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Bacterial Hyperthermostable Alkaline Lipase Production by *B. stearothermophilus* Isolated from Oil Polluted Soil.

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Abstract

Lipases are ubiquitous enzymes which catalyze the hydrolysis of fats into fatty acids and glycerol at the water lipid interface and reversing the reaction in non-aqueous media. Lipases occupy a place of prominence among biocatalysts owing to their novel and multifold applications in oleochemistry, organic synthesis, detergent formulation and nutrition. This paper aims to isolate, purify of thermoalkaliphilic bacteria capable of producing thermoalkalistable lipase from oil polluted soil samples collected from Khormah Governorate district and also recycling of some agricultural industrial and environmental wastes. Forty-five bacterial isolates were exhibited highest productivity for thermoalkaliphilic lipase between 65-70°C and pH 11.5. The most potent bacterial isolate was identified as *B. stearothermophilus*-KKSA12. Maximum lipase activity of the culture medium was obtained after 24 h. incubation period; at 70°C; 11.5 pH; 1.12% (SHW)%(w/w); 2ml inoculum size; at shaking incubation condition; with lactose and sucrose sugars as best carbon sources; ammonium nitrate as best nitrogen source; with 1% yeast extract as vitamin source; with Tween 80as best surfactant; with 250 ml flask capacity; ammonium sulphate as best metallic ion at 100 ppm; and finally with folic acid as best vitamin at 500 ppm. Highest lipase activity was determined at pH 8.0 with *p*-nitrophenyl palmitate (PNPP) as substrate.Although lipase showed variable specificity / hydrolytic activity toward a number of P-nitrophenyl esters, it was preferentially active towards long -carbon chain acyl esters (C_{12}-C_{16}).The high temperature stability and ability to hydrolyze long length esters support the potential of this lipase enzyme as a vigorous biocatalyst for industrial applications especially in bio-detergent technology.

Keywords: Microbial enzymes, Bacterial lipase, Thermoalkaliphilic enzymes, Bio-detergent.

Introduction

Lipase (triacyl glycerol ester hydrolase, EC 3.1.1.3) is a water –soluble enzyme that catalysis the hydrolysis of fats to produce mono glycosides, diglycerides free fatty acids and glycerol **(Gupta, 2018).** Lipases constitute the most important group of biocatalysts for biotechnological application. Enzymes or microbial cells are used as biological catalysts due to their high specificity and economic advantages without any environmental impact. The enzymes from microorganisms used in various industries such as dairy, food, detergents, textile, pharmaceutical, cosmetic and biodiesel industries, and in synthesis of fine chemicals, agrochemicals and new polymeric materials. Research on microbial lipases, has increased due to their great commercial potential **(Ferreira-Dias *et al*., 2013; Sharma *et al*., 2014; Abrunhosa *et al*., 2013; Andualema and Gessesse, 2012;Femi-Ola *et al*., 2018).**

Enzymes work better at gentle and available temperatures and other environmental conditions, as the result it could be helpful to save energy and prevent pollution **(Gupta, 2018)**.

Microbial lipases are of major interest because of the diversity of catalytic activities, high yields, ease of genetic manipulation, rapid growth, and inexpensive culture **(Hansan *et al*., 2006).** Lipases belong to the triacylglycerol acylhydrolase group (EC 3.1.1.3) and act on long-chain fatty acid glyceroyl esters, which have very low solubility in water. The latter group often shows interfacial water-lipid activation **(Chahinian *et al*., 2005; Kurtovic *et al*., 2009; Gurung *et al*., 2013; Tyagi *et al*., 2014).**Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis**(Joseph *et al*., 2008).**

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physic-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration **(Gupta *et al*., 2004)**. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods **(Saxena *et al*., 1999).**

Each lipase has a number of unique characteristics such as substrate specificity, regio-specificity, and chiral selectivity and some enzymes are important for the industrial production of free fatty acids, synthesis of useful esters and peptides **(Hansan *et al*., 2006).** A major requirement of a commercial lipase is its thermalstability and performance at high temperature that would increase the reaction conversion rates, substrate solubility, and also prevent contamination by microorganisms. Recently, there has been a great demand for thermostable enzymes in industry, and a number of thermostable lipases from various sources have been purified and extensively characterized **(Li and Zhang, 2005; Kanwar *et al*., 2016).** As lipase-producing microbes are widely distributed in nature, there is an immense need to explore natural habitats to isolate thermally stable lipase producing microbes.

