.90	2.666	2.35	8.59	2 330	0.00	8.23
3.0	2.502	0.00	7.00	0.446	2.15	8.40	2.381	0 00	8.13
4.0	2.585	0.00	7.60	2.321	2.00	8.60	2.390	0.00	8.30

(1) Culture conditions: Incubation period, 3 days; temperature, 30°C; initial pH, 6.
(2) Culture conditions: Incubation period, 1 day; temperature, 30°C; initial pH, 6.
(3) Culture conditions: Incubation period, 2 day; temperature, 30°C; initial pH, 6.
Lipase activity = µmol FFA / ml / min, Growth = O.D. 600 nm,
Final pH = Arbitrery units.

Effect of nitrogen source:

Peptone and urea of the original medium were considered as control of the bacterial medium. The control was replaced by peptone, tryptone, yeast extract, urea, sodium nitrate and ammonium sulfate (provided equalization of N levels in all treatments, approximately).

From the data presented in Table 16 it could be observed that the original nitrogen source urea with peptone (control) of the medium gave the highest yield of growth and lipase.

Table 16: Effect of nitrogen source on lipase production by different lipolytic bacteria.

microorganism	*Bacillus cereus* (1)			*Pseudomonas fluorescens* (2)			*Pseudomonas aeruginosa* (3)		
Nitrogen source	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
Urea	1.953	1.00	4.91	2.00	3.00	9.24	2.099	4.70	2.29
Sodium nitrate	1.480	0.70	5.08	1 300	0.45	5.15	1.260	0.50	5.17
Ammonium sulfate	1.125	1.45	6.48	1 629	0.55	5.22	1.792	3.40	5.11
Peptone	2.306	1.75	6.52	2.534	3.00	5 91	2.240	1.60	6 08
Yeast extract	2.049	1 15	6.64	2.636	1.45	7.38	2 431	1.75	7 42
Tryptone	2.300	2.45	7.02	2.390	2.60	6.71	2 251	4.40	6.41
Control (urea+peptone)	2.727	3.10	6.55	2.628	5.03	7.98	2.508	5 35	8.10

(1) Culture conditions: Incubation period, 3 days; temperature, 30°C; initial pH, 6.
(2) Culture conditions: Incubation period, 1 day; temperature, 30°C; Initial pH, 6.
(3) Culture conditions: Incubation period, 2 day; temperature, 30°C; Initial pH, 6.
Lipase activity = µmol FFA/l/ml/min, Growth = O.D. 600 nm,
Final pH = Arbitrary units.

At the same time, all used organic and inorganic nitrogen sources were affecting growth and lipase production. Sodium nitrate gave the least amount of lipase and the pH became the most acidic with all nitrogen sources used in the experiment.

These results are in parallel to those of Shady (1993) who reported that addition of casein as a sole nitrogen source was found to be superior relative to other nitrogen sources for *P. aeruginosa* lipase production, while the addition of peptone as a sole nitrogen source to the basal medium for *B. subtilis*, gave the highest level of lipase production followed by wheat bran. Other citations reported the use of complex materials like wheat flour and soymeal to cover N-sources i.e. Songgang *et al.* (1997) who found that wheat flour can be considered as optimal nitrogen source for lipase production by *P. pseudoalcaligenes*. Lin *et al.* (1995) reported that soymeal was found to be essential.

Nitrogen source concentration:

An experiment was carried out to study the effect of nitrogen content elevation through utilizing various concentrations of urea and peptone which were found to be the best nitrogen sources for tested bacterial strains.

The results given in Table 17 indicate that addition of urea and peptone to the culture medium affected both growth and lipase production. The lipase yield reached its highest proliferation at 0.92% nitrogen content with lipase activity of 4.98 µmol FFA / ml / min after 3 days of incubation and this was nearly 178% that of control with 0.52% nitrogen in case of *B. cereus*. Lipase activity for *P. fluorescens* was 8.50 units which is near to 243% of the control level with growth nearly equal to 108.2%, while for *P. aeruginosa* higher lipase activity proliferation was reached to 218% compared to control, and growth was increased by 174%.

Yasuhide *et al.* (1978) showed that addition of 0.2% urea to the production medium stimulated lipase production by *Saccharomyces lipolytica*.

Table 17: Effect of nitrogen concentration on lipase production by lipolytic bacteria.

microorganism	*Bacillus cereus* (1)			*Pseudomonas fluorescens* (2)			*Pseudomonas aeruginosa* (3)		
Nitrogen concentration %	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH	Growth	Lipase activity	Final pH
0.24	2.300	0.20	6.03	2.599	0.40	6.84	1.750	1.2	7.50
0.32	2.500	0.32	6.50	2.597	0.75	7.39	2.600	1.5	7.55
0.44	2.405	1.20	7.02	2.598	1.00	8.05	2.450	2.5	7.70
**0.52	2.671	2.80	7.33	2.400	3.50	7.98	2.300	5.0	7.73
0.60	2.602	3.00	7.40	2.602	5.40	8.84	3.000	4.2	8.61
0.68	2.898	3.00	7.43	2.898	6.22	9.01	3.400	5.7	8.53
0.76	2.560	3.40	7.80	2.655	7.50	9.06	3.500	6.5	8.77
0.84	2.700	3.50	8.03	2.671	8.00	8.94	4.000	9.9	8.88
0.92	2.597	4.98	8.00	2.597	8.50	8.92	4.003	10.9	8.97
1.00	2.500	4.75	8.00	2.580	8.20	8.90	4.000	10.0	8.99

(1) Culture conditions: Incubation period, 3 days; temperature, 30°C; initial pH, 6.
(2) Control. conditions: Incubation period, 1 day; temperature, 30°C; initial pH, 6.
(3) Culture conditions: Incubation period, 2 day; temperature, 30°C; initial pH, 6.
Lipase activity = µmol FFA/ml/min, Growth = O.D. 600 nm,
Final pH = Arbitrary units.

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عزل وتعريف وإنتاج الليبيز من البكتيريا المنتجة للإنزيم

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استهدفت الدراسة عزل وتعريف بعض البكتيريات المنتجة لإنزيم الليبيز من التربة، وقد تم عزل سبعة أنواع كان أكثرها نشاطا في إنتاج الإنزيم *P.aeruginosa* *B.cereus* , *P.fluorescens,*

وقد وجد أن امثل فترة تحضين لإنتاج الإنزيم هي ١، ٢ ،٣ يوم لبكتيريات *P.fluorescens* *P.aeruginosa*, *B.cereus* على الترتيب ،كما تم الحصول على أعلى إنتاج من الإنزيم عند ٣٠°م وتركيز ايون أيدروجين pH ٦ من العزلات الثلاث وان بيئة الزرع المحتوية على ١%جلوكوز ،١% زيت زيتون ، ٩٢ر٠% نيتروجين (اليوريا و البيتون) هي الأمثل للحصول على أعلى نشاط لإنزيم الليبيز حيث بلغ ٩ر١٠ ،٥ر٨ ، ٩٨ر٤ وحدة/ملل من رائق المزرعة للبكتيريات التالية على الترتيب: *P.aeruginosa*, *P.fluorescens*, *B.cereus* .