# Bioremediation of Khormah Slaughter House Wastes by Production of Thermoalkalistable Lipase for Application in Leather Industries

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**Abstract:** Thermoalkalistable lipases enzymes occupy a very important group used for bioremediation of environmental pollutants, act as biocatalysts and many other industrial application. One of the major industrial microorganism is the *Bacillus* sp. which are abundance in the desert localities. The aim of this research was to isolate, characterize of lipase producing bacteria from Khormah slaughter house wastes (SHW), Taif, Makkh, KSA, thermoalkalstable lipase production and its purification. SHW was found to be the most successful natural source for thermoalkalstable lipase production. Twenty eight lipase producing bacterial isolates were isolated. One strain was selected as a best thermoalkalistable lipase producer identified by morphological, physiological and biochemical characteristics as *Bacillus stearothermophilus* KGSHW-1.The identification was confirmed by molecular biology technique. Different factors such as temperature, pH, incubation period, nutritional requirements, substrate concentration, inoculum size, vitamin requirements, effect of metallic ions, effect of incubation condition and bottle capacity were studied for improving thermoalkalstable lipase production. An optimum conditions for production of thermoalkalstable lipase by *B. stearothermophilus* KGKSA-1 was achieved at 60ºC, after 24 h. of fermentation at pH 10. Beef extract, vitamin B6 were the best carbon source and vitamin source, while potassium nitrate served as a source for nitrogen. Tryptophan was served as best amino acids, substrate concentration 75 (%,w/v) inoculated by 1 ml (7 x $10^{10}$ CFU.$ml^{-1}$) under shaking conditions in 2.5% of 250 ml capacity. $BaCl_2$ was induced the enzyme production. This enzyme was purified using acetone concentration 60% precipitation and gel filtration column using Sephadex G200 and 100 respectively. The purification fold steps for precipitation by acetone, dialysis against sucrose, Sephadex G200 and Sephadex G100 were found 2.086, 1.43,14.9 and 39.8 respectively. Sodium dodecyl sulphate revealed that the monomeric lipase has 60 kDa of its monomeric lipase. These results show a great potential for the use of this produced enzyme in industry and other future studies.

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## 1. Introduction

Lipases (Triacylglycerol hydrolases EC3. 1. 1.3) are diverse group of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides to liberate fatty acids and glycerol, showing activity on a great variety of substrates **(Jaeger *et al.*,1999).** Due to the fact that extracellular thermoalkalistable lipases are quite stable under extreme environments (pH, temperature…etc), substrate specificity and active in organic solvent, these enzymes occupy a very important place in the bioremediation of food wastes and generally considered as most important group of biocatalyst for biotechnological applications. Other applications include the pharmaceutical (resolution of racemic mixtures), dairy (hydrolysis of milk, fat), detergent (removal of oil/fat stains), cosmetic, oleochemical, fat processing, food flavoring, leather processing (removal of lipids from hides and skin), textile, paper industries, medical (diagnostic tool in blood triglyceride assay) industries and waste water treatment (degreasing of lipid clogged drains) **(Posorsk,1984; Macrae and Hammond,1985; Sharma *et al.*, 2001;Snellman and Colwell, 2004).** Lipases can be naturally and readily found in the earth's flora and fauna. However, most commercial lipases are produced from microbes. Alkaliphilic microorganisms are widely distributed in nature and alkaliphilic *Bacillus* strains are often good source of alkaline extracellular enzymes **(Abdul-Hamid *et al.*,2003; Littechild *et al.*, 2007;Ghori *et al.*, 2011).**

Microbial lipases are usually extracellular enzymes, which are produced by bacteria, fungi and actinomycetes. Bacterial strains are generally more used as sources of lipases because they offer higher activities compared to other microorganisms. Bacterial enzymes are often more useful than enzymes derived from plants and animals because of the great verity of catalytic activities available, the

high possible yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. They are also more stable and safer than their production is more convenient. Microbial lipases have already established their vast potential regarding usage in numerous applications. Many microorganisms are capable of producing lipase and *Bacillus* sp. is the most widely studied group. *Bacillus* species lipases have been purified and biochemically characterized from many *Bacillus species such as Bacillus subtilis, Bacillus pumilus, Bacillus stearothermoleovorans* and their genes have been cloned and sequenced **(Kim *et al.*, 2002)**.

*Bacillus* lipases attract attention because they have unique protein sequences and many uncommon biochemical properties **(Kim *et al.*,2002)**. In contrast many bacteria other than *Bacillus* sp. have been studied for their lipase production ability **(Gupta *et al.*,2004)**, among them are *Arthrobacter* sp. **(Sharma *et al.*,2009)**, *Pseudomonas* sp. **(Meghwanshi *et al.*,2006)** and *Staphylococcus* sp. **(Horchani *et al.*,2009).** One of the major industrial microorganism is the *Bacillus* species which are in abundance in the desert regions. Thermophilic bacteria is an important source of thermostable enzymes **(Rahman *et al.*, 2007;Zuridah *et al.*, 2011; Patil *et al.*, 2011)** and desert environments are usually their favorable habitats. In Kingdom of Saudi Arabia (KSA), Makkah region Khormah governorate slaughter house throw huge amount of wastes. These wastes have been explored their potential as a source of thermophilic bacteria. Thermophilic organisms are defined as those that have their optimal growth temperature between 50 and 80ºC **(Brown, 2005)**. Potential enzymes such as amylase, protease, xylanase and chitinase have been identified in thermophilic microorganisms including the lipase enzyme **(Dominguez *et al.*, 2005).**

In order to be used in industry, lipases need to be thermostable and maintain their activity in organic solvents **(Nawani and Kaur,2001,2007).** Thermostability is required since many processes use temperatures around 50 ºC due to the high melting points of the lipids that are used as substrates **(Lima *et al.*,2003, 2004;Hasan *et al.*, 2006,2007).** Lipases are of significant important in leather industry. Degreasing of leather during its processing in an important use of lipases in tanning industry in the process of bating , removal of fat and protein debris by chemical processes in both polluting and laborious . Lipase can play distinct role in resolving such problems of leather industry and tanneries. Therefore, researchers in search of novel lipases with high catalytic rates from microbial sources **(Ghori *et al.*, 2011).**

Because of the numerous potential uses of lipase enzyme, this study present a preliminary investigation on lipase produced from *B. stearothermophilus* isolated from Khormah governorate slaughter house wastes (KGSHW) as a local environment. The most potent resultant culture was investigated to determine optimal bacteria lipase production which include temperature, pH, incubation period, nutritional requirements, substrate concentration, inoculum size, vitamin requirements, effect of metallic ions, effect of incubation condition and bottle capacity were studied for improving lipase production. Purification of the produced thermoalkalistable lipase under all optimal conditions.