Microbial lipases have gained huge attention from industry because of their flexibility in temperature, pH and organic solvent **(Verma *et al*., 2012).** Lipases have been widely used for biotechnological and industrial applications such as in the cosmetic industry, biodiesel, degreasing of leather and in the pharmaceutical and food industry. Lipase also used in the oil processing, environmental management and the production of surfactants, detergents, and pesticides.
Lipases operating chemical reaction to elevated temperature has the following advantages: - a higher diffusion rate, increased solubility of lipids and other hydrophobic substrates in water, decreased substrate viscosity, increased reactant solubility, higher temperature faster reaction rates, reduced risk of microbial contaminations **(Gupta, 2018).** The temperature stability of lipases has been regarded as the most important characteristic for use in industry. Cold-adapted lipases are largely used in the detergent industry, where cold washing reduces both energy consumption and the wear and tear of textile fibers. These lipases are also preferred in the food industry, as these enzymes can be inactivated at reasonably low temperatures, thereby conserving the nutritional quality of the food. Lipase specificity to catalyze triglycerides in particular has attracted application in nutraceutical industry. Specific structured triacylglycerols (SSTs) is a product mainly produced via lipase reactions in which the chemical catalysts are unable to do. They are also used in environmental applications such as wastewater treatment and the bioremediation of fat-contaminated cold environments **(Lu *et al*., 2010; Ray, 2012; Weerasooriya and Kumarasinghe, 2012; Parapouli *et al*., 2018).**

The main research published many papers for microbial lipase production and its application **(Bayoumi *et al*., 2012; Abol Fotouh *et al*., 2016).** The objective of this study was to isolate, identify and

characterize a novel bacterium that produces a thermostable lipolytic enzyme.

Materials and Methods

I. Culture media:

i) Isolation media:

i.a) Isolation medium was prepared. It contains the following: Yeast extract 5 and olive oil 10. The mineral salt solution contained (Per liter of distilled water): KNO_3 2.0 g; $MgSO_4.7H_2O$ 0.5 g; K_2HPO_4 1.0 g; $ZnSO_4.7H_2O$ 0.44 g; $FeSO_4.7H_2O$ 1.1 g and $MnSO_4.7H_2O$ 0.2 g. The medium adjusted at pH 10.5 before sterilization. This is a selective medium for isolation and also used for production and symbolized medium (C) **(Bayoumi *et al*., 2012; Abol Fotouh *et al*., 2016).**

i.b) Czapek-Dox's agar medium: Czapek-Dox's agar medium contained the following (g/l): $NaNO_3$ 3; K_2HPO_4 1; KCl 0.5; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.01; Sucrose 30 and Agar 20. All the ingredients dissolved in 100 ml tap water and the pH adjusted at 9.5 before sterilization. This medium supplemented with 10 ml tributyrin grinding with 10 ml Arabic Gum instead of sucrose to obtain a selective medium for isolating lipolytic microbial isolates.

i.c) Nutrient broth medium: Nutrient broth medium was prepared for isolating lipolytic bacterial isolates as well as sub-culture and preservation of the organism. It was contained (g/l): Peptone 5; Beef extract 3 and NaCl 5. pH was adjusted at 9.5 before sterilization and used as a broth medium for sub-culture and preservation of the bacterial isolate and also used for lipase production medium and symbolized medium (N).

