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# Production, Purification and Characterization of Thermoalkalophilic Lipase for Application in Bio-detergent Industry

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**Abstract:** One hundred and fifty-three aerobic thermophilic bacterial isolates were isolated from some desert soil samples collected from different localities in Egypt. *Bacillus licheniformis* B42, and *Geobacillus stearothermophilus* B78 were selected as the most potent bacterial isolates for lipase production from slaughter house wastes (SHW) as cheap raw substrate. Culture conditions in the growth and thermoalkalophilic lipase production by two most potent strains were investigated using partially modified thermus medium at pH 9 and temperature 55°C.Thermoalkalophilic lipase productivity of the two most potent bacterial isolates was affected by pH, bottle capacities, substrate concentration, carbon source, nitrogen source and inoculum size metal ion, vitamin, and amino acid supplements. Maximum thermoalkalophilic lipases production by *B. licheniformis* B42 and *G. stearothermophilus* B78 was obtained from SHW at pH 9,9; temperature, 55,55°C; substrate concentration, 7.5,12.5 %; carbon source, fructose, glucose; nitrogen source, non of the tested nitrogen source, ammonium molybdate; inoculum size, 1.5, 2.5 ml each ml contained 0.55 x $10^{11}$ CFU; vitamins, thiamine, pyridoxine; amino acid, DL-tyrosine, DL-tyrosine; flask capacities, 250; 250 ml respectively. The overall purification protocol steps of the lipase produced by *B. licheniformis* B42 under all nutritional and environmental optimal conditions resulted in raising the purification fold to 27.33 times. Thiamine exhibited the highest concentrations of the partially purified lipase by *B licheniformis* B42. The kinetic characterization of the partially purified enzyme exhibited activity at temperature, 50-60°C; thermostability, 60°C; pH, 9-9.5; pH stability, 9.5; incubation period, 36h; and substrate concentration, 0.1%(tributyrin). The partially purified thermoalkalophilic lipase exhibited good stability towards chlorine. The purified lipase exhibited not only stable but enhanced maximal activity by Triton X100. Unfortunately, the partially purified thermoalkalophilic lipase was unstable in the presence of commercial detergents whereas calcium chloride was able to restore and enhance enzyme activity. The thermoalkalophilic lipase secreted by both strains is industrially important from in terms of its abilities to function in alkaline pH and to show stability in broad pH ranges, in addition to its stability towards Triton X100,SDS, and $H_2O_2$, suggesting that it is a potential candidate as an additive in detergent formulations.

**Keywords:** Thermoalkalophilic lipase, *Bacillus*, *Geobacillus*, Bio-detergent Technology

## INTRODUCTION

Lipases (Triacylglycerol acylhydrolase; EC 3.1.1.3) constitute a group of enzymes defined as carboxyesterases that hydrolyze (and synthesize) long chain acylglycerols at the lipid-water interface[2,4,27]. Microbial lipases have been widely used for biotechnological applications in fat, food ingredients, detergents, dairy and textile industries, production of surfactants, and oil processing[1,3,15,29,30]. Most lipases are mesophilic enzymes, which cannot hydrolyze a substrate that exists in solid form at room temperature. Thermophilic lipases show higher thermostability, higher activity at elevated temperatures, and often shows more resistance to chemical denaturation. This makes them ideal tools in industrial and chemical processes where relatively high reaction temperatures and / or organic solvents are used. The industrial demand for the thermostable continues to stimulate the search for microorganism's produces of thermostable enzymes. Thermostable enzymes are usually derived from thermophilic strains, which may be expected to

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produce intrinsically more heat stable enzymes. A small number of thermophilic lipase producing bacteria have been described in the last decades[5,14].

As each industrial application may require specific properties of the biocatalysts, there is still an interest in finding new lipases that could create novel applications[11].

In this study, we describe the production, purification and characterization of thermoalkalophilic lipase produced by thermophilic strains for application in bio-detergent industry.

## MATERIALS AND METHODS

**Construction of Standard Curves:**

**Stock lipase solutions:** A stock solution (200,000µg/ml) of the purified lipase supplied by Sigma was prepared in Tris-HCl buffer (0.2 M) at pH 9.0. The protein content was given by the producing company. Descending dilutions were prepared.

**Construction of the Standard Curves:** After preparing the required dilutions for lipase, only 0.1 ml of each dilution was transferred to each well in the Tributyrin Clearing Zone (TCZ) plates. Five wells were used for each dilution. Incubation was performed at 55ºC for 18 h. Then mean diameters of clearing zones (mm) were determined for each particular lipase concentration. A standard curve was constructed relating to lipase concentrations applied against their corresponding mean diameters of clearing zones (mm). The obtained standard curves were used for estimating the lipase activities in terms of µg/ml and then units (U). One unit is defined as the amount of enzyme protein (mg) required to exert one unit of clearing zone (mm) in one unit of time under all the specified conditions of enzyme assay (clearing zone technique).

**Microorganisms:** An alkaliphilic *B. licheniformis* B-42 and *G. stearothermophilus* B-78 strains, which produce extracellular alkaline lipase, were originally isolated from different desert soil samples collected from different localities of Al-Ain El-Sokhna area. Humus samples were also collected from El-Hawamdya City, Giza governorate, South Egypt. Two strains were identified on the basis of various morphological, physicho-chemical, and biochemical characteristics following the criteria laid down in Bergey`s Manual of Systematic Bacteriology[33], and Schallmey *et al.,*[28]. Growth curves of two bacterial strains were determined by measuring the optical density during cell growth at 600 nm against the fresh medium.

**Production Medium:** Alkaline thermostable lipase production was determined by applying a modified form of basal medium given by Vincent[35]. It contained following (g/l): SHW, 10; NaCl, 6; $(NH_4)_2SO_4$, 1; yeast extract, 1; $KH_2PO_4$, 0.5; $MgSO_47H_2O$, 0.1; $CaCl_2.6H_2O$, 0.1, $FeSO_4.7H_2O$, and distilled water up to one liter. The initial pH of the culture medium was about 9. The SHW basal medium (SHWBM) was sterilized for 20 min at 120 °C at 1.5 psi.

**Assay Medium for Lipase by Tributyrin Clearing Zone (TCZ):** This medium was used for assay of enzyme productivity according to the Tributyrin Clearing Zone (TCZ) technique according to Elwan *et a1*.,[7]. It contained 0.2% (v/v) of emulsified tributyrin and supplemented with 1.5 % agar for solidification. pH was adjusted at 9 using Tris-buffer(0.2 M). At the end of incubation period, it was easy to obtain the diameters of the clearing zones by a special grade scale and then calculating the mean diameters of clearing zones in term of (mm).

**Optimization of Fermentation Conditions for Alkaline Thermostable Lipase Production:** Lipase production was optimized under SSF on SHW.