## 2. Materials & Methods

**Sample collection, sampling sites and bacterial isolation:**

Samples of fat, meat, water and polluted soil were collected from various locations at Khormah governorate slaughter house, Makkah, KSA. Samples were taken and transported in sterile plastic bags to laboratory and stored at 4ºC when not used immediately. The slaughter house wastes mainly included fatty and meat pieces were found to be attached to general viscera of slaughtered animal. They were dried at a temperature of 70ºC for 8 hours and kept in plastic bags at a refrigerator.

One ml or one gram (from each site or samples) were suspended in 10 ml of sterile double saline solution. After shaking, 5 ml of each suspension was transferred into a 250 ml Erlenmeyer flask containing 100 ml of an enrichment medium with the following composition:(g/L;w/v):Yeast extract,5;olive oil, 10 ml. The mineral salt solution contained (g/L): $KNO_3$,2.0; $KH_2PO_4$,1.0; $MgSO_4.7H_2O$,0.5; $ZnSO_4.7H_2O$, 0.44 ; $FeSO_4.7H_2O$, 1.1 and $MnSO_4.7H_2O$, 0.2. The pH of the medium was adjusted raised to 10 using 1 N NaOH. Plates were incubated at 60ºC and growth checked every day for growth. Developed bacterial colonies were purified and transferred to the nutrient agar medium slants for storage. In addition, bacterial colonies were sub-cultured on nutrient agar medium plates for identification and lipase production. Nutrient agar medium containing (g/L): tryptone ,10.0, yeast extract,5.0; NaCl, 5.0;Agar-agar,20.0 **(Buchanan,1974)**.

**Construction of standard curve of lipase enzyme**

A stock solution of (200,000 µg/ml) of lipase (Sigma) was prepared in glycine NaOH buffer at pH 10. Descending dilutions were prepared. Lipolytic activity determination was performed by colorimetric assay according to **(Castro-Ochoa *et al.*, 2005)** with slight modifications.

**Solution A:** Stock solutions of *para*-Nitrophenyl laurate were freshly prepared in isopropanol at a concentration of 0.3% (w/v). This solution was emulsified by 3 min. sonication pulses of 0.3 s at 50 watts using a Lab sonic 1510 sonicator (B. Braun).

**Solution B:** This solution is 0.1% (w/v) Arabic gum, 0.4 % triton-X-100 (v/v) emulsified in 1 M phosphate buffer at 8 pH value. Then, 450 µl of a 1:10 dilution of solution (A) in solution (B) were pre-incubated for 5 min. at the assay temperature before adding 50 µl of lipase concentration previously prepared. This mixture was incubated at 30°C for 30 min., and the reaction was terminated by addition of 50 µl of 0.1 M $Na_2CO_3$ and 450 µl of distilled water. Released *p*-nitro phenol was immediately determined by measuring the absorbance at $\lambda$ = 410 nm. Proper blanks were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*-NP-derivatives. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 mol of *p*-nitrophenol (molar absorption coefficient $4.6mM^{-1}$ $cm^{-1}$) from *p*NP-laurate in 30 min under test conditions.

**Protein determination**

Total protein content for all enzymatic preparations was determined by the method of **Lowry *et al.*, (1951)** using Bovine Serum Albumin as standard.

**Production medium:**

Czapek- Dox's **(Dox,1910)** mineral salt medium was found to be the most convenient medium for production of lipase enzyme. It was altered to contain (g/l); yeast extract,20 ; $NaNO_3$,2 ;$KH_2PO_4$,1 ;$MgSO_4.7H_2O$,0.5 ;KCl,0.5 and $CaCl_2$,1 and supplemented with 1% of fatty wastes. The pH values of all above media were raised to 10 by 6N NaOH before sterilization.

**Screening and identification of thermoalkaliphilic bacteria**

Thermoalkaliphilic bacterial isolates, the subject of the present investigation were isolated from several localities around Khormah slaughtered house such as slaughter house wastes desert soil, polluted water. Lipolytic activity was detected on Czapek-Dox's mineral salt medium supplemented with 2% fatty wastes. Clear zones around the isolated colonies were detected which demonstrated lipase production **(Akatsuka *et al.*,2003)**. Colonies with highest clear zone of hydrolyzing lipase on the plate were selected as potential lipase producing strain .Based on the lipase secretion on the Czapek- Dox's mineral salt medium supplemented with 2% fatty wastes agar plates , the best enzyme secreting organism was selected for lipase production. Identification of the thermoalkaliphilic bacterial isolate was carried out by morphological, biochemical and physiological characteristics. Identification was confirmed by 16s ribosomal DNA gene sequencing technique according to **Woo *et al.*, (2000**).

**Cell growth determination**

Biomass concentration was measured via turbidity at 600 nm and obtained values were converted to concentration by using a previously determined calibration curve.

**Fermentation technique**

Ten gram of substrate was moistened with 10 ml of diluents in 250 ml of conical flask. The flasks were autoclaved at 15 $lb/inch^2$ pressure for 15 min. The flasks were cooled at room temperature. One ml of bacterial inoculum was aseptically transferred to each flask. The flasks were then placed in an incubator at 60ºC for 48 hrs. The flasks were run parallel in triplicate. After 48 hrs. , 100 ml of buffer (pH 10)was added to each flask. The flasks were placed on rotary incubator shaker at 200 rpm for one hour. After one hour the ingredients of the flasks were filtered and centrifuged. The supernatant was used for estimation of lipase production.

**Parameters controlling the optimum production of thermoalkaliphilic lipase(s) enzyme**

**Effect of incubation temperature on lipase production:**

The most potent thermoalkaliphilic bacterial isolate was grown on Czapek-Dox's mineral salts medium supplemented with 2% slaughter house wastes at temperatures; 40, 50, 60, 65,70,75,80 and 85°C for 48 hrs. The crude enzyme preparation was obtained by centrifugation at 1000 rpm, at 4 ºC for 15 min. The crude enzyme was assayed by colorimetric technique. All the experiments were conducted in triplicate and the average values were reported.