II. Production medium:

a) Production medium was prepared. It contains the following (g/100 ml): Sun flower oil 1.5; Peptone 0.5; Glucose 1; KH_2PO_4 0.25; KCl 0.05 and $MgSO_4.7H_2O$ 0.05. The pH adjusted to 9.5. This medium used for production of lipase by the bacterial isolate and symbolized Medium (A).

b) Luria-Bertani (LB) medium: LB medium was prepared: LB medium used having the following composition (g/l): Tryptone 10; yeast extract 5 and NaCl 10. This medium used for production medium and symbolized medium (B).

c) Production medium was prepared: It contains the following (g/l): Tryptone 10; yeast extract 5; NaCl 10 and NH_4Cl 1. During cultivation, the basal medium supplemented with varying concentrations of olive oil and n-hexadecane, as indicated. This medium used for lipase production and symbolized medium (D).

d) PY medium: PY medium was prepared: a PY medium was used having the following composition: (g/100 ml): K_2HPO_4, 0.2; KH_2PO_4, 2; poly-peptone 4; beef extract 0.5; yeast extract, 0.5; glucose 2; $MgSO_4.7H_2O$ 0.01; $ZnSO_4.7H_2O$ 0.0001; $MnSO_4.7H_2O$ 0.0001 and $FeSO_4.7H_2O$ 0.001. The pH adjusted to 9.5 with 2N NaOH before sterilization. This medium used for lipase production and symbolized medium (E).

III. Isolation of thermoalkaliphilic lipolytic producing bacterial isolates:

The thermoalkaliphilic bacterial isolates were isolated from the seven crude oil polluted soil samples as follows: Ten grams of sandy and/or clay soil polluted by crude oil mixed with 10 gm of different wastes and incubated for 10 days at 70^{o}C after adjust pH 10-11. Then take 1 ml from each sample and dissolved in 50 ml nutrient broth medium, then incubated for 2 days at the same pH and temperature **(Bayoumi *et al*., 2012; Abol Fotouh *et al*., 2016).** Then take 50 µl from each sample and cultured, Dox Czapeks Dox's agar medium, and Nutrient agar medium. All the previously mentioned media supplemented with tributyrin emulsified with Arabic-Gum as the carbon source and incubated for 1, 2, 3, 4 and 5 days at pH 10.5 and temperature 65-70^{o}C.

The purification of thermoalkaliphilic lipolytic producing bacterial isolates carried out according to the agar streak technique. It has used to obtain pure cultures of the microorganisms on the surface of plates.

IV. Identification of the most potent bacterial isolates: The bacterial isolates subjected to screening in relation to thermoalkaliphilic lipolytic producing bacterial isolates and screening again in relation to the production of thermoalkalostable lipase with different wastes as only source of carbon. The most potent lipolytic bacterial isolates subjected to characterization using the standard identification keys.

V. Methods of lipase assay:

(1) Titrimetric assay: Lipase activity as assayed by alkali titration using olive oil as substrate.

The assay mixture consisting of: Olive oil 3.5 ml, phosphate buffer (0.1M; pH 8) 3.5 ml and crude enzyme preparation 2 ml. The assay mixture was incubated for 30 min. at 60^{o}C with stirring at 250 rpm. The reaction mixture was terminated by the adding 10 ml acetone: ethanol (1:1). The amount of liberated fatty acids during incubation period was tittered with 0.04N KOH in the presence of 25 µl of phenolphthalein (0.2% w/v in ethanol) as indicator. One unit of enzyme was defined as: 1µ moles of free fatty acids released per ml: per min. under the previously mentioned assay conditions.

(2) Colorimetric method for lipase assay: The thermoalkalostable extracellular lipase released into the medium was assayed quantitatively by using *p*-nitrophenyl palmitate (PNPP) as the substrate, with some modifications:

The assay mixture consisting of:

1- Ten ml of isopropanol containing 30 mg of p-nitrophenyl palmitate.
2- Ninety ml of 0.05M phosphate buffer (pH 8) containing 207 mg of sodium deoxycholate and 100 mg Arabic-Gum.
3- The previously mentioned mixture was mixed and used as freshly prepared substrate solution.
4- Take 2.4 ml from freshly prepared substrate solution was pre-warmed at 60^{o}C and mixed with 0.1 ml enzyme solution.
5- The enzyme mixture incubated at 60^{o}C for 30 min.
6- The O.D. 410 measured against an enzyme free control.