- Effect of different temperatures in SSF for lipase production was studied by incubating the production medium at different temperatures.
- Effect of different pH values was carried out to determine the optimum pH value for lipase productivities by *B.licheniformis*B-42 and *G. stearothermophilus* B-78. The pH was adjusted at different pH values for the production media using 6 N NaOH or 6 N HCl.
- Effect of different substrate concentrations on the productivities at 55°C and pH 9 was studied for alkaline thermostable lipase. Different concentrations of SHW were applied (g/bottle, w/v).
- Effect of various supplements on alkaline thermostable lipase production was studied by adding different carbon sources (1% w/w), nitrogen sources as equivalent amount of nitrogen present in ammonium sulphate (0.1%; w/v) in the basal medium. Peptone was introduced as organic nitrogen source at the level of 0.1 % ; metal ions (25-1000 ppm); vitamins (100ppm); and amino acids (added at an equimolecular amount of nitrogen located in the best inorganic nitrogen source).
- Effect of different inoculum size of the *B.licheniformis*B-42 and *G. stearothermophilus* B-78 was used.
- Effect of different incubation periods was carried out by allowing the *B.licheniformis*B-42 and *G. stearothermophilus* B-78 strains to grow on SHW and incubated for different incubation periods.

Alkaline thermostable lipase production using SSF in different production vessels: Enzyme production were studied in various sized Erlenmeyer flasks using SHW in different production vessels viz. 100, 250,500, 1000, and 2000 ml.

**Lipase Production by *B.licheniformis*B-42:** *B.licheniformis*B-42 was allowed to grow under the optimal static natural substrate under solid state fermentation conditions on slaughter house wastes for lipase production. The optimum lipase production medium was contained , 10 g of slaughter houses wastes per flask of 1000 ml capacity were used and supplemented by 20 ml of production medium, which contained (g/l, w/v): NaCl, 6; $KH_2PO_4$, 0.5; $MgSO_47H_2O$, 0.1; $CaCl_2.6H_2O$, 0.1; yeast extract, 1 in addition to fructose, ammonium sulphate, EDTA(25 ppm), and thiamine. pH was adjusted at 9; inoculated with 1.5 ml bacterial suspension and incubated at 55ºC for 72 h.

**Alkaline Thermostable Lipase Purification:** The purification method that consisted of ultrafilteration, Sephadex G200 and Sephadex G100 gel-chromatography was applied. *B. licheniformis* B42 was allowed to grow under the optimal cultural and environmental conditions on SHW medium.

**Step 1:** Culture supernatant (1000 ml) was obtained by centrifugation of the culture broth after extraction at 5000 x g, 5°C, for 25 min. Ultrafilteration was performed using a Millipore system (membrane pore size of 30.000 Da).

**Step 2:** Ammonium sulphate saturation was carried out according to chart of Gomori[9].

**Step 3:** The concentrate was chromatographic on Sephadex G200 (2.5 x 50cm) equilibrated with (0.2 M) Tris–HCl buffer, pH 9 and then the enzyme was eluted with the same buffer. The flow rate was adjusted to 20 ml/ hours, the fraction volume of 5 ml was collected.

**Step 4:** The enzyme containing fractions were applied to Sephadex G100 column (2.5 x 50 cm) previously equilibrated with the above buffer.

The protein concentrations of the enzyme samples were measured using Lowry method[20] with bovine serum albumin as standard. All measurements were performed three times and the mean values were calculated.

**Amino Acid Analytical Data of The Purified Lipase:** The hydrolyzed protein amino acids have been determined in the central Lab. for Food, Agricultural Research Center according to the methods described by Pellet and Young[25]. LKB Alpha plus high performance Amino Acid Analyzer LKB Biochrom. LTD England were used for this purpose. Retention time and area were determined using Hewlett Pakard 3390 recording integrator. The concentration of each amino acid GM/16 G.M., nitrogen was calculated by a specially designed program.

**Factors Affecting the Purified Alkaline Thermostable Lipase Activities:** The kinetic studies on the purified lipase fractions obtained after purification on sephadex G200 were carried out in terms of the effect of incubation temperatures, heat stability, pH, pH stability, stability with chlorine, oxidizing agents stability and surfactants compatibility with commercial detergents stability in the presence of $CaCl_2$ and commercial detergents.

**Effect of Temperature and Stability:** The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range of 40-100 °C at pH 9 using Tris-buffer (0.2M). The thermostability of the enzyme was tested by incubating the enzyme preparation at varying temperatures ranging from 50-90°C for two hours.

**Effect of pH and stability:** pH activity and stability profiles were studied at pH range of 6-10.5 using different buffers at 55°C for 18 h . For stability studies, one volume of enzyme was mixed with one volume of respective buffer and incubated up to 3 h at 55°C.

**Stability with Chlorine:** This experiment was carried out according to the design set by Singh *et al.*,[32]. The partially purified lipase was incubated with different concentrations of chlorine viz. 5, 7, 10, and 15 µl/ml, then the lipase was inoculated into assay medium plates at different time intervals viz. 15, 30, 45, and 60 min.

**Effect of Oxidizing Agents and Surfactants:** This experiment was carried out according to the design set by Moreira *et al.*,[22]. The purified lipase was incubated with different concentrations of some oxidizing agents and surfactants viz., sodium dodecyl sulphate (SDS), sodium perborate, hydrogen peroxide, Tween 20, Tween 40, Tween 80, Triton X100 and sodium cholate at 1, 2, 3, 4, 5, 7.5, and 10 (% v/v or w/v) for 2 hours.

**Compatibility of the Purified Lipase with Various Commercial Detergents:** The commercial detergents : Rabso, Ariel, Omo, Lang, Persil, Xtra, Ariel automatic,

and Xtra automatic were added separately to partially purified lipase in a concentration of 7 mg/ml, while the liquid detergents viz., General and Pril were added in concentration of 7 µl/ml.

**Stability of the Purified Lipase in the Presence of $CaCl_2$ and Commercial Detergents:** This experiment was performed to investigate the effect of $CaCl_2$ as a stabilizer on the stability of lipase in the presence of various commercial detergents. The purified lipase was supplemented with 7 mg/ml of solid detergents or 7 µg/ml of liquid detergents in addition to $CaCl_2$ which was added at different levels to attain concentration of 250, 500, 1,000, 2.500, 5.000, and 10.000 ppm.

## RESULTS AND DISCUSSIONS

**Results:** One hundred and fifty-three bacterial isolates were grown at 55°C and at pH 9 in order to produce a thermoalkalostable lipase to be used as additive to detergent formulations. A screening test of lipolytic productivity of all bacterial isolates resulted in the fact that only two bacterial isolates were found to be the best lipolytic enzymes producer (Table 1). From the industrial point of view, in order to produce low cost thermoalkalostable lipase, the two most potent bacterial isolates viz. B42, & B78 were allowed to grow on SHW under sub merged fermentation (SmF). The purpose of the present study was also to determine the best factors controlling the lipase production by *B. licheniformis* B42 and *G. stearothermophilus* B78. The optimum temperature for lipase production by both *B. licheniformis* B-42 and *G. stearothermophilus,* B-78 grown on the SHBM was found to be 55˚C. This temperature led to the highest yield of lipase(s) viz., 2.167 and 2 (U/ml) respectively. However the elevation of incubation temperature above 55˚C was accompanied by suppression of lipase(s) production as compared to the optimal value (Figure 1). The optimum initial pH value for lipase(s) production by both bacterial strains was 9. The enzyme yields reached up to 12.7, and 54.45 unit/ml for both *B. licheniformis* B-42 and *G. stearothermophilus* B-78 respectively. Below or above this optimal pH value, the enzyme productivity decreased gradually (Figure 2).

