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Production of Lipases by Certain Thermo Tolerant Bacteria

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Lipases are hydrolytic enzymes which hydrolyse triglycerides to free fatty acids and glycerol. Bacterial lipase producers were isolated from certain loamy arid soils. Eight thermotolerant bacteria able to produce lipases were identified down to their species level. Identification studies have revealed that 5 isolates were belonging to *Bacillus stearothermophilus*, while the others were belonging to *B. megaterium*; *B. licheniformis* and *B. subtilis*. The impact of the incubation period ,medium pH, temperature , the source of carbon for the lipase production was detected. Results have shown that the optimal conditions for Lipases production by most of these bacteria were incubation temperature of 50°C, incubation period of 72 hours, pH 7.0, presence of 0.1% yeast extract, and when sucrose plus tributyrin were supplied as carbon sources. Production of lipases by the used heat resistant bacteria could be of academic and applied importance especially for manufacture of detergents.

Keywords: Lipase, Thermotolerant bacteria, Loamy arid soils.

Lipases (triacyglycerol acylhydrolase E C 3.1.13) are a biotechnologically important group of enzymes that act on the carboxyl ester bonds existing in triacylglycerols and release the fatty acids and glycerol[1]. They have acquired specific interest over few decades due to their capability to act in microaqueous environments and stimulate esterification, transesterification, aminolysis, acidolysis reactions[2,3].

Lipases are ubiquitous enzymes that are exceedingly spreaded in plants, animals, and microbes[4]. Lipases isolated from fungi are best studied among all microbial lipase. Though, bacterial strains are being permanently screened and improved for lipase production. Bacterial lipases were first detected in the year 1901 in the strains, *Serratia marescens* and *Pseudomonas aeruginosa*[5]. Ever since that lipase production by many different bacterial species has been extensively studied and notified. There are various documents available on the production of bacterial lipase particularly from *Pseudomonas* and *Bacillus sp.*[6], *Pseudomonas flourescens*[7], *Bacillus pumilus*[1], *B. thermocatenulatus*[8], *B. subtilis*[9], *B. licheniformis*[10], *B. coagulans* [11], *B. cereus*[4] and *B. halodurans*[12]. Other genera like *Acinetobacter*[13], *Serratia marescens*[14], *Achromobacter, Achromobacter*, *Alcaligenes* and *Chromobacterium*[15] too have been studied. However, until recently ,little attention was devoted to detect the lipolytic activities of thermotolerant micro-organisms. Therefore, this paper is an attempt to throw some light upon the identities and lipolytic activities of certain thermotolerant bacteria isolated from certain loamy and soils.

MATERIALS AND METHODS

Microorganisms used :these were isolated from certain loamy arid soils, Hilla, Iraq.

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Isolation, Purification, and Identification of used bacteria

Dox's yeast extract-tributyrin agar medium[16] was used for such a purpose. It contains : $NaNo_3$ 0.2gm; K_2HPO_4 0.1 gm; $MgSO_4.7H_2O$, 0.05gm; KCl,0.05gm ; $FeSO_4.7H_2O$,0.001gm;yeast extract,0.1 gm; agar ,2.0 gm and 100 ml distilled water. Tributyrin emulsion in warm distilled water (0.1%, v/v) was used as a carbon source. The pH value was adjusted to 7.2 before autoclaving. The medium was then autoclaved at 1.5 atm for 15 minutes.

Media and technique used for identification of the used bacterial isolates were similar to those recommended in the Manual of Microbiological Methods[17], the keys of Bergey's Manual of Determinative Bacteriology[18] and Macfaddin[19].