One enzyme unit defined as: mol of p-nitrophenol enzymatically released from the substrate ml/min. Each of the assayed performed in triplicate unless otherwise stated and mean values presented, as well as standard deviation (S.D) were calculated and then taken as an indication of lipase activities. Lipase activities estimated with the help of the standard curve pure lipase enzyme.

VII. Parameters controlling the alkalothermostable lipase enzyme productivity by the most potent selected bacterial isolate:

1- Different pH values.
2- Different incubation periods.
3- Different incubation temperatures.
4- Different substrate concentrations.
5- Different inocula sizes.
6- Incubation conditions.
7- Different harvesting the yield.

Results

I. Isolation, characterization, screening and selection of the most potent thermoalkaliphilic lipase producers isolates:

1.1. Isolation of the thermoalkaliphilic microorganisms from different collected wastes: Forty-five thermoalkaliphilic bacterial isolates were the subject of the present investigation. All bacterial isolates were isolated from crude oil polluted soil samples.

1.2. Screening of thermoalkaliphilic lipase producers bacterial isolates in relation to lipid clearing zoon and temperature: Only three bacterial isolates exhibited highest activity for thermoalkaliphilic lipase production after their incubation period at temperature ranging from 65-70°C. One bacterial isolate was exhibited highest production of thermoalkaliphilic lipase producers but less than the above three bacterial isolates at the same temperature and also exhibited no growth at 30°C, one bacterial isolate was exhibited weak clear zoon at temperature ranging from 65-70°C and also exhibited no growth at 30°C and four bacterial isolates were exhibited growth only at 30 and 65°C. The different thermophilic bacterial isolates capable of grown at high temperatures (65-70°C) on lipid contacting media.

1.3. Screening of lipolytic thermoalkaliphilic bacterial isolates with best different wastes: The most potent lipolytic thermoalkaliphilic five selected from all forty-five bacterial isolates which exhibited highest clear zones were allowed to grow on all the previously mentioned wastes as mentioned in the production medium to obtain the most potent bacterial isolates, which have the highest ability to produce thermoalkalostable lipase enzyme with the best substrates used.

1.5. Thermoalkalostable lipase production in relation to different media by the most potent bacterial isolate: The effect of different media used only with the bacterial isolateKKSA12without any modifications. Results revealed that, the lipase productivity were increased when using the different media with addition of slaughterhouse wastes on the different media used by thisbacterial isolate (data not shown).

II-Characterization of most potent bacterial isolates by morphological, physiological and biochemical characteristics of bacterial sample:

The bacterial isolate (KKSA12) suggested to be *Bacillus stearothermophilus* according to key of "Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The ability of the most potent bacterial isolate to produce enzymes such as, amylase, pectinase, gelatinase and cellulase were studied. While when allowed the most potent bacterial isolate 12 to grow on slaughterhouse wastes and tap H_2O only at pH 11.5 and incubation temperatures 60^oC exhibited the same result when allowed to grow on the medium A with slaughterhouse wastes. It is means that the bacterial isolate has the ability to use the slaughterhouse wastes as the sole carbon and nitrogen sources to produce lipase under the same conditions used in this experiment. On the other hand, with add yeast extract in concentration 1% this increase lipase productivity with fish wastes only and tap water at pH 11.5 and temperature 60^oC.

III. Effect of different environmental and nutritional conditions on controlling thermoalkalostable lipase production by *B. stearothermophilus*-12:

1. Different pH values: The most potent thermoalkaliphilic lipolytic bacterial isolates *Bacillus stearothermophilus*-KKSA12 were incubated for 48 hours at different pH values using 1N NaOH and HCl. Viz. pH 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 13, 14. Results were recorded in table (1). It appeared from the results that pH 11.5 was the optimum pH values for the highest thermoalkalostable lipase productivity by *B. stearothermophilus*-KKSA12. An increase or decrease beyond or below this pH value led to a decrease in the lipase productivity.