The maximum lipase productivity was attained in the presence of 1.5 (12.5 %) g SHW/Baxter bottle (7.5%) in the case of *B. licheniformis* B-42**.** *G. stearothermophilus* B-78, on the other hand, produce the maximum yield at SHW concentration of 2.5 (12.5 %) g / Baxter bottle(Fig.3). These results mean that , lipase biosynthesis depends not only on the substrate concentration but also on the kind of the producing strains. The maximum lipase productivity reached its

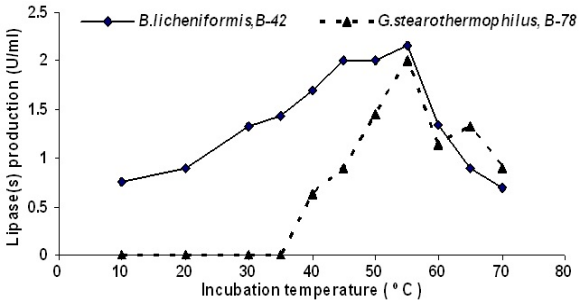


**Fig. 1:** Effect of different incubation temperatures on lipase(s) productivity by *B. licheniformis,* B-42 and *G. stearothermophilus,* B-78.

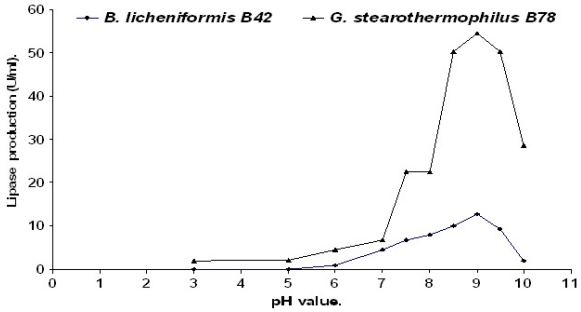


**Fig. 2:** Effect of different initial pH values on lipase(s) productivity by *B. licheniformis* B-42 and *G. stearothermophilus,* B-78.

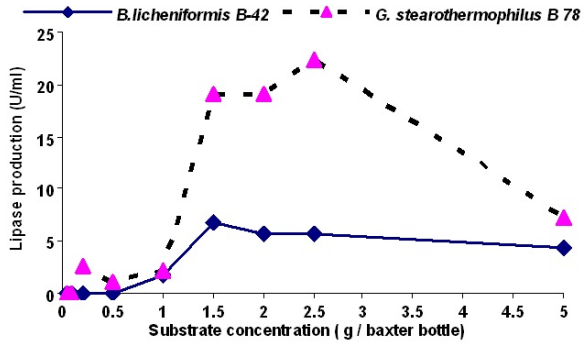


**Fig. 3:** Effect of different substrate concentrations on lipase(s) productivity by *B.licheniformis* B-42 and *G. stearothermophilus* B-78.

maximal value 251.78 U/ml by supplementing the production medium with fructose in the case of *B. licheniformis* B- 42. Also, data proved that glucose was the best carbon source for lipase(s) production by *G. stearothermophilus* B-78 where it reached its maximal value 251.78 U/ml (Fig.4) .

None of the tested nitrogenous sources induced lipase productivity by *B. licheniformis* B-42 as compared to control. Ammonium molybdate gave the

**Table 1:** Relation of lipase production to potent bacterial isolate growing on SHW at 55 ºC for 48 h. using TCZ technique.

| No. | Number of bacterial isolate | SHW | No. | Number of bacterial isolate | SHW |
|---|---|---|---|---|---|
| | | Lipase production (mm) | | | Lipase production (mm) |
| 1 | 15 | 12.0 ± 0.0 | 11 | 78 | 13.8 ± 0.0 |
| 2 | 38 | 12.1± 0.22 | 12 | 80 | 13.1 ± 0.0 |
| 3 | 42 | 14.0 ± 0.0 | 13 | 89 | 12.0 ± 0.0 |
| 4 | 47 | 12.0 ± 0.0 | 14 | 90 | 12.0 ± 0.0 |
| 5 | 57 | 12.6 ± 0.0 | 15 | 95 | 12.2 ± 0.0 |
| 6 | 60 | 12.8 ± 0.0 | 16 | 106 | 12.8 ± 0.0 |
| 7 | 64 | 12.6 ± 0.0 | 17 | 108 | 12.0 ± 0.0 |
| 8 | 70 | 12.0 ± 0.0 | 18 | 110 | 12.0 ± 0.41 |
| 9 | 71 | 13.0 ± 0.0 | 19 | 141 | 13.5 ± 0.0 |
| 10 | 72 | 13.5 ± 0.0 | 20 | 147 | 13.7 ± 0.20 |

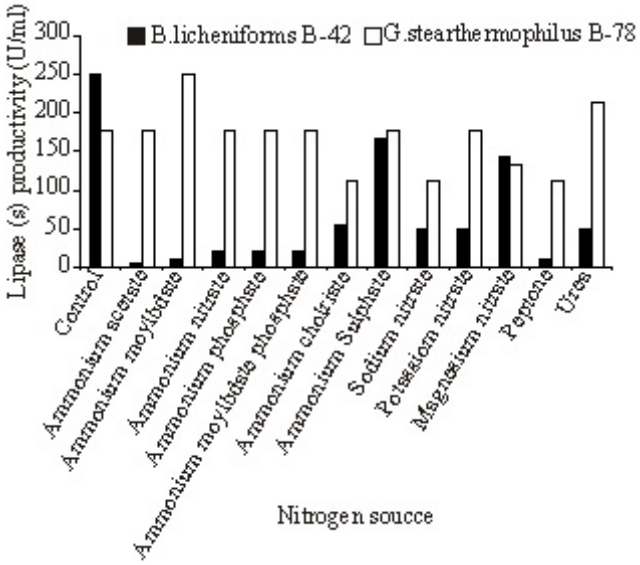


**Fig. 4:** Effect of different substrate concentrations on lipase(s) productivity by *B.licheniformis* B-42 and *G. stearothermophilus* B-78