**Effect of different pH values and buffers on lipase production**

Production medium was prepared and pHs were designed at the following values: 7, 8, 9,10,11,12 by using 6N NaOH and 6N $H_2SO_4$.For determination of the best buffer that promotes the highest yield of thermoalkaliphilic lipase(s). Production medium was prepared by using each of Glycine-NaOH buffer (0.2 M) (at pH values; 8.6, 9, 9.2, 9.4, 9.6, 10, 10.4, 10.6), Carbonate-bicarbonate buffer (0.2 M) (at pH values; 9.2, 9.5, 9.8, 10.0, 10.2, 10.3, 10.6, 10.7) and Boric acid-Borax buffer (0.2 M) (at pH values; 9.3, 9.5, 9.7, 9.9, 10.1). Selection of these buffers was dependent mainly on their ability to provide the proper alkaline pH range.

**Effect of fermentation period on lipase production**

One ml of an 18 h. culture of *Bacillus* sp. was inoculated into sterilized 250 ml Erlenmeyer flask containing the lipase production medium. It was incubated for 6, 18, 24, 42, 48, 66, 72 hours.

**Effect of nutritional factors of lipase production**

To evaluate the effect of carbon sources on the lipase production, the following were added to lipase production medium at (1% w/v) glucose, galactose, xylose, ribose, rhamnose, melezitose, sorbitol, mannitol, inuline, sucrose, lactose, maltose, cellobiose, dextrine, starch, pectin, molasse and beef extract. All the carbon sources were sterilized separately through a 0.22 µm membrane filter and then added to the mixture.

Nitrogen sources were added to the production with an equimolecular amount . Different nitrogen sources were used to determine the favorable nitrogen source that fulfills the highest yield of thermoalkalistable lipases. The control was devoid from any nitrogen source. These nitrogen sources including ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium molybdate, ammonium phosphate, sodium nitrate, sodium nitrite, urea, calcium nitrate, potassium nitrate and peptone.

Amino acids were supplemented to the production medium with equimolecular amounts to determine the optimum one(s) that fulfill the highest yield of thermoalkaliphilic lipases. These amino acids included lysine, ornithine, arginine, valine, phenylalanine, histidine, aspartic acid, tryptophan, glycine, threonine, alanine, serine, glutamic acid, cysteine, proline, citruline, methionine, cystine, asparagine and glutamine. The control was devoid from any nitrogen sources.

The lipase production medium was supplemented with different vitamins individually to determine the best vitamin that can improve the production. The control was devoid from any vitamin supplement and the separately vitamins added were; Ascorbic acid, Nicotinic acid, Riboflavin, Myristic acid, and Vitamin B6, each vitamin was applied in concentrations of 25, 50, 100, 250, 500 ppm.

**Effect of substrate concentration**

The effect of different substrate concentrations on the productivity of thermoalkaliphilic lipases was observed. The production medium was supplemented with different concentrations of slaughter house wastes including: 0.125, 0.250, 0.5, 1, 2, 5, 10, 20, 50 and 75 %( w/v).

**Effect of different metallic ions**

Different metallic ions were applied for studying their effects on the production of thermoalkaliphilic lipases by the two isolates to be either activators or inhibitors. Metal ions were supplemented separately in the form of EDTA, $MgSO_4$, $CuSO_4$, $BeSO_4$, $NiSO_4$, $CaSO_4$, $FeSO_4$, $MnSO_4$, $ZnSO_4$, $Na_2MoO_4.2H_2O$, $MnCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, $CoCl_2$, $MgCl_2$, $CuCl_2$, $FeCl_3$, and $Na_2S_2O_3$ at concentration of 500ppm. The control was devoid of any metal ions and all previously mentioned optimum parameters were applied.

**Purification of thermoalkaliphilic lipases**

The crude enzyme preparation (1000ml) was fractionated with acetone organic solvent (20,40,60 and 80 %) **(Deutcher, 1993)**. The protein precipitate was re-dissolved in a minimal volume of double – distilled water and the resulting enzyme was dialyzed overnight against the buffer (phosphate buffer pH8 1.0M) at 6 ºC. The dialyzed sample was subjected to Sephadex G200. Fifty fractions were collected (each of 5 ml).Both enzyme activity and protein content were determined for each separate fraction **(Soliman, 2003)**. Fractions of the sharp peak obtained after applying sephadex G 200 column were collected and tested for their activity and protein content. The peaks exhibiting lipase activity were pooled together and used as a purified enzyme and applied on Sephadex G100 by similar technique. Crude of thermoalkalistable and this partially purified enzyme solution was used for investigating the effects of temperature, pH, various metal ions on enzyme activity and its application in leather industries well published in other paper.

**Sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE)**

For the characterization of proteins and evaluation of the protein enrichment process SDS-PAGE was performed in a discontinuous SDS-PAGE vertical slab gel electrophoresis apparatus as described by **Laemmli *et al.*, (1970)** and **Sambrook *et al.*, (1989).**

## 3. Results and Discussion

**Standard curves and isolation of microorganisms**

Lipases are the special kind of esterases belong to subclass 1 of hydrolytic enzyme class 3 and have been assigned sub-sub class 3.1.1 due to their specificity for carboxylic acid ester bonds. The biological function of lipase is to catalyze the hydrolysis of triglycerols to give free fatty acids, diglycerols , mono-acylglycerols and glycerol. Most commercial lipases are produced from microbes **(Jaeger *et al.*,1999).** Lipase occurs widely in nature : however microbial lipases are commercially significant because of low production cost, greater stability and wider availability than other sources. Few review articles were published (**Pahoja and Sethar,2002;Gupta *et al.*,2004; Aravindan *et al.*,2007; Patil *et al.*, 2011)** on lipase sources along with its industrial applications. Lipases producing bacteria have been found in diverse habitats such as soil contaminated with oil , dairies, industrial wastes, oilseeds and decaying food, compost heaps, coal tips and hot springs. **Vijayaraghavan *et al.*,(2011)** mentioned that, several lipases from

*B.thermocatenulatus* **(Schmidt-Dannert *et al*.,1996)**, *B. thermooleovorans* **(Lee *et al*.,1999),** *B.stearothermophilus* **(Kim *et al*.,1998),** *B. subtilis* **(Eggert *et al*.,2000),** *B.megaterium* **(Jaeger *et al*.,1999;Ruiz *et al*.,2001, 2002;Jaeger and Eggert, 2001,2002)** have been well described.