Quantitative Determination of lipolytic activity of the used bacteria

The above mentioned medium without agar was used as a production medium for such a purpose. Sucrose (1%, w/v, in the production medium) was used instead of tributyrin as a carbon source. The constituents of 30 ml of this medium were dispensed in the form of 27 ml in bottles of 100 ml capacity, and then autoclaved at 1.5 atm for 15 minutes. Three ml of a standardized bacterial suspension (4 x 10^6 cells/ml) in a physiological saline solution (0.85% NaCl, w/v, in distilled water) were used as an inoculum for each bottle, thus restoring the volumes of the media to their original ones. Triplicates were made throughout for each treatment. The inoculated bottles were then incubated at the required temperature for the required incubation period under a static culture condition.

The bacterial culture in each bottle at the end of the incubation period was filtered through a sintered glass funnel (G6). The filtrate was then concentrated (x10) by using a dialysis bag and sucrose crystals. This was done in a refrigerator in order to prevent dialysate deterioration. The dialysate was then used for quantitative determination of lipolytic activity. This was carried out by using tributyrin cup-plate clearing zone assay method as qualified by Morikawa *et al*[20]. In this method, 0.2% tributyrin (v/v was emulsified in phosphate buffer, and then supplemented with 2% agar "B.D.H" (w/v) for solidification. Equal amounts of assay substrate (20ml) were transferred into Petridishes of 12cm diameter. Cup-shaped pores in the assay substrate were made (3 pores for each dish) by using a sterile cork borer. Equal amounts from each culture dialysate (0.1ml) were transferred to each pore. The dishes were then incubated at 30°C for 6 hours. A standard curve for assaying lipases concentration was constructed by using down concentrations of "Sigma" pancreatic lipase (mg/ml). 0.1 ml from each concentration was transferred into a pore as mentioned before and the clearing zones were measured after 6 hours incubation period. The incubation temperature of the standard curve dishes was 30°C. Logs of "Sigma" lipase concentrations were plotted against the mean values of diameters of clearing zones in mm. Amounts of lipases concentration in the dialysates (incubated at 30°C for 6 hours) were calculated by extrapolation and obtaining their antilogs.

For setting up the experimental work, the following treatments were made:

Effect of incubation temperature on lipases production

Dox's Sucrose liquid medium, as mentioned before (at pH 7.2) was used for such a purpose. The inoculated media were incubated at 50°C and/or 55°C for 24 hours. All other microbiological techniques and estimation of lipolytic activity in cell-free culture dialysate (x10) were as mentioned before.

Effect of incubation period on lipases production

Dox's sucrose liquid medium, as mentioned before(at pH 7.2), was used for such a purpose .The inoculated media were incubated at 50°C for 24, 48,72 and 96 hours. All other microbiological techniques and estimation of lipolytic activity in cell-free culture dialysate (x 10) were as mentioned before.

Effect of pH value on lipases production

Dox's sucrose liquid medium, as mentioned before, was used for such a purpose. The pH values were adjusted at 6.5 ,7.0, 7.5 and 8.0 before autoclaving. The inoculated media were incubated at 50°C for 72 hours. All other microbiological techniques and estimation of lipolytic activity in cell-free dialysate (x 10) were as mentioned before.

Effect of supplying yeast-extract on lipases production

Dox's sucrose liquid medium, as mentioned

before, was used for such a purpose. Yeast-extract "B.D.H", was supplied at concentrations of 0.0% (cont.), 0.05%, 0.1% and 0.5%(w8 v, in the production medium) .The pH value was adjusted at 7.0 before autoclaving. The inoculated media were incubated at 50°C for 72 hours. All microbiological techniques in cell-free dialysate were as mentioned before.

Effect of supplying various carbon sources on lipases production

Dox's liquid medium, as mentioned before, supplied with sucrose (1%, w/v), sucrose (1%) plus tributyrin (0.1%, v/v), and tributyrin (0.1%) were supplied singly as carbon sources. The production medium was supplied with 0.1% yeast extract (w 8v), and the pH value was adjusted at 7.0 before autoclaving. The inoculated media were then incubated at 50°C for 72 hours. All other microbiological techniques and estimation of lipolytic activity in cell-free dialysate (x10) were as mentioned before.