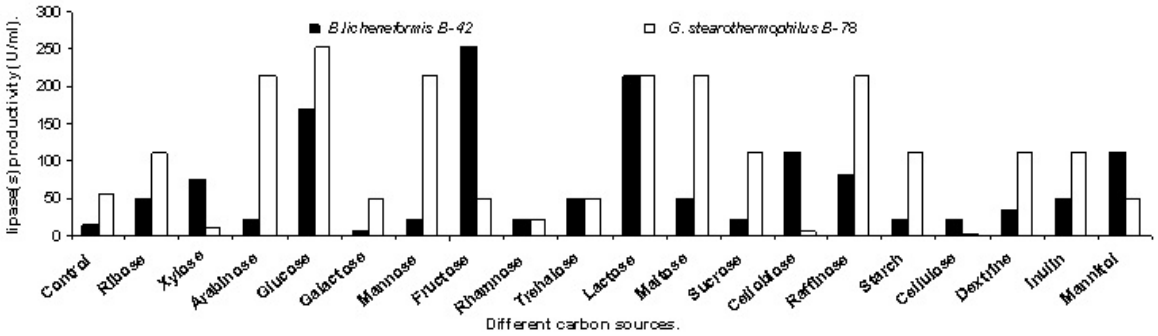


**Fig. 5:** Effect of different carbon sources on lipase (s) productivity by B.licheniformis B-42 and G. stearothermophilus B-78.

highest lipase yield by introducing into production medium specialized for *G. stearothermophilus* B-78, where it reached up to 251.78 U/ml (Fig.5). Addition of 1.5 ml of bacterial suspension to a Baxter bottle containing 20 ml gave the maximum lipase(s) production represented as 50.23 U/ml for *B. licheniformis* B-42**.** On the other hand, the volumes of inoculum below or above 1.5 ml gave gradually decreased values as compared to the optimal one. The maximum lipase(s) yield reached up to 251.78 U/ml by *G. stearothermophilus* B-78 at an inoculum size of 2.5 ml/Baxter bottle (Fig. 6).The highest yield of lipase production was obtained at the end of an incubation period of 72 h. for the two most potent bacterial strains since the productivity reached up to 935.4 U/ml and 1517.15 U/ml for *B. licheniformis* B-42 and *G. stearothermophilus* B-78 respectively (Fig.7). A sharp decrease in lipase production occurred after 72 h of cultivation which could be due to proteolysis degradation of enzyme system.

The maximum lipase productivity reached up to 590.24 U/ml in the presence of thiamin application for *B. licheniformis* B-42. Also, data revealed that the maximum lipase yield reached up to 2118.5 U/ml in the presence of pyridoxine for *G. stearothermophilus* B-78. DL-isoleucine was considered to be the best amino acid used for lipase production by *B. licheniformis* B-42 where this production reached its maximal value 935.4 U/ml**.** This highest stimulatory effect was followed by DL-tyrosine and DL-alanine, while glycine, DL-threonine, L-methionine, DL-aspartic acid, L-glutamine, L-lysine, and L-phenylalanine gave the same results as control. Data indicated that DL-tyrosine exhibited the highest enzyme productivity by *G. stearothermophilus* B-78 where production reached its maximal value 251.78 U/ ml. L-glutamine and L-arginine were also found induce higher enzyme productivity than control (Fig.8).

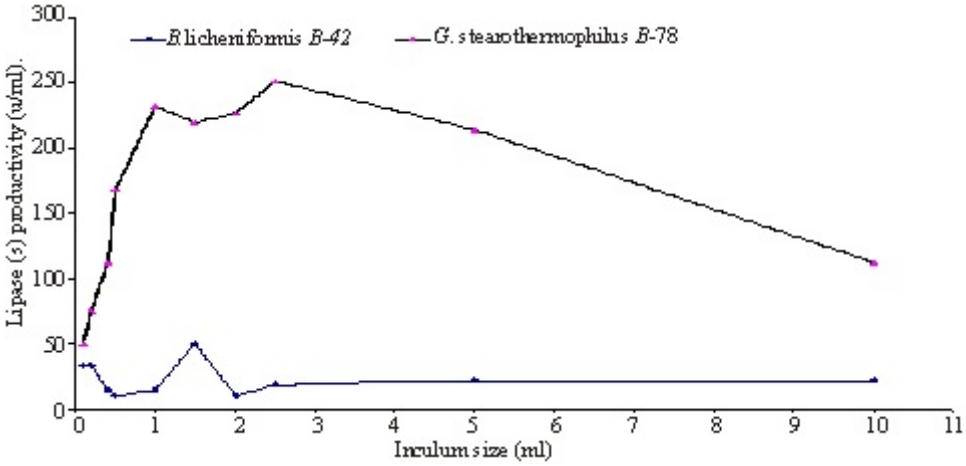


**Fig. 6:** Effect of different inoculum size on lipase (s) productivity by *B .licheniformis* B-42 and G. *stearothermophilus* B-78.

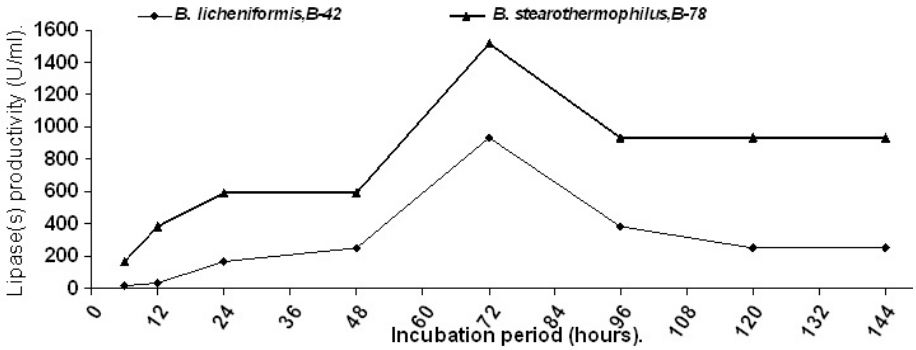


**Fig. 7:** Effect of different incubation periods on lipase(s) productivity *B. licheniformis* B-42 and G. *stearothermophilus* B-78.

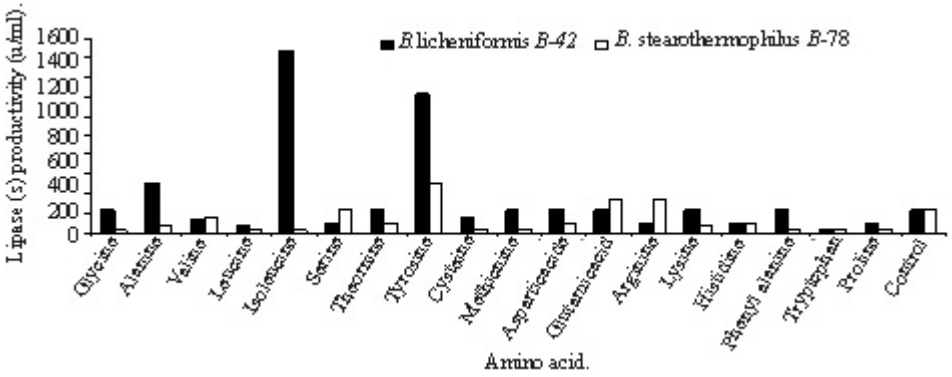


**Fig. 8:** Effect of different amino acids application on lipase(s) productivity by *B. licheniformis* B-42 and G. *stearothermophilus* B-78.