**3.1. Construction of Standard curve of lipase enzyme**

The productivity of lipase enzyme was estimated by colorimetric assay method depending on the estrification effect of lipase enzyme on *p*-nitrophenyle laurate and liberation of colored *p*-nitrophenol which is directly proportional with the density of lipase enzyme. The assay was achieved on spectrophotometer with absorbance at 410 nm. Descending dilutions of lipase enzyme (SIGMA) were prepared covering the range of (3125 – 800 000 µg protein / ml). The protein content was determined using the method of **Lowry *et al*., (1951)** where the samples were read at 750nm calorimetrically. However, data of this method covered the range of (0.1 - 1 mg/ml) using the protein of egg albumin.

**3.2.Localities of thermoalkaliphilic microbial isolates**

In view of the variety of applications, there has been a renewed interest in the development of source of lipases. Bacteria, yeast and moulds can produced lipase but the availability of lipase with specific characteristics is still a limiting factor **(Kashmiri *et al*.,2006; Nwuche and Ogbonna,2011; Patil *et al*.,2011)**.Thus, this lead to the search for new lipases amongst the bacteria isolated from different waster and soil temperature. Twenty eight thermoalkaliphilic bacterial isolates were picked up from different soil, water and slaughter house waste samples on the isolation medium (A) according to **(Lima *et al*., 2003)** and nutrient agar medium (**Shiriling and Gottlieb, 1966)** at a temperature of 60°C and pH value of 10. Based on the calorimetrically , it was found that only one out of twenty eight bacterial isolates were best lipase producers. All cultures were tested at 60ºC at pH 10 for 48 hrs.

**3.3. Screening of thermoalkaliphilic lipase(s) forming bacterial isolates and selection for the most potent producing ones:**

All the purified bacterial isolates were allowed to grow on medium **(**B**)** which was deprived from agar. The pH value was adjusted at 10, and the incubation was performed at 60°C for 48 hrs. Screening was achieved in three-way programs including monitoring of lipase production by all the isolated thermoalkaliphilic bacteria on the medium (B) which was supplemented with 1% of olive oil, 1% of slaughter house wastes and 1% of oily restaurant wastes separately. Only the group that was supplement with slaughter house wastes had the ability to support the production of lipase enzyme by 7 isolates out of twenty eight bacterial isolates. The isolate number (18) - obtained from sandy soil obtained from Khormah, Khormah governorate slaughter house waste and isolate number (1) - obtained from slaughter house wastes derived from El Khormah governorate shown to be the most potent lipase producing isolates.

**3.4.Identification of both the two most potent thermoalkaliphilic lipase(s) producing isolates**

The most potent thermoalkaliphilc bacterial isolates (SHW-1) was subjected to the common morphological, physiological and biochemical investigations such as: Cultural characteristics, Gram stain, spore stain, and utilization of many carbon sugars according to the identification keys described in Bergey's manual of systematic bacteriology **(Whitman, 2009)**. For further confirmed identification of the most potent isolate viz (SHW-1), 16S ribosomal RNA gene sequencing technique was performed. The sequence of the PCR product was compared with known 16S rRNA gene sequences in the Gene Bank database by multiple sequence alignment on: http://www.ncbi.nlm.nih.gov/genbank/ . The results showing the sequence of isolate (SHW-1) had matching with genome of ***B. stearothermophilus* ste.a.ro.ther. mo'phi.lus**. Gr. n. *stear* fat; Gr. n. *therme* heat; Gr. adj. *philos* loving; N.L. adj. *stearothermophilus* (presumably Intended to mean) heat- and fat-loving- with the sequence similarity of 99.2%. **Massadeh and Sabra (2011)** produced and characterized of lipase from *B. stearothermophilus.*

**3.5. Parameters Controlling lipase enzyme produced by the thermoalkaliphilic *B. stearothermophilus* KGSHW-18**

In order to improve growth and / or favor the enzyme secretion , the influence of some key variable such as medium composition, and culture conditions were assessed. On the other hand, some factors such as culture pH, culture temperature and the addition of lipid compounds seemed to have great influence on the behavior of the microorganism. The characterization of lipase is based on pH, temperature of the production medium and enzyme. Many bacterial lipases are well studied compared to plant and animals. The organisms are normally grown nutrient medium containing carbon source (oil, sugar and mixed carbon sources), nitrogen, phosphorous sources and mineral salts, whereas the production of lipases mostly depends on inducer such as triglycerides, bile salts and glycerol. **Patil *et al*.,(2011)** mentioned the characteristics of some bacterial lipases in pH, temperature and molecular weight from both gram positive and gram negative

bacteria. It is evident from the **Patil *et al.*, (2011)** results that , pH range is between 4.0 to 10.0; temperature range is between 27 to 80 ºC, whereas molecular weight various from 11 to 176kDa. Enzyme stability , which includes thermostability, oxidative stability and resistance to undesired aggregation and precipitation, is critical in industrial applications of biocatalysts and in biotechnology all of these factors contribute to the "robustness" of a protein.

**3.5.a. Optimum incubation temperature and pH**

Lipases are active over broad pH and temperature range and they have molecular weight ranging from 94 to 840 kDa. From available literature it can be interoperated that generally lipases have neutral pH optima but the pH and temperature optima of lipases depends on the habitat of its sources. Lipases posses stability over a wide range from pH 4 to 11 and temperature optima in the range from 10 to 96ºC **(Patil *et al.*,2011).** Incubation temperature also plays an important role in the metabolic processes of an organism. Increasing temperature increased the rate of all physiological processes but beyond certain limits it started decreasing . A range of 40-85 ºC was employed in the present study. Maximum lipase production was achieved at 60 ºC. Data was recorded in table (1) explained that the production of *Geobacillus stearothermophilus* KGSHW-1. lipase(s) enzymes reached their optimal value 1350.73 (U/ml) at an incubation temperature 60°C. **Cherif *et al.*,(2011)** mentioned that, the increase in temperature seemed to have a negative effect on biomass production and also lipolytic activity. Decrease in lipase production can be associated to either decrease in cell growth or inactive nature of enzyme itself. **Shafei and Rezkallah (1998)** have reported similar results. **Kim *et al.*, (2000)** reported a *B. stearothermophilus* that showed optimum growth at 55 ºC , but showed almost no activity at the same temperature. On the other hand **Sifour *et al.*,(2010)** reported a *B. stearothermophilus* that produced high yields of lipase at 55 and 60 ºC.