Expression of results

Yield of lipases were expressed as mg/ml culture dialysate (x10).

RESULTS AND DISCUSSION

Eight isolates were obtained from the used soils. For identification of these isolates, tables of family Bacillaceae in Bergey's Manual of Determinative Bacteriology[18], Macfaddin[19] have been consulted. These isolates were identified down to their species level as in the following : 5 were identified as *Bacillus stearothermophilus* and they were given the names; *B.stearothermophilus 1 ,B. stearothermophilus 2, B. stearthermophilus 3 ,B. stearothermophilus 4, and B. stearothermophilus 5*. While, three isolates were identifies as *B.*

Table 1. Effect of incubation temperature on lipases production

Micro-organisms	*Lipase concentrations (mg/ml culture dialysate, x 10) at :	
	50°C	55°C
Bacillus stearothermophilus, 1	1500± 10	515± 12
Bacillus stearothermophilus, 2	1423± 20	665 ± 30
Bacillus stearothermophilus, 3	722 ± 10	473 ± 09
Bacillus stearothermophilus, 4	1579 ± 27	630 ± 33
Bacillus stearothermophilus, 5	624 ± 13	571 ± 16
Bacillus megaterium	1470 ± 17	627 ± 22
Bacillus licheniformis	627 ± 13	466 ± 24
Bacillus subtilis	591 ± 16	417 ± 12

± = Standard error of mean

* Mean values of three determinations.

Table 2. Effect of incubation period on lipases production

Micro-organisms	*Lipase concentrations (mg/ml culture dialysate, x 10) after :			
	24 hours	48 hours	72 hours	96 hours
Bacillus stearothermophilus, 1	1431 ± 31	1492 ± 12	1575± 15	625 ± 16
Bacillus stearothermophilus, 2	1233 ± 23	1247 ± 33	1322 ± 42	1200 ± 11
Bacillus stearothermophilus, 3	684 ± 14	674 ± 33	726 ± 31	652 ± 23
Bacillus stearothermophilus, 4	1350 ± 46	1370 ± 72	1379 ± 35	1425 ± 34
Bacillus stearothermophilus, 5	577 ± 11	577 ± 32	670 ± 22	667 ± 18
Bacillus megaterium	1425 ± 34	1425 ± 63	1477 ± 23	732 ± 25
Bacillus licheniformis	623 ± 11	645 ± 09	650 ± 54	645 ± 22
Bacillus subtilis	525± 12	555 ± 13	585 ± 23	570 ± 22

± = Standard error of mean

* Mean values of three determinations.

Table 3. Effect of pH value on lipases production

Micro-organisms	*Lipase concentrations (mg/ml culture dialysate, x -10) at pH : 6.5	7.0	7.5	8.0
Bacillus stearothermophilus, 1	1575 ±45	1576 ± 50	1500 ± 46	591 ± 32
Bacillus stearothermophilus, 2	1320 ±34	1327 ± 34	1245 ± 23	654 ± 43
Bacillus stearothermophilus, 3	675 ±21	732 ± 13	625 ± 32	600 ± 24
Bacillus stearothermophilus, 4	1377 ±52	1474 ± 23	1375 ± 24	700 ± 34
Bacillus stearothermophilus, 5	652 ±11	659 ± 21	652 ± 24	402 ± 14
Bacillus megaterium	735 ±24	1485 ± 34	1491 ± 16	650 ± 21
Bacillus licheniformis	600 ±30	668 ± 33	667 ± 24	505 ± 33
Bacillus subtilis	593 ±44	660 ± 31	592 ± 22	450 ± 11

± = Standard error of mean
* Mean values of three determinations.