One hundred ml bottle capacity was the best volume for maximum lipase productivity by *B. licheniformis* B-42 whereas, 250 ml bottle capacity was considered the favorable volume for maximal lipase yield by *G. stearothermophilus* B-78.

From the above results we can conclude that, the lipase production by two bacterial strains under the optimum conditions (Table 2) increased many time compared to the amount obtained at the first screening of production from 2.167 to 1517 U/ml (700 fold) by *B. licheniformis* B42 and from 2 to 112.46 U/ml (56.2 fold) by *G. stearothermophilus* B78.

In a trial to precipitate lipase by ammonium sulphate, results revealed that 80 % saturation was proved to be the best concentration for maximal specific activity. The most active protein fractions, raise the purification fold many times from the origin i.e. 20.81 fold. The purification fold of alkaline thermostable lipase increased to 23.43 after applying Sephadex G200 column chromatography technique (Fig. 11). The overall purification steps protocol resulted in raising the purification fold to 27.33 times for lipase after applying Sephadex G100 (Fig.12). This would possibly be due to removal of inhibitory and /or high molecular weight contaminants during these purification stages.

Only one active peak for lipase was obtained in fractions (6-13). The fraction number (10) reached the highest specific activity up to 29220.68 $U/mg^{-1}$.protein (Fig.9). A sharp peak was obtained in lipase purification and specific activity reached its maximal value up to 29220.68 $U/mg^{-1}$ protein for lipase. A summary of the purification steps of lipase produced by *B. licheniformis* B-42 is given in table (3).

*B. licheniformis* B-42 lipase contains 13 amino acid. Aspartic acid and threonine represented the highest concentration (i.e.182.511 and 181.194 µg/ml) respectively These amino acids were followed by serine (164.6 µg/ml). Amino acids aspartic acid, threonine, serine, glutamic acid, glycine, histidine, alanine, leucine, phenylalanine, valine, arginine, lysine, isoleucine, were detected in the partially purified lipase by 182.511; 181.194; 164.608; 136.977; 129.711; 117.169; 111.205; 100.309; 72.0224; 66.1079; 64.9647; 59.1062; and56.3964 respectively.

The aim of the following experiments was to investigate some properties of the partially purified lipase produced by *B. licheniformis* B-42 incubated under all optimal nutritional and environmental fermentation conditions. These properties include:- effect of incubation temperature, thermostability, pH value, pH stability, incubation period, purified enzyme concentration, substrate concentration on purified lipase activity.

Lipase produced by *B. licheniformis* B-42 reached its maximal activity at 50-60ºC where it reached up to 50.23 U/ml. Below or above this range the activity decreased (Fig.11). The purified lipase exhibited its maximum activity at 50ºC where it reached up to (590.24 U/ml) (Fig 12).

The best pH value for the purified lipase activity was 9.5 where it reached up to 50.23 U/ml. The maximum activity for lipase was at pH 9.5 where it reached up to 112.46 U/ml. The enzyme was still active up to pH of 10.5(Fig.13 &14).

The purified lipase exhibited its maximal activity at 36 h incubation period where it reached up to 1517.15 U/ml. A continuous increase of enzyme activity was due to the increase of enzyme concentration units, where it reached up to 251.78 units /ml for the purified lipase. One tenth (%) tributyrin was the best substrate concentration for the purified lipase activity, where it reached up to 112.46 U/ml. It was found that the enzyme activity decreased above this particular concentration (0.1)% for lipase.

**Table 2:** A summary of the optimal nutritional and environmental parameters controlling the lipase production by *B. licheniformis* and *G. stearothermophilus*.

| No. | Parameter | Lipase production | |
|---|---|---|---|
| | | *B. licheniformis* B-42 | *G. stearothermophilus* B-78 |
| 1 | Temperature | 55 | 55 |
| 2 | pH value | 9 | 9 |
| 3 | Substrate concentration | 1.5 | 2.5 |
| 4 | Carbon source | Fructose | Glucose |
| 5 | Nitrogen source | Control | Amm.molybdate |
| 6 | Inoculum size (ml) | 1.5 | 2.5 |
| 7 | Incubation period (h) | 72 | 72 |
| 8 | Vitamin (100ppm) | Thiamine | Pyridoxine |
| 9 | Amino acid | Isoleucine | Control |
| 10 | Bottle capacity (ml) | 100 | 250 |

**Table 3:** A summary of purification steps of lipase produced by *B. licheniformis* B42 allowed to grow on SHW substrate under 55 °C.

| Purification step | Volume (ml) | Protein content (mg/ml) | Total protein (mg) | LipaseActivity (U/ml) | Total activity | Specific activity ($U/mg^{-1}$ protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| CFF | 1000 | 0.473 | 473 | 590.24 | 590240 | 1247.86 | 1.0 | 100 |
| $(NH_4)SO_4$ fractionation | 100 | 0.823 | 82.3 | 17825 | 1782500 | 21658.5 | 17.3 | 301 |
| Dialysis against sucrose | 3.0 | 2.3 | 6.9 | 59707.6 | 179122.8 | 25959.8 | 20.81 | 30.1 |
| Sephadex G200 | 5.0 | 0.0725 | 0.3625 | 2118.5 | 10592.5 | 29220.68 | 23.43 | 1.0 |
| Sephadex G100 | 5.0 | 0.0058 | 0.029 | 197.7 | 988.5 | 34086.2 | 27.33 | 0.16 |

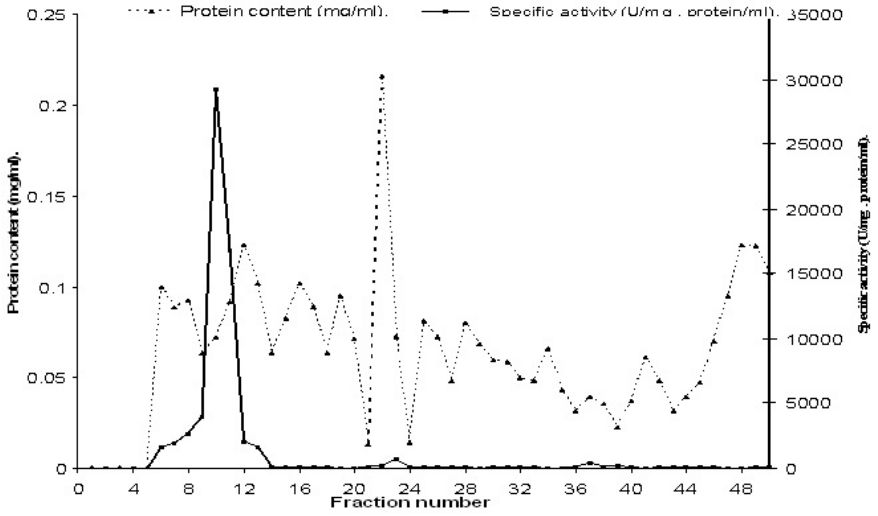


**Fig. 9:** Fractionation pattern of lipase(s) produced by *B. licheniformis* B-42 at 55ºC using sephadex G-200 column chromatography technique.