The effect of pH on the growth and enzyme production by *Geobacillus stearothermophilus* KGSHW-1 was assessed in a wide range of pH. Lipolytic productivities were found at maximum at initial pH 10, since the lipase yields reached up to 1477.9 (U/ml) .Below and above this particular value, the enzyme productivity decreased gradually. From this result , it was concluded that the microorganism needs neutral or alkaline pH values . For pH values outside this range, cell growth seems to be completely inhibited a fact which reveals the importance of studying this factor in cultures of G. stearothermophilus strain and the controlling the pH variations during its cultivation. **Kim *et al.*,(2000)** isolated of bacterial lipase from *B. stearothermophilus* characterized by pH,7.4 ; temperature 68 and its molecular weight 43kDa. The culture medium for Bacillus cells growth is usually neutral to slightly alkaline **(Gupta *et al.*,2004;2005).** As it was demonstrated in a previous paper, the enzymatic synthesis can be greatly associated with cell growth . Thus , the highest values of enzyme production, maximum at pH 10, were also reached in the same range of pH values where the microorganism showed optimal growth.

**3.5.b. Best buffer**

pH plays a pivotal role in the biosynthesis of enzyme. Data recorded in table (2) showed that the usage of any of the three: Carbonate-bicarbonate, NaOH- Glycine or Borax- Sodium borate buffers had no positive effect on the productivity of lipase(s) enzyme by *B. stearothermophilus* SHW-1 since the enzyme(s) yields were less than harvested by using 6N NaOH for initial pH adjustment and used as control. The enzyme secretion was found to be high at pH 10.

**3.5.c. Optimum incubation period and substrate concentration**

Incubation period also affects the lipase production. Lipases are stable and rugged enzyme that act on lipids as well on wide variety of natural and artificial reactant since it has ability to catalyze diversified reaction **(Patil *et al.*,2011).** Lipases are often crucial to their application in industries and laboratories. Different lipases appear too specific in splitting various fatty acids. In this study it could be concluded from the results recorded in table (3) that the maximum lipase(s) productivity (1734.2 U/ml) was obtained after only 24 hrs. Beyond these maximum values, decline in the enzyme(s) productivity could be observed. It might be due to the exhaustion of nutrients in the substrate , which resulted in the inactivation of enzymes. This finding is in accordance with **Korn and Fujio(1997). Tsujisaka *et al.*,(1977)** the specificity of lipase is controlled by the molecular properties of enzyme, structure of substrate and factors affecting binding of enzyme to the substrate. Lipases act on substrate in specific and non-specific manner, resulting in complete of hydrolysis of triglycerides into free fatty acids and glycerol or along with triglycerides, monoacylglycerides and diacylglycerides, fatty acids and glycerol's are also formed **(Aravindan *et al.*,2007).** It could be concluded best substrate concentration recorded in table (3) showed that the maximum lipase(s) productivity by *B. stearothermophilus* KGSHW-1 was obtained in the presence of (75%) of slaughter house wastes reaching (11205.84 U/ml). Similar results were obtained with *Bacillus* sp.**(Gupta *et al.*,2004;2005).** This study

revealed that enzyme secretion was associated with the growth of organism. **Iftikhar *et al.*,(2011)** isolated fifteen bacterial strains capable of producing lipase enzyme from oily products.

**Table (1):Relation of different incubation temperatures to lipases productivities by *B. stearothermophilus* KGSHW-1.**

| Incubation temperature ( C°) | Lipase production (U/ml) | Initial pH value | Lipase production (U/ml) |
|---|---|---|---|
| 40 | 915.1 ± 0.001 | 7 | 980.52 ± 0.015 |
| 45 | 950.2±002 | 7.5 | 1111.52 ± 0.015 |
| 50 | 977.07 ± 0.003 | 8 | 1112.4 ± 0.020 |
| 55 | 1200.2 ±002 | 8.5 | 1210.3±0.0015 |
| 60 | 1350.73 ± 0.003 | 9 | 1332.63 ± 0.001 |
| 65 | 1296.79 ± 0.004 | 9.5 | 1350.2±0.002 |
| 70 | 1225.37 ± 0.013 | 10 | 1477.89 ± 0.001 |
| 75 | 1123.68 ± 0.005 | 10.5 | 1350.2±0.002 |
| 80 | 1043.59 ± 0.012 | 11 | 1366.3 ± 0.002 |
| 85 | 1012.82 ± 0.012 | | |

**Table (2): Effect of using carbonate – bicarbonate, sodium hydroxide-glycine and borax-sodium borate buffer on lipases productivities by *B. stearothermophilus* SHW-1.**

| $Na_2$-bicarbonate Buffer | Lipase production (U/ml) | NaOH-Glycine buffer | Lipase production (U/ml) | Borax-sodium borate buffer | Lipase production (U/ml) |
|---|---|---|---|---|---|
| 9.2 | 1012.82 ± 0.020 | 8.6 | 908.57 ± 0.001 | 9.3 | 918.7 ± 0.002 |
| 9.5 | 1033.23 ± 0.015 | 9 | 1044.59 ± 0.03 | 9.5 | 1000 ± 0.013 |
| 9.8 | 1086.08 ± 0.001 | 9.2 | 1055.33 ± 0.001 | 9.7 | 1035 ± 0.011 |
| 10 | 1119.08 ± 0.0003 | 9.4 | 1142.64 ± 0.005 | 9.9 | 1056 ± 0.001 |
| 10.2 | 1188.13 ± 0.002 | 9.6 | 1166.66 ± 0.002 | 10.1 | 1081 ± 0.002 |
| 10.3 | 1212.09 ± 0.001 | 10 | 1255.17 ± 0.004 | 10.2 | 1116 ± 0.002 |
| 10.6 | 1256.17 ± 0.004 | 10.4 | 1334.63 ± 0.001 | Control | 1605 ± 0.003 |
| 10.7 | 1398.85 ± 0.002 | 10.6 | 1213.09 ± 0.002 | | |
| Control | 1605.03 ± 0.004 | Control | 1605.03 ± 0.001 | | |