Table (1): Different pH values in relation to bacterial thermoalkalophilic lipase productivity using *B. stearothermophilus*-KKSA12.

Different pH values	Thermoalkalophilic lipase productivity (Unit/ml).
7.5	2.46 ± 0.11
8	3.84 ± 0.12
8.5	4.32 ± 0.12
9	5.52 ± 0.12
9.5	14.77 ± 0.32
10	15.35 ± 0.12
10.5	16.08 ± 0.02
11	19.05 ± 0.03
11.5	**29.58 ± 0.05**
12	24.99 ± 0.02
13	18.48 ± 0.03
14	17.74 ± 0.04

2. Incubation period: The thermoalkalostable lipase productivity was detected at time intervals of 6, 12, 18, 24, 30,36,42,48 and 72 hours incubation on the previously mentioned production medium and incubated at 60°C. Data recorded in table (2) the relation between lipase productivity and times of incubation. Data showed that, the level of thermoalkalostable lipase productivity by *B. stearothermophilus*- KKSA12 increased gradually with increasing the incubation period up to a maximum of 24 hours. Beyond or below this maximum value a decline in the enzyme productivity could be observed.

3. Incubation temperatures: Different incubation temperatures viz. 50, 55, 60, 65, 70, 75, 80 and 85°C in relation to thermoalkalostable lipase productivity by thermoalkalostable lipolytic *B.stearothermophilus*-

KKSA12, were incubated for 24 hours. Results were recorded in table (3) it appeared from the results that 70°C was the optimum temperature for the highest lipase productivity. An increase or decrease beyond or below this temperature led to a decrease in lipase productivity.

4. Substrate concentrations: The effect of different substrate (Slaughterhouse wastes) concentrations used as a sole carbon source in fermentation medium on thermoalkalostable lipase productivity by the most potent bacterial isolate was studied. The results were recorded as shown in table (4), showed that, high level of thermoalkalostable lipase productivity was detected at substrate (Slaughterhouse wastes) concentration of 1,125 % *Bacillus stearothermophilus*-KKSA12. An increase or decrease beyond this concentration led to a decrease in lipase productivity.

5. Inoculum size: The different inoculum sizes from bacterial isolates were adjusted by inoculated broth medium (Nutrient broth) with *Bacillus stearothermophilus*-KKSA12 for 24 hours and then read at absorbance 650 nm against blank (medium without inoculation). Each one ml heavy bacterial cell medium contained about, 0.40±0.00. The used inocula sizes were: 50, 100, 500 µl, 0.05, 0.1, 0.5,1, 2, 3, 4 and 5 ml. The optimum bacterial inoculum size was 2 ml of the applied heavy cell medium. At this particular inoculum size, the highest yield of thermoalkalostable lipase productivity was recorded 40.49 Unit/ml. Results were recorded in table (5).

Table (2): Incubation period in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Incubation period (Hours)	Thermoalkalophilic lipase productivity (Unit/ml).
6	11.65 ± 0.004
12	12.44±0.002
18	13.14 ± 0.012
24	**34.66 ± 0.005**
30	35.55±0.012
36	30.12±0.012
42	29.39 ± 0.02
48	25.12±0.001
72	21.99 ± 0.04

Table (3): Incubation temperature in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Incubation temperature (°C)	Thermoalkalophilic lipase productivity (Unit/ml).
50	24.95 ± 0.01
55	26.42 ± 0.01
60	33.09 ± 0.23
65	35.57 ± 0.01
70	**37.05 ± 0.01**
75	30.39 ± 0.02
80	24.23 ± 0.02
85	22.05 ± 0.01

Table (4): Different substrate (Slaughterhouse wastes) in relation to bacterial thermoalkalophilic lipase productivity produced by *Bacillus stearothermophilus*-KKSA12.