Chlorine acted as activator for lipase activity, where chlorine concentration up to 10 µl /ml increased the lipase activity and the remaining activity was 149.6% as compared to control. Increasing chlorine concentration up to 15µl/ml indicated an obvious decrease in lipase activity up to 66.8 % remaining (Table 4).

The application of Triton X100 exhibited a very high enhancement in lipase activity in which the maximum stimulation occurred with 7.5-10 %

**Table 4:** Different chlorine concentrations in relation to the activity of the purified lipase produced by *B. licheniformis* B42.

| Exposure time (min.) | Chlorine concentration (µl/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | | 7 | | 10 | | 15 | |
| | Enzyme activity (U/ml) | Remaining activity (%) | Enzyme activity (U/ml) | Remaining activity (%) | Enzyme activity (U/ml) | Remaining activity (%) | Enzyme activity (U/ml) | Remaining activity(%) |
| 0 | 75.16 | 100 | 75.16 | 100 | 75.16 | 100 | 75.16 | 100 |
| 15 | 112.46 | 149.6 | 112.46 | 146.6 | 112.46 | 146.6 | 50.23 | 66.8 |
| 30 | 112.46 | 149.6 | 112.46 | 146.6 | 112.46 | 146.6 | 50.23 | 66.8 |
| 45 | 112.46 | 149.6 | 112.46 | 146.6 | 112.46 | 146.6 | 50.23 | 66.8 |
| 60 | 112.46 | 149.6 | 112.46 | 146.6 | 112.46 | 146.6 | 50.23 | 66.8 |

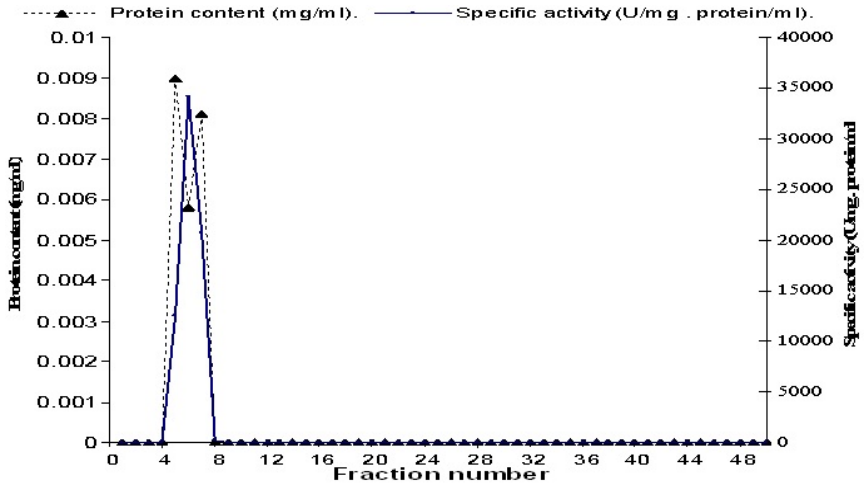


**Fig. 10:** Fractionation pattern of lipase(s) produced by *B. licheniformis* B-42 at 55ºC using sephadex G-100 column chromatography technique.

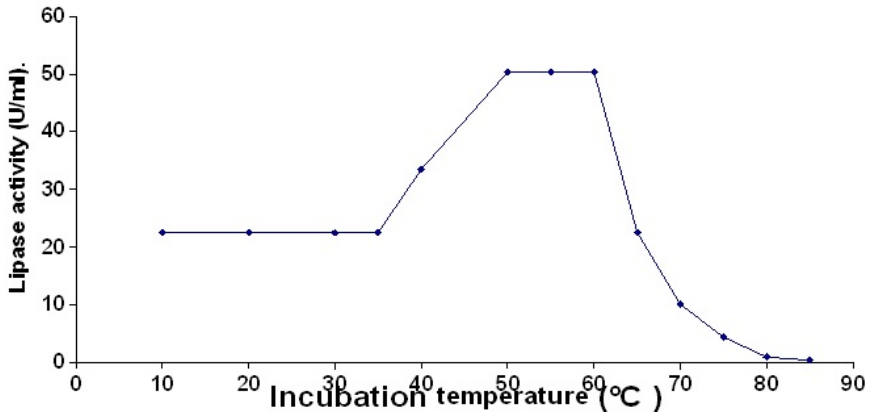


**Fig. 11:** Effect of different incubation temperatures on the purified lipase produced by *B. licheniformis* B-42.

(v/v)Triton, where the activity increased to 6607.9 %. Also, lipase was stable in all applied concentrations of SDS, and sodium cholate. Lipase was unstable in the presence of the remainder of the oxidants and surfactants used in this study, except Tween 40, and Tween 80, where lipase was found to be stable at high concentrations 7.5-10 (% v/v).

Unfortunately, a high percentage of the lipase activity was lost in presence of commercial detergent. This percentage reached up to 99.1 % in the presence Omo. Calcium chloride acted as a stabilizer for lipase activity where, lipase was stable in the presence of Rabso, Omo, Lang, Xtra automatic, and Pril by $CaCl_2$ application at 10000 ppm concentration. To overcome