**Table (3): Relation of different incubation periods to lipases productivities by *B. stearothermophilus* W-1**.

| Incubation period (hr) | Lipase production (U/ml) | Substrate concentration | Lipase production (U/ml) |
|---|---|---|---|
| 6 | 1008.8 ± 0.001 | 0.125 | 1700.88 ± 0.004 |
| 12 | 1250.2±0.015 | 0.25 | 1594.94 ± 0.003 |
| 18 | 1366.3 ± 0.012 | 0.5 | 10378.62 ± 0.002 |
| 24 | 1734.2 ± 0.002 | 1 | 4058.06 ± 0.004 |
| 30 | 1724.2±0.015 | 2 | 1744.89 ± 0.005 |
| 42 | 1709.4 ± 0.004 | 5 | 1380.33 ± 0.002 |
| 48 | 1494.7 ± 0.002 | 10 | 2055.42 ± 0.003 |
| 66 | 1428.1 ± 0.004 | 20 | 2329.99 ± 0.052 |
| 72 | 1355.7 ± 0.002 | 50 | 7075.75 ± 0.003 |
| 75 | 1222 ± 0.004 | 75 | 17205.84 ± 0.012 |

**3.5.d. Best nutritional conditions**

The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes **(Gupta *et al*.,2004).** These enzymes are generally produced in the presence of lipid or any other inducer and glycerol **(Ito *et al*.,1998; Sharma *et al*.,2009).** After studying the efficacy of certain twenty various carbon source on the lipase(s) productivity by the bacterial strain, it was obvious that beef extract had a great inductive effect on *B. stearothermophilus* KGSHW-1 productivity, where the lipase(s) yields reached (16275.52 U/ml). The effect of different organic and inorganic nitrogen sources on lipase(s) productivity by the thermoalkaliphilic bacterial strain was studied. Eleven different nitrogen sources were applied and the data shown in table (4) revealed that the maximum productivity of lipase(s) by the strain *B. stearothermophilus* KGSHW-1 was induced by potassium nitrate reaching (15024.67 U/ml). Among twenty two amino acids introduced into the utilized production medium to grow the of thermoalkaliphilic strain, amino acid tryptophan was the best for optimum productivity of lipase(s) by *B. stearothermophilus* KGSHW-1 reaching (16275.52 U/ml). All results were listed in table (4).

**3.5.e. Optimum inoculum size and incubation condition:**

The number of cells in inoculum had great influence on the production of lipase by G. stearothermophilus KGSHW-1.As the number of cells increased , it consumed majority of the substrate for growth purpose, hence enzyme synthesis decreased. It was obvious from the results recorded in table (5) showed that the optimum inoculum size applied for the best lipase(s) productivity from the *B. stearothermophilus* KGSHW-1 was 1%(v/v) - of 7 x $10^{10}$ CFU.$ml^{-1}$ with lipase(s) yields of (95484.04 U/ml). Shaking condition was more inductive for productivities of lipase(s) enzymes by *B. stearothermophilus* KGSHW-1 the enzyme yields reached (49611.33 U/ml). It was designed to determine the best revolution rate for optimizing productivity of lipase(s) enzyme by the thermoalkaliphilic strain. Data recorded in table (5) showed that the best revolution rate to attain the highest productivity of lipase(s) by the *B. stearothermophilus* KGSHW-1 was 60 rpm where the enzyme yield reached 70744.75 ( U/ml).

**3.5.f. Optimal vitamin requirement and metallic ions:**

The effect of applying of different vitamins separately on lipase(s) productivity by the thermoalkaliphilic bacterial strain was studied. Data recorded in table (6) showed that vitamin $B_6$ was the best inductive agent *B. stearothermophilus* KGSHW-1. The maximal lipase(s) productivity reached (28203.43 U/ml) at a concentration of 100 ppm. Results listed in table (6) showed that, the lipase(s) productivity by *B. stearothermophilus* W-1 had the highest yield by applying barium chloride where it reached the value 28486.77 (U/ml). The activity of lipases may be inhibited or stimulated by cofactors. Divalent cations such as calcium often stimulated enzyme activity due to the formation of calcium salts of long chain fatty acids (Macrae and Hammond,1985). Calcium stimulated lipases have been reported in the case of *Acinetobacter* sp. RAG1**(Snellaman and Colwell,2004).** In contrast , the lipase from *Pseudomonas aeruginosa* 10145 is inhibited by the presence of calcium ions. Further lipases activity is inhibited drastically by heavy metals like $Ca^{+2}$,$Ni^{+2}$,$Hg^{+2}$ and $Sn^{+2}$ and slightly inhibited by $Zn^{+2}$ and $Mg^{+2}$ **(Patkar and Bjorkling,1994).**

*Pseudomonas aeruginosa* KKA-5 lipase hydrolyze castor oil in the presence of various metal chlorides. $CaCl_2$ , $AlCl_2$, $CrCl_3$ and $MgCl_2$ displays enhanced hydrolysis capability. When $Cr^{+3}$ were used , hydrolysis of castor oil was four times faster than that of calcium and 1.6 times faster with regards to $Al^{+3}$. The presence of chloride salts of $Mg^{+2}$,$Cu^{+2}$,$Ca^{+2}$,$Hg^{+2}$ and $Fe^{+2}$ result in a profound increase in the hydrolytic activity of the purified lipase. Interestingly , $Hg^{+2}$ ions resulted in a maximal increase in lipase activity but $Co^{+2}$ ions showed as antagonistic effect. The EDTA at concentration of 150mM markedly inhibited the activity of lipase.