Table 4. Effect of supplying yeast extract on lipases production

Micro-organisms	*Lipase concentrations (mg/ml culture dialysate, x 10) at yeast extract of : 0.0%	0.05%	0.1%	0.5%
Bacillus stearothermophilus, 1	1125 ± 11	1430 ± 23	1576 ± 13	619 ± 10
Bacillus stearothermophilus, 2	1341 ± 19	1410 ± 27	1500 ± 33	966 ± 22
Bacillus stearothermophilus, 3	735 ± 44	741 ± 55	862 ± 36	576 ± 52
Bacillus stearothermophilus, 4	1470 ± 33	1477 ± 19	1621 ± 44	1474 ± 23
Bacillus stearothermophilus, 5	667 ± 11	674 ± 32	825 ± 42	727 ± 31
Bacillus megaterium	1417 ± 33	1426 ± 25	1513 ± 26	1170 ± 31
Bacillus licheniformis	672 ± 15	675 ± 32	900 ± 34	832 ± 21
Bacillus subtilis	666 ± 44	666 ± 25	750 ± 42	625 ± 33

= Standard error of mean
* Mean values of three determinations.

Table 5. Effect of supplying sucrose, sucrose plus tributyrin, and tributyrin, singly as carbon sources on lipases production

Micro-organisms	*Lipase concentrations (mg/ml culture dialysate, x 10) for Sucrose plus : Sucrose	Tributyrin	Tributyrin
Bacillus stearothermophilus, 1	1590 ± 35	1980 ± 44	1431 ± 24
Bacillus stearothermophilus, 2	1450 ± 33	1848 ± 32	1480 ± 16
Bacillus stearothermophilus, 3	880 ± 41	1140 ± 43	870 ± 33
Bacillus stearothermophilus, 4	1701 ± 22	2092 ± 25	1660 ± 44
Bacillus stearothermophilus, 5	830 ± 11	966 ± 10	799 ± 33
Bacillus megaterium	1600 ± 44	1978 ± 22	1530 ± 41
Bacillus licheniformis	880 ± 55	1144 ± 23	790 ± 43
Bacillus subtilis	780 ± 33	858 ± 42	648 ± 52

± = Standard error of mean
* Mean values of three determinations.

megaterium, *B. licheniformis* and *B. subtilis*. The following deals with the descriptions of these isolates:

***Bacillus stearothermpohilus* isolates**

These were characterized by the following characters: Cells are gram positive and spore formers. Spores are elliptical in shape and terminal in position. Sporangia definitely swollen. They produce acid without gas from glucose, arabinose and xylose, but not from mannitol when ammonium sulphate was used as a source of nitrogen[18].They were unable to produce both acetylmethyl-carbinol and indole but able to reduce nitrates to nitrites and to hydrolyse casein and starch . they were able to liquefy gelatin and coagulate milk. They showed no growth in 7% NaCl broth medium, and no bacterial growth happened under reduced oxygen tension (candle jar technique), and catalase test was positive. Bacterial growth was excellent at 65°C, but moderated at 30 & 75°C. No growth happened below 30°C, and 8or above 75°C on Dox's agar medium plus yeast extract or Dox's agar medium containing tributyrin, as carbon sources. These isolates differed in their cell diamensions (measured from negatively-stained preparations). They were of 0.60 x 20.8 mm; 0.68 x 2.7mm; 0.66 x 2.8µm ; 0.77 x 3.0 µm; and 0.80 x 3.8 mm for isolates 1, 2, 3, 4 and 5 respectively. They were differed in their ability to produce lipases (Tables 1-5).

***Bacillus megaterium* isolate**

Cells are gram positive, of 1.3 x 2.4mm, motile, and spore formers (with oval-shaped spores). They produce acid without gas from media supplemented singly with arabinose and xylose. Cells are able to assimilate nitrate without accumulation of nitrite in the medium. They liquefy gelatin and coagulate milk. No bacterial growth happened under reduced oxygen tension (candle jar technique) and catalase test was positive. Cells are able to grow at 45-55°C,but not below or above this range of temperature. Its ability to produce lipases differed from the above mentioned isolates (Tables 1-5).