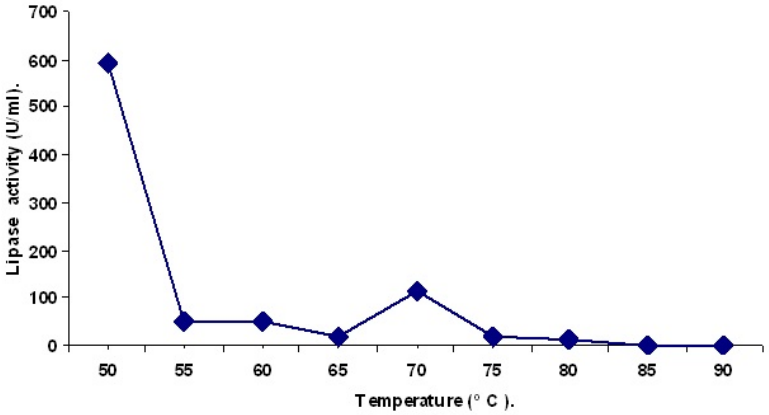


**Fig. 12:** Thermostability of the purified lipase produced by *B. licheniformis* B-42.

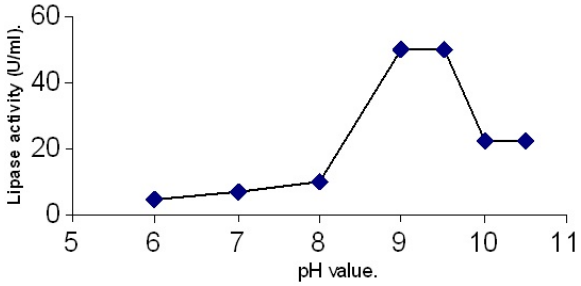


**Fig. 13:** Effect of different pH values on the activity of purified lipase produced by *B. licheniformis* B-42.

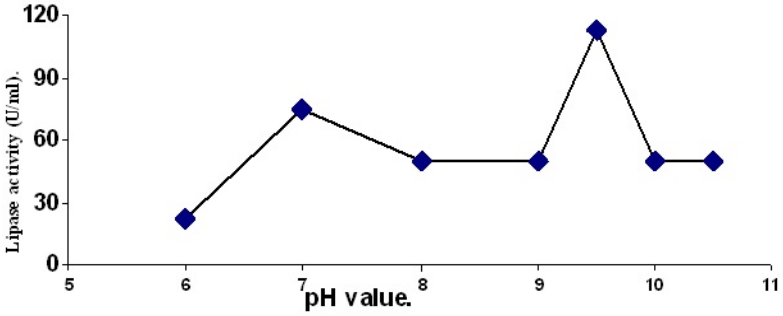


**Fig. 14:** pH stability of the purified lipase produced by *B. licheniformis* B-42.

the inhibition of lipase activities in the presence of commercial detergent, a trial to supplement the enzyme-detergent complex with different concentration of $CaCl_2$ was carried out. Interestingly, the lipase activity was not only recovered (restored) but also stimulated with increasing $CaCl_2$ concentration in the presence of some applied detergents (Table 5).

**Discussion:** Enzyme production is a growing field of biotechnology and the world market for enzymes is over $1.5 billion and it is anticipated to double by the year 2008[19]. Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. The main objective of the present work was to investigate of screening, production, purification, and characterization of thermoalkalostable lipase for application in detergent technology has been undertaken. Taking account of its commercial importance, cost effective production of the lipase is indispensable. The productivity of any cultivation is principally affected by the medium composition and the different fermentation process parameters. To meet the demand of industrial application, low cost medium is required for the fermentation. Since the contents of synthetic medium such as nutrient broth as well as other components are very expensive these contents may be replaced by more economically available or industrial byproducts for the reducing of the cost of the medium. The nature of solid substrate is the most important factor in solid

**Table 5:** Effect of $CaCl_2$ as a stabilizer on the activity of the purified lipase in the presence of various detergents.

| $CaCl_2$ | Lipase activity (U/ml) | | | | | |
|---|---|---|---|---|---|---|
| Detergent | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| Powder detergent | | | | | | |
| Rabso | 4.477 | 10.02 | 14.99 | 22.44 | 22.44 | 50.23 |
| Ariel | 0.89 | 2.0 | 10.02 | 22.44 | 22.44 | 22.44 |
| Omo | 0.89 | 0.89 | 0.89 | 50.23 | 50.23 | 50.23 |
| Lang | 0.89 | 2.99 | 4.47 | 22.44 | 22.44 | 112.46 |
| Persil | 0.89 | 2.0 | 4.47 | 22.44 | 22.44 | 22.44 |
| Xtra | 0.89 | 4.477 | 10.02 | 10.02 | 10.02 | 10.02 |
| Ariel automatic | 4.477 | 4.477 | 4.477 | 10.02 | 10.02 | 10.02 |
| Xtra automatic | 1.336 | 1.336 | 2.0 | 4.477 | 4.477 | 112.46 |
| Liquid detergent General | 0.89 | 2.99 | 50.23 | 50.23 | 50.23 | 112.46 |
| Pril | 0.0 | 0.0 | 17.97 | 17.97 | 45.76 | 45.76 |

state fermentation (SSF). This not only supplies the nutrients to the culture but also serves as an anchorage for the growth of microbial cells[32]. However, the selection of SHW for the biosynthesis of lipase was based on the following factors:(i) They represent the cheapest wastes in Egypt; (ii) they are available at any time of the year; (iii) their storage represents no problem; and (iv) they are not affected by the exposure to environmental conditions. Interestingly, SHW are serious pollutants for the environment, many pathogenic microorganisms can grow on them. As this may cause many diseases for man and animals, their use for lipase production helps in avoiding the spread of diseases. The pH of the culture medium plays a critical role for the optimal physiological performance of the bacterial cells and the transport of various nutrient components across the cell membrane aiming at maximizing the alkaline enzyme yields. Thus, the pH of the fermentation medium had a marked effect on the cell growth and enzyme production. Furthermore, the optimal pH values may be affected by the incubation temperature in many thermophiles. Concerning lipase production in this study it has been found that, the yield was reached its maximum at pH 9 by both potent strains.

In agreement to the present results Nawani *et al.,*[23] found that, the optimum conditions for production of a thermostable lipase by *Bacillus* sp. at temperature 60 ºC and pH 8.0. Hassan *et al.,*[8,12] also found, the maximum lipase production by *Bacillus* sp. FH5 was optained after 48 h at pH 8.0. Among all the tested carbon gave the maximum activity and all the tested nitrogen sources gave maximum production /activity. One of the most fascinating data, the optimum pH value for the growth of both thermoalkalophilic bacterial strains under study viz., *B.licheniformis* B-42 and *G. stearothermophilus* B-78 was at pH 9 for all the produced thermostable lipases under investigation. The optimum pH values for lipase production by *B. stearothermophilus* was recorded between 9-10 Kim *et al.,*[16]. These results were showed that, the microbial enzyme production depends not only on species or strain variation but also on other factors such as incubation period and temperatures. On the other hand lipase production would also depend on three other factors (i) protease formation, (ii) pH stability of the lipase; and (iii) reactivity of the lipase at different pH values[21,26].

Results were recorded in this study means that, lipase biosynthesis depends not only on the substrate concentration but also on the kind of the producing strain. Fructose and glucose were induce lipase production by *B. licheniformis* B42, and *G. stearothermophilus* B78 respectively. In accordance to our results, fructose was reported be the best carbohydrate for the production of an extracellular lipase by *Rhodotorula glutinis* Paparaskevas *et al.,* [24]. In view of other workers, Sugihara *et al.,*[34] reported that, lipase production from *Bacillus* sp. in the presence of 1% olive oil in the culture medium. Kim *et al.,*[16] reported that, production of a highly alkaline thermostable lipase by *Bacillus stearothermophilus* L1 in a medium that contained beef tallow and palm oil. A thermophilic bacterium, *B. thermooleovorans* ID-1 isolated from hot springs in Indonesia showed extracellular lipase activity and high growth rates on

lipid substrates at elevated temperature Lee *et al.,*[17].

A sharp decrease in lipase production occurred after 72 h. of cultivation which could be due to proteolytic degradation of enzyme system. The decrease of lipase productivity at the latter fermentation stage, could be explained by pH inactivation, proteolysis or both, and the increase of protease activity was result of the release of intracellular protease. Non of the tested nitrogen sources induced lipase production by *B.licheniformis* B-42 while ammonium molybdate gave the highest yield by *G. stearothermophilus* B-78.