Lipase activity was enhanced in the presence of $K^{+}$ and $Ca^{+2}$ and $Mg^{+2}$ ions, but inhibited by $Hg^{+2}$ ions **(Sharma *et al*.,2009;2001).** The addition of $Mg^{+2}$ did not significantly stimulate lipase production. While many other metal ions including $Ca^{+2}$,$Mn^{+2}$,$Ba^{+2}$,$Zn^{+2}$, metal ions, including $Ca^{+2}$, $Mn^{+2}$, $Ba^{+2}$, $Zn^{+2}$, and $Cu^{+2}$ exerted inhibitory effects. However , lipase production was decreased slightly, to approximately 5% with the addition of K+ and 30% decrease was observed in lipase production in an absence of potassium ions. The absence of magnesium ions ($Mg^{+2}$) in the basal medium was also shown to stimulate lipase production. The lipase activity in presence of a metal ion whose activity was taken as 100% and the relative activities at 1mM of $Cu^{+2}$,$Hg^{+2}$,$Pb^{+2}$,$Co^{+2}$,$Cd^{+2}$ and $Li^{+}$ were 0.44,24.4,36.2,49.1,64.2,90.0 and 98.2 % respectively. **Annamalai *et al*.,(2011)** found that, metal ions, $Ni^{2+}$, $Mn^{2+}$, $Hg^{2+}$, $Fe^{2+}$,$Fe^{3+}$and $Co^{2+}$ were slightly inhibited enzyme activity and no effect was found with $Cu^{2+}$.

**Table (4): Effect of applying various carbon, nitrogen and amino acid sources on lipases productivities by *B. stearothermophilus* KGSHW-1.**

| Carbon sources | Lipase activity (U/ml) | Nitrogen sources | Lipase activity (U/ml) | Amino acids | Lipase activity (U/ml) |
|---|---|---|---|---|---|
| Rhamnose | 7800.76 ± 0.011 | Sodium nitrite | 14715.57 ± 0.004 | Control | 3859.32 ± 0.004 |
| Xylose | 1366.3 ± 0.001 | Pot. nitrate | 15024.67 ± 0.003 | Ornithine | 1044.59 ± 0.071 |
| Galactose | 10915.54 ± 0.031 | Amm. nitrate | 10539.38 ± 0.004 | Histidine | 935.35 ± 0.003 |
| Melezitose | 8756.48 ± 0.004 | Peptone | 11079.38 ± 0.002 | Arginine | 1005.77 ± 0.002 |
| Mannitol | 1788.003 ± 0.032 | Amm. phosphate | 9488.56 ± 0.002 | Lysine | 1046.76 ± 0.062 |
| Glucose | 1738.2 ± 0.022 | Sodium nitrite | 14715.57 ± 0.004 | Phenylalanine | 1194.08 ± 0.032 |
| Cellobiose | 1449.63 ± 0.003 | Calcium nitrate | 14366.81 ± 0.005 | Alanine | 1825.08 ± 0.007 |
| Maltose | 899.56 ± 0.002 | Urea | 13130± 0.005 | Aspartic acid | 875.07 ± 0.003 |
| Ribose | 9488.56 ± 0.003 | Amm. chloride | 14080.49 ± 0.004 | Glycine | 1635.25 ± 0.008 |
| Sucrose | 1366.3 ± 0.003 | Amm.molybdate | 12555.56 ± 0.007 | Cysteine | 1025.97 ± 0.081 |
| Dextrin | 6267.39 ± 0.002 | Amm.sulphate | 13199.78 ± 0.004 | Threonine | 1169.07 ± 0.072 |
| Starch | 4017.72 ± 0.003 | | | Citruline | 1333 ± 0.002 |
| Inuline | 2628.8 ± 0.004 | | | Serine | 1093.51 ± 0.005 |
| Pectin | 2935.99 ± 0.003 | | | Valine | 1438.22 ± 0.046 |
| Sorbitol | 3669.95 ± 0.003 | | | Proline | 1805.32 ± 0.073 |
| Lactose | 3579.64 ± 0.0004 | | | Tryptophan | 16275.52 ± 0.064 |
| Beef extract | 16275.52 ± 0.002 | | | Glutamic acid | 888.43 ± 0.009 |
| Sugar cane molasses | 1399.83 ± 0.004 | | | Methionine | 1317.4 ± 0.002 |
| Cellulose | 4660.66 ± 0.005 | | | Asparagine | 1585 ± 0.001 |

**Table (5):Effect of applying various inocula sizes, incubation conditions and shaking speed on lipases productivities by *B. stearothermophilus* KGSHW-1.**

| Inoculum Size (%) | Lipase production (U/ml) | Incubation condition | Lipase production (U/ml) | Revolution speed (rpm) | Lipase production (U/ml) |
|---|---|---|---|---|---|
| 0.25 | 5477.46 ± 0.018 | Static | 28488.77 ± 0.001 | 60 | 70744.75 ± 0.0095 |
| 0.5 | 49619.33 ± 0.003 | Shaking (100 rpm) | 49611.33 ± 0.012 | 90 | 46959.41 ± 0.00 |
| 1 | 95484.04 ± 0.012 | | | 120 | 44235.88 ± 0.002 |
| 2 | 33095.93 ± 0.005 | | | | |
| 4 | 30098.77 ± 0.017 | | | | |
| 5 | 6928.05 ± 0.002 | | | | |
| 10 | 8218.56 ± 0.039 | | | | |

### 3.5.g. Optimum bottle capacity

Data recorded in table (8) showed that the optimum productivity of lipase(s) by the strain *B. stearothermophilus* KGSHW-1 was in the 250 ml Erlenmeyer flask containing with 2.5% of its capacity where the enzyme yield reached (63378.33 U/ml). Data recorded in table (8) showed a summary of the optimal environmental and nutritional conditions for optimum productivities of lipase(s) enzyme by both the two thermoalkaliphilic *B. stearothermophilus* KGSHW-1 grown on slaughter house wastes. According to **Davranov (1994)** extensive and persistent screening for new microorganisms and their lipolytic enzymes will open new, simple routes for synthesis processes and consequently new and faster ways to solve environmental problems.

### 3.6.Purification and molecular weight of thermoalkaliphilic lipase

A summary of the purification steps of lipase enzymes produced by both the two thermoalkaliphilic strain *B. stearothermophilus* KGSHW-1 were presented in table (9). The protein sample related to the strain *B. stearothermophilus* KGSHW-1 also was detected in one sharp band of molecular weight 60 kD, compared with the utilized protein marker.