***Bacillus licheniformis* isolate**

Cells (0.66 x 3.0 mm) are Gram-positive, motile, and spore formers (of swollen sporangia). Cells produce acid from media containing xylose, arabinose and/or mannitol. They are capable to grow at 40-55°C, and in nutrient broth medium containing 7% NaCl. Cells are able to coagulate milk and liquefy gelatin. Its ability to produce lapases differed from the above mentioned isolates (Tables 1-5).

***Bacillus subtilis* isolate**

Cells (of 0.75 x 3.0 mm) are Gram-positive, motile, spore formers. They produce acid from media containing xylose, arabinose and/orf mannitol. They are capable to grow at 5-55°C, and to grow in nutrient broth containing 7% NaCl. Cells are able to hydrolyse starch and utilize nitrate causing accumulation of nitrite in the medium. They can't grow under oxygen tension (candle jar technique), and catalase test was positive. Its ability to produce lipases differed from the above mentioned isolates (Tables 1-5).

Concerning the ability of these isolates to produce lipases, it was found (Tables 1-5) that lipase activities were detected in the culture filtrate of each of the used bacteria.

As for the impact of the incubation temperature on lipases production (Table 1), the results obtained have revealed that the maximal yield of lipases production occurred at an incubation temperature of 50°C and/or above 55°C (not tabulated). Therefore, this range of temperature (45-55°C) conditioned induction of lipases by these isolates. Among the used 8 isolates, 4 isolates (*Bacillus stearothermophilus* 4; *B. stearothermphiilus* 1; *B. megaterium* and *B. stearothermophilus* 2 produces 1579 ± 27, 1500 ± 10, 1470 ± 17 and 1423 ± 20 g/ml culture dialysate, x 10 respectively) were the most potent lipolytic ones.

As for the works of other investigators, Ghaima[21] reported that they maximal yield of lipases production by *Bacillus cereus* was achieved at 25-45°C temperature range. The optimum incubation temperature of the lipase production of 55.25U/ml was found at 35°C. Enzyme production was reduced when the incubation temperature was increased above 35°C. Loperena[22] found the highest activity of lipase in 31 yeasts isolated from various environments of the Antractic continent as they were incubated at 20°C rather than 4°C.

Sooch and Kauldhar[23] reported that the extreme activity of the lipase from *Pseudomonas sp. BWS-5* was gained at temperature 37°C .Norman *et al*.[24] as well obtained the maximum activity of lipase from *P. aeruginosa BN1* at 37°C.

The higher production of lipase (147.36 IU/ml) from *P.aeruginosa* S*RT9* was also carried out at37°C by Prita[25].

The maximum activity of lipase was spotted at temperature 37°C by *Staphylococcus* [26].

Misbah and Haq[27] recorded the impact of the temperature on the lipase of *Penicillium fellutanum* ,the temperature of incubating chamber was diversed from 25 to 45°C with an increasing of 5°C. The crude enzyme manifested maximum activity (401.37U/gds) at 30°C. It was observed that increase or decrease in temperature caused the production of enzyme with much low activities . At temperature below 30°C, there was a slight decrease in the lipase activity, while above 30°C the drop in enzyme production was decline.

Temperature is a crucial parameter that has been controlled and it differs from organism to organism. Temperature effects excretion of extracellular enzymes by changing the physical characteristics of the cell membrane[28,29].

Concerning the impact of various incubation periods on the production of lipase. The effect of different incubation periods on lipases production, the results obtained (Table 2) have revealed that, except *B. stearothermpohilus* 4 (produce 1425 ± 34 mg/ml culture dialysate after 96 hours), the maximal yield of lipases production were achieved after incubation period of 72 hours. The most potent lipases producer was *B. stearothermphilus*, (produce 1575 ± 15 mm/ml culture dialysate, x 10). This finding is agreeable to that obtained by Elwan *et al*[30] ,who found that *Thermoactinomyces vulgaris* produced its maximal yield of lipases after 48-72 hours. Also, with study by[27],who found the production of lipase by *Penicillium fellutanum* investigated after 24h up to 144h and thee result observed that incubation period affected the production of lipase very significantly (p Â 0.05) and the extreme lipase activity (373.56 U/gds) was indicated after 48h of incubation time. After long incubation time, lipase production was turned down as its activity was found 68.65 U/gds after 6 days (144h) of incubation .