Slaughterhouse wastes concentrations (%) (w/w)	Thermoalkalophilic lipase productivity (Unit/ml).
0.125	15.24±0.01
0.25	24.23 ± 0.01
0.5	25.68 ± 0.007
1	36.78 ± 0.006
1.125	**38.77±0.01**
1.5	34.56 ± 0.012
2	29.39 ± 0.12
2.5	28.35±0.01
5	24.21 ± 0.00
10	22.41 ± 0.12
15	20.22±0.01
20	19.49 ± 0.032

Table (5): Different inoculum size in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Inoculum size (ml)	Thermoalkalophilic lipase productivity (Unit/ml).
0.05	11.15 ± 0.05
0.1	14.12 ± 0.05
0.5	24.42 ± 0.05
1	28.28 ± 0.04
2	**40.49 ± 0.08**
3	33.08 ± 0.19
4	31.01±0.01
5	30.05 ± -0.01

6. Incubation conditions:

Effect of incubation conditions on thermoalkalostable lipase productivity. Data recorded in table (6) showed that, shaking states exhibited slightly increased of lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Table (6): Incubation conditions in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Incubation conditions		Thermoalkalophilic lipase productivity (Unit/ml).
Shaking	25	40.22 ± 0.07
	50	42.12±0.01
	75	44.11±0.05
	100	56.12±0.01
Static		35.52 ± 0.05

7. Different carbon sources in relation to thermoalkalostable lipase production:

The effect of nine different carbon sources on lipase productivity by *B. stearothermophilus*-KKSA12 was studied. As shown in table (7). Data indicated that, lactose and sucrose were exhibited the highest lipase productivity by *Bacillus stearothermophilus*-KKSA12. All the other carbon sources tested in this study found to exhibit an obvious inhibitory effect on all determined enzyme productivity comparable to control.

8. Different nitrogen sources: The effect of eleven different nitrogen sources on the lipase productivity by *B. stearothermophilus*-KKSA12 was studied. As shown in table (8), it was clear that all the different nitrogen sources exhibited various inductive degrees lower than control (100%).

9. Yeast extract concentrations: The purpose of this experiment was to investigate the best concentration of yeast extract that induced the highest thermoalkalostable lipase productivity by *B. stearothermophilus*-KKSA12. Thus, different yeast extract concentrations were applied viz. 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5% (% w/v). Results recorded in table (9). indicated that, 1% yeast extract concentration was exhibited the highest thermoalkalostable lipase productivity by *B. stearothermophilus*-KKSA12.

Table (7): Different carbon sources in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

No.	Carbon source	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
Control		35.32 ± 0.03	100
Monosaccharaides			
1	Glucose	37.53 ± 0.01	106.257
2	D (+) Xylose	36.88 ± 0.01	104.416
3	D (+) Mannose	27.90 ± 0.04	78.992
4	D (-) Galactose	39.00 ± 0.03	110.41
5	D (-) Fructose	36.04 ± 0.05	102.03
Disaccharides			
6	**Lactose**	**44.94 ± 0.02**	**127.236**
7	**Sucrose**	**44.94 ± 0.02**	**127.236**
8	Maltose	43.71 ±0.06	123.75
Polysaccharides			
9	Cellulose	27.18 ± 0.002	76.953
10	Starch	25.48 ± 0.001	72.140
Polyhydic alcohol			
11	Mannitol	27.92±0.06	79.048
12	Sorbitol	18.55±0.03	52.519

Table (8): Different nitrogen sources in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

No.	Nitrogen sources	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
	Control	44.94 ± 0.03	100
1	Peptone	41.98 ± 0.01	93.413
2	**Amm. nitrate**	**44.93 ± 0.16**	**99.977**
3	Amm. chloride	44.18 ± 0.08	98.308
4	Amm. sulphate	35.13 ± 0.08	78.170
5	Pot. nitrate	38.28 ± 0.13	85.180
6	Amm. monohyd. phosphate	33.09 ± 0.07	73.631
7	Sodium nitrate	41.99 ± 0.04	93.435
8	Tryptone	24.12 ± 0.03	53.671
9	Malt extract	23.99 ± 0.06	53.382
10	Urea	36.79 ± 0.12	81.864
11	Amm. molybdate	33.09 ± 0.12	73.631