The application in the detergent industry does not require high-purity for enzymes and generally requires use of the crude or partially purified enzyme preparation. Precipitation of enzymes was carried out firstly by ammonium sulphate since it is highly soluble in water, cheap, and has no deleterious effect of structure of protein, so far all these reasons, precipitation by ammonium sulphate was selected as a first step of purification program. The alkaline thermostable partially purified lipase purification fold increased 23.43 and 27.33 times after applying Sephadex G200 and G100 respectively. This would possibly due to removal of inhibitory and/ or high molecular weight contamination during these purification stages. On the other hand, Saxena *et al.,*[27] used, 85% ammonium sulphate fractionation for alkaline lipase purification. Sharma *et al.,*[30] used ammonium sulphate saturation for alkaline lipase purification by *Bacillus* Sp. RSJ-1. In view of lipase purification by many authors, the extracellular lipase by *B.stearothermophilus* MC7 was purified to 19.25- fold with 10.2% recovery and a specific activity of about 12 U(mg protein)-1 Kambourova *et al.,*[14]. Hiol *et al.*[13] purified extracellular lipase by ammonium sulphate precipitation, sulfopropyl sepharose chromatography, sephadex G-75 gel filtration and a second step sulfopropyl sepharose chromatography with 1200 fold. A thermostable lipase produced by a thermophilic *Bacillus* sp. J33 was purified to 175-fold by ammonium sulphate and phenyl sepharase column chromatography Nawani and Kaur,[23]. Lee *et al.,*[17,18] purified a lipase from *B.thermoleovorans* ID-A (BTIDA), and *B.thermoleovoran*-ID-B(BTIDB) with a purification fold of 300 and 108 respectively while the over all yield was 16 and 3.2% respectively. Sharma *et al.,*[30] purified a thermostable alkaline lipase from *Bacillus* sp. RSJ-1 and resulted in 201- fold purification with 19.7% final yield. Also 19- purification fold was attained by lipase purification using ammonium sulphate fractionation, exchange chromatography and gel filtration Gopinath *et al.,*[10]. Amino acid analytical data of purified lipase produced by *B.licheniformis* B-42 revealed that, aspartic acid and therionine exhibited the highest concentrations.

In order to assess the utility and compatibility of enzymes with the commonly used detergents, its properties such as pH and temperature stability …..etc should be determined. The optimal temperature and pH were 55-60°C, and pH 9.5 also the enzyme was found to be thermostable up to 60°C and it had a wide pH range (8-10.5) for its maximal activity at pH 9.5. Lee *et al.,*[18] purified two thermostable lipases (A&B)from *Bacillus thermooleovorans* ID-1 with an optimal temperature of 60-65ºC and 60ºC for lipase A and B respectively while the pH optima were 9 and 8-9 respectively. Lipase A retained 75% of its activity when incubated for 30 min. at 60ºC. Optimum temperature and pH *Bacillus* sp. RSJ-1 lipase were 50ºC and pH 8 and it was stable at 50ºC for 60 min. The enzyme showed good stability after for 2h in an alkaline pH range Sharma *et al.,*[30]. Lipase from *B.stearothermophilus* was stable up to 55ºC for 30 min. Kim *et al.,*[16]. Kambourova *et al.,*[14] purified lipase from *Bacillus stearothermophilus* MC7 that expressed maximum activity at 75-80ºC and had pH optimum within the range of 7.5-9.0 and was stable alkaline pH range (7.0-11.0).

Thermal stability of lipase activity is obviously related to its configuration and subsequently, the melting point. Detergent may contain chlorine, which degrade proteins into smaller peptide chains, thereby lowering binding energies and affecting desertion from the surface. Hence enzymes used as cleaning agents should be stable in the presence of chlorine. Interestingly, chlorine stimulate lipase activity where it enhanced up to 149.6 % or its activity with 10µl chlorine/ml for 60 min. Triton X100 was enhance lipase activity by 6607.9% and stable toward SDS and sodium cholate (up to 10%), and to Tween 40 and Tween 80 at 7.5-10% (v/v). On the other hand, sodium perborate, $H_2O_2$, Tween 20 were inhibited lipase activity. Unfortunately, lipase activity was completed inhibited in the presence of all tested commercial detergents. To overcome the lipase activity inhibition, a trial to supplement the enzyme –detergent complex with different concentration of calcium chloride was carried out. Interestingly, the enzyme activity not only recovered but also stimulated with increasing calcium chloride concentration in the presence of some applied detergents. Lee *et al.,*[17,18] isolated two lipases from *B. thermooleovorans* ID-1 lipase A was inhibited by divalent ions including $Cu^{+2}$, $Hg^{+2}$ and $Co^{+2}$. In contrast lipase B was slightly activated by $Ca^{+2}$, $Na^{+}$, $Co^{+2}$ and $Mn^{+2}$ ions. EDTA treatment strongly inhibited both enzymes. *B.stearothermophilus* MC7 lipase was inhibited by divalent ions of heavy metals, entirely by $Cu^{+2}$ and strongly by $Fe^{+2}$ and $Zn^{+2}$ Kambourova *et al.,*[14]. Concerning alkaline- thermostable lipase

activity in this study, it was found Triton X100, enhance lipolytic activity by 6607.9%. Also lipase was stable towards SDS and sodium cholate (up to 10%) and to Tween 40 and Tween 80 at 7.5-10% (v/v). On the other hand, sodium perborate $H_2O_2$, and Tween 20 inhibited enzyme activity. Thermostable lipase from *Bacillus stearothermophilus* Pl was stable for 1 h in 0.1% Triton X100 and activity reduced slightly with SDS and Tween 20. while concentration of 1%, SDS and Tween strongly inhibited lipase Sinchaikul *et al.,*[31]. Sharma *et al.,*[30] found that, *Bacillus* sp. RSJ-1 lipase retained 100, 92 and 82% of its activity in the presence of Triton X100, Tween 80 and Tween 20, respectively, but the enzyme was stable at low concentration of $H_2O_2$ up to 0.2% while with 1% $H_2O_2$ the enzyme retained 47% of its original activity. In conclusion, results of the present study suggested the possibility of *B. licheniformis* B 42 and *G. stearothermophilus* B78 to produce enzymes by SSF and SmF using a cheeps substrates for enzyme production. This enzyme was stable over a wide range of pH and temperature and also showed compatibility with various commercial detergents tested, it were used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent (Rabso) could significantly improve the cleansing performance towards different stains. Considering the over all properties of different alkaline enzymes of microbial origin and the thermostable alkaline enzymes from our strains *B. licheniformis* B 42 and *G. stearothermophilus* B78 are better as regards to pH and temperature stability, stability in the presence of surfactants, detergents compatibility and above all bleach stability for a potential application not only in heavy-duty detergents for fabrics but also in bleaches and detergents for automatic dishwashing machines.

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