**Table (6):Effect of applying different concentrations of various vitamins and metallic ions on lipases productivities by *B. stearothermophilus* KGSHW-1.**

| Vitamin conc. (ppm) | | Lipase production (U/ml) | Metallic Ion (500 ppm) | Lipase production (U/ml) |
|---|---|---|---|---|
| Nicotinic acid | 25 | 4019.72 ± 0.004 | Control | 16198.37 ± 0.091 |
| | 50 | 4188.54 ± 0.002 | $Na_2MoO_4.2H_2O$ | 13595.42 ± 0.061 |
| | 100 | 4267.94 ± 0.053 | $BeSO_4$ | 4109.2 ± 0.003 |
| | 250 | 4440.88 ± 0.004 | $CuSO_4$ | 17285.53 ± 0.004 |
| | 500 | 5210.57 ± 0.002 | $CaSO_4$ | 11646.04 ± 0.0095 |
| | 1000 | 5680.38 ± 0.002 | $NiSO_4$ | 27238.76 ± 0.0027 |
| Riboflavin | 25 | 6588.49 ± 0.0095 | $ZnSO_4$ | 5447.17 ± 0.003 |
| | 50 | 7133.35 ± 0.003 | $FeSO_4$ | 12679.64 ± 0.035 |
| | 100 | 10599.18 ± 0.012 | $MnSO_4$ | 12070.63 ± 0.084 |
| | 250 | 9535.09 ± 0.035 | $MgSO_4$ | 25145.73 ± 0.042 |
| | 500 | 11880.18 ± 0.092 | $FeCl_3$ | 8633.15 ± 0.00 |
| | 1000 | 12740.57 ± 0.002 | $MnCl_2$ | 13995.3 ± 0.017 |
| Vitamin B6 | 25 | 19301.07 ± 0.053 | $CaCl_2$ | 7468.1 ± 0.053 |
| | 50 | 19512.29 ± 0.080 | $MgCl_2$ | 12928.62 ± 0.0095 |
| | 100 | 28203.43 ± 0.052 | $ZnCl_2$ | 4558.18 ± 0.014 |
| | 250 | 26410.73 ± 0.091 | CoCl2 | 26169.33 ± 0.001 |
| | 500 | 26199.24 ± 0.002 | $BaCl_2$ | 28486.77 ± 0.003 |
| | 1000 | 26719.83 ± 0.006 | $CuCl_2$ | 14945.77 ± 0.002 |
| Myristic acid | 25 | 14366.81 ± 0.005 | EDTA | 16280.52 ± 0.002 |
| | 50 | 11080.38 ± 0.003 | $Na_2S_2O_3$ | 18730.26 ± 0.013 |
| | 100 | 9490.56 ± 0.003 | | |
| | 250 | 12366.78 ± 0.004 | | |
| | 500 | 11134.88 ± 0.004 | | |
| | 1000 | 13218.44 ± 0.007 | | |
| Ascorpic acid | 25 | 20793.92 ±0.015 | | |
| | 50 | 25268.7 ± 0.027 | | |
| | 100 | 14150.04 ± 0.038 | | |
| | 250 | 11999.52 ± 0.055 | | |
| | 500 | 17113.64 ± 0.069 | | |
| | 1000 | 17315.19 ± 0.003 | | |

**Table(7):Relation of bottle capacity to lipases productivities *B. stearothermophilus* KGSHW-1.**

| Bottle Capacity | Content vol. (%) | Lipase Production (U/ml) |
|---|---|---|
| 1000 ml | 2.50% | 15877.83 ± 0.001 |
| | 5% | 20689.27 ± 0.009 |
| 500 ml | 2.50% | 13195.78 ± 0.041 |
| | 5% | 24158.33 ± 0.002 |
| 250 ml | 2.50% | 63378.33 ± 0.064 |
| | 5% | 15795.69 ± 0.003 |
| 100 ml | 2.50% | 6656.66 ± 0.012 |
| | 5% | 9729.62 ± 0.093 |

**Table (8): A summary of the optimal environmental and nutritional parameters controlling the productivities of lipases produced by *B. stearothermophilus* KGSHW-1.**

| Parameter | *B. stearothermophilus* KGSHW-1 | Parameter | *B. stearothermophilus* KGSHW-1 |
|---|---|---|---|
| Temperature (°C) | 60 | Substrate concentration (%) w/v | 75 |
| pH | 10 | Inoculum size (%) v/v | 1 |
| Incubation period (hrs) | 24 | Vitamin (ppm) | Vitamin B6 |
| Carbon source | Beef Extract | Metallic ion (500 ppm) | $BaCl_2$ |
| Nitrogen source | Potassium nitrite | Shaking/ Static conditions | Shaking (60rpm) |
| Amino acid | Tryptophan | Bottle capacity | 2.5% of 250 ml capacity |

**Table (9):A summary of purification steps of lipase produced by *B. stearothermophilus* KGSHW-1.**

| Purification Step | Volume (ml) | Protein content (mg/ml) | Total Protein (mg) | Lipase Activity (U/ml) | Total Activity (U) | Specific Activity (U/mg protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| CFF | 500 | 0.436 | 218 | 6753.2 | 3376580 | 15489 | 1 | 100 |
| Precipitation by Acetone | 100 | 0.704 | 70.40 | 22650 | 226502 | 32311 | 2.086 | 77 |
| Dialysis against sucrose | 7 | 1.146 | 8.099 | 42703.41 | 298923.8 | 36908.72 | 1.44 | 8.4 |
| Sephadex G-200 | 5 | 0.0431 | 0.2155 | 9972.8 | 49863.75 | 231942 | 14.92 | 2.4 |
| Sephadex G-100 | 5 | 0.0060 | 0.0253 | 3209 | 16045 | 617108 | 39.88 | 0.5 |

## 4. Conclusion

Most commercial lipases produced from microbes. In this study, thermophilic and alkaliphilic bacteria with lipase enzyme producer were identified from Khormah governorate slaughter house wastes, soil and water samples collected from different localities around this slaughter house. The slaughter house wastes diversity is large and that most microbiologists seek to isolate thermophiles and alkaliphiles from "hot" environments seems to be worth since these environment have proved to be the home for bacteria and useful enzymes. Morphological, biochemical , physiological and molecular biology techniques can be used to identify both the bacteria and the enzymes being expressed. The results obtained in this study permit to conclude that pH is a highly significant factor in growth of newly isolated *B. stearothermophilus* strain, with an optimal growth and lipolytic enzyme production at pH and a wide range of temperature from (40to 85ºC). The study of the characteristics of this lipase showed its potential for the use in many application , as it is thermostable , pH tolerant and can withstand a number of meta ions.

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