10. Effect of different surfactant on thermoalkalostable lipase production: The aim of this experiment was to study the ability of thermoalkaliphilic lipolytic bacterial isolate viz. *B. stearothermophilus*-KKSA12 to produce the enzyme in the presence of surfactants because of the fact that a good enzyme detergent should be stable in the presence of surfactants. Data recorded in table (10) showed that, the enzyme productivity was exhibited very slightly increased in the presence of tween 20. But in the presence of triton X-100 and/or tween 80 exhibited inhibitory effect on enzyme production.

12. Effect of different flask capacities: The effect of different flask capacities on thermoalkalostable lipase productivity by *Bacillus stearothermophilus*-KKSA12, was studied. Data in table (11) indicated that the differences in volumes (capacities) of the fermentation flask have an obvious effect on lipase productivity. The best fermentation volume for maximum lipase production was 250 ml capacity for *B. stearothermophilus*-KKSA12.

Table (9): Different yeast extract concentrations in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Yeast extract concentration (%).	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
Control	41.23 ± 0.01	100
0.5	44.94±0.01	108.99
1	**53.33±0.05**	**129.34**
1.5	40.55±0.02	98.350
2	36.05±0.01	87.436
2.5	27.99±0.003	67.887
3	24.96±0.011	60.538
3.5	20.99±0.014	50.909
4	23.01±0.012	55.808
4.5	21.25±0.005	51.540
5	21.26±0.02	51.564

Table (10): Surfactants in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Surfactants	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
Control	42.9 ± 0.007	100
Triton X-100	36.04 ± 0.014	84.00
Tween 20	**45.66 ± 0.05**	**106.43**
Tween 80	42.99 ± 0.01	100.20

13. Metallic ions:

Effect of different metallic ions on lipase production was studied in concentration of (100 ppm). Data recorded in table (12) exhibited that $(MH_4)_2SO_4$ recorded the highest lipase productivity by *B. stearothermophilus*-KKSA12.

Table (11): Different flask capacities in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

Flask capacity (ml)	Thermoalkalophilic lipase productivity (Unit/ml).
100 (1.25 gm)	70.40 ± 0.04
100 (2.5 gm)	63.48 ± 0.02
250 (2.5 gm)	60.99 ± 0.01
250 (5 gm)	**65.04 ± 0.05**
500 (5 gm)	64.02 ± 0.01
500 (10 gm)	60.28 ± 0.01
1000 (5 gm)	55.02±0.00
1000 (10gm)	44.01±0.01

14. Vitamins:

The effect of vitamins on thermoalkalostable lipase productivity by *B. stearothermophilus*-KKSA12 was studied. For this purpose, five vitamins were introduced in the production medium at four different concentrations, viz. 100, 250, 500 and 1000 ppm. Results for lipase productivity by *Bacillus stearothermophilus*-KKSA12 were recorded in table (13). Data indicated that, folic acid at concentration of 500 ppm was found to induce the highest lipase production.

Table (12): Metallic ions in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

No.	Metallic ions (100 ppm) concentration	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
Control		50.85 ± 0.01	100
1	NaSO4	48.63 ± 0.01	95.634
2	CuSO4	50.02 ± 0.02	98.36
3	$MnSO_4$	54.05 ± 0.02	106.29
4	$ZnSO_4$	83.50 ± 0.00	164.20
5	$Fe_2(SO_4)_3$	73.04 ± 0.02	143.63
6	$CaSO_4$	20.29 ± 0.05	39.901
7	**$(NH_4)_2 SO_4$**	**83.67 ± 0.01**	**164.542**
8	MgSO4	75.77 ± 0.01	149.00
9	$Pb(NO_3)_2$	25.91 ± 0.01	50.953
10	NH_4Cl_2	44.19 ± 0.00	86.902
11	$ZnNO_3$	18.28 ± 0.01	35.948
12	$AgNO_3$	31.60 ±0.02	62.14
13	$NaNO_3$	44.78 ± 0.03	88.06
14	$CsCl_2$	39.74 ± 0.12	78.151
15	$CoCl_2$	39.02 ±0.01	76.735
16	$MnCl_2$	37.77 ± 0.003	74.277
17	KCl	39.01 ± 0.01	76.715
18	NaCl	47.16 ±0.02	92.743
19	$CdCl_2$	53.33 ±0.003	104.877
20	$CuCl_2$	27.18 ±0.02	53.451
21	$CaCl_2$	22.48 ±0.01	44.208
22	$ZnCl_2$	14.59 ±0.003	28.692
23	EDTA	29.39±0.02	57.79

Table (13): Different vitamins in relation to bacterial thermoalkalophilic lipase productivity by *Bacillus stearothermophilus*-KKSA12.

No.	Vitamins (ppm)	Thermoalkalophilic lipase productivity (Unit/ml).	Final activity (%)
	Control	74.74 ± 0.02	100
1	**Nicotinicacid**		
	100	70.84 ± 0.00	94.781
	250	74.54 ±0.002	99.732
	500	84.16 ± 0.01	112.60
	1000	84.44 ± 0.02	112.978
2	**Histidine**		
	100	75.29 ±0.003	100.73
	250	70.66 ± 0.01	94.54
	500	64.44 ± 0.02	86.218
	1000	60.48 ± 0.01	80.920
3	**Riboflavin**		
	100	75.01 ± 0.01	100.361
	250	77.48 ± 0.02	103.66
	500	75.66 ± 0.00	101.230
	1000	70.11 ±0.001	93.805
4	**Folicacid**		
	100	75.29 ±0.002	100.73
	250	75.28 ±0.003	100.722
	500	**87.15 ± 0.002**	**116.60**
	1000	78.24 ± 0.01	104.682
5	**Ascorbicacid**		
	100	82.68 ± 0.20	110.623
	250	77.50 ± 0.03	103.69
	500	75.29 ± 0.01	100.73
	1000	75.00 ± 0.02	100.34

Table (14): A summary of the optimal nutritional and environmental parameters controlling thermoalkalophilic lipase productivity by *B.stearothermophilus*, KKSA12.

No.	Parameter	Optimal conditions
1	pH value.	11.5
2	Incubation period (hours).	24
3	Incubation temperature (°C).	70°C
4	Substrate concentration (%).	1.125
5	Inoculum size (ml).	2 ml
6	Incubation conditions.	Shaking
7	Carbon source.	Sucrose and lactose
8	Nitrogen source	Control and Ammonium nitrate
9	Yeast extract concentration (%).	1ml
10	Surfactants.	Tween 20
11	Erlenmeyer flask capacity (ml).	250 ml Erlenmeyer flask capacity &5gm solid wastes.
12	Metallic ions.	$(NH_4)_2SO_4$ (100 ppm)
13	Vitamins.	Folic acid (500 ppm)

Discussion

Lipases are enzymes which are used in a variety of biotechnological applications being able to catalyze a broad spectrum of reactions. This fact has led to an increase interest in the isolation of novel microbial lipases with particular properties, in order to be exploited as biocatalysts in various industrial applications. In a world of diminishing resources and increasing needs, every opportunity for recycling waste materials must be sought **(Haddadin *et al.*, 2009; Shivika and Shamsher, 2014; Verma and Prakash, 2014; Femi-Ola *et al*., 2018).**

One of the main goals of enzymes research is industrial applications. Nowadays, we are surrounded by enzymes as well as chemicals produced by enzymes in our daily life. The majority of the industrial enzymes are of microbial origin **(Kameshwar *et al*., 2014; Abol Fotouh *et al*., 2016; Sreelatha *et al*., 2017; Sharma *et al*., 2016).**

Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification **(Femi-Ola *et al*., 2018; Kameshwar *et al*., 2014; Gupta, 2018; Parapouli *et al*., 2018).**

The main object of the present work was an investigation of screening and production of thermoalkalostable lipase enzyme from crude oil polluted soil