With regard to the effect of pH values on lipases production, the results (Table 3) obtained have revealed that, in most cases, the high yield of lipases production was achieved at pH 7.0, and the most potent lipases producer was *B. stearothermophilus*, (produce 1576 ± 50 mg/ml culture dialysate, x 10). This finding is agreeable to that recorded by Ghaima *et al*.[21] who present the production of lipase by *B. cereus w*as detected between pH 5.0-9.0. Also, accordance with that recorded by Veerapagu *et al*.[29], who found the bacteria *Pseudomonas gassardii* has optimum lipase production that production that turned down with excess in pH from 7.0 to 10.0.

Sooch and Kauldhar[23] observed the maximum activity of the lipase from *Pseudomonas sp.BWS-5* was obtained at pH 6.5. Noman *et al*[24] alsoalso found the maximum activity of lipase from *P. aeruginosa BN-1* by using medium of pH at 6.5. Commonly, Bacterial species prefer pH around 7.0 for superior production of lipase.

Alteration in the external pH might affect the ionization of nutrient molecules and thus reduce their accessibility to the organism. In addition to that, severe variations in pH can also affect the microbial cells by obstruct the plasma membrane and disrupting their metabolisms[28].

Concerning the effect of supplying yeast extract to Dox's liquid medium on lipases production, it was found (Table 4) that the maximal yield of lipases was achieved when the medium was supplied with 0.1% yeast extract, below and above this concentration, the yield of lipases decreased. Therefore, yeast extract as vitamins source may be of an important role for lipases production when supplied to the production media, as mentioned in treated media in comparison to that of the controlled ones. This indicate that these isolates required vitamins B-group for enhancement lipases production. This finding still needs further investigations, regarding the role of vitamins and other growth substances on lipases production by these micro-organisms and other thermotolerant ones.

As for the effect of supplying the carbon sources ;sucrose, sucrose plus tributyrin, or tributyrin, on lipases production by the used bacteria, it was found (Table 5) that the maximal yield of lipases was achieved when the production medium was supplied with sucrose plus tributyrin as carbon sources. In all cases the yield of lipases was decreased when tributyrin and sucrose were supplied singly as carbon sources. However, in most cases, sucrose was more favourable than

tributyrin for lipases production. Enhancement of lipases production by these bacteria, at the presence of sucrose plus tributyrin as carbon sources, could be explained by the fact that these kinds of lipases are belonging to the inductive type but not to the constitutive one.

Decreasing in the lipase production in the presence of sugars as carbon sources could be lead to catabolite suppression by readily obtainable carbon sources in the medium[31,32].

Lipase production is affected by the type of carbon source¸ the medium pH, the temperature of the growth,and the incubation period[33].

Finally, the optimal conditions for lipases production, by most of the used bacteria, in their culture media, were incubation temperature of 50 °C, incubation period of 72 hours, pH 7.0, presence of 0.1% yeast extract as vitamins source, and when sucrose plus tributyrin were supplied as carbon sources. Accordingly, it was concluded that these bacteria were thermophilic or at least thermotolerant micro-organisms, and its biological processes can be carried out safely at incubation periods of high temperatures. The lipases produced were at least partially an extracellular enzymes becaue the activity of such group of enzymes was detected in the cell-free culture dialysate.

CONCLUSIONS

In conclusions, this paper has revealed certain fundamentals regarding the lipases production by certain thermotolerant bacteria present in certain loamy arid soils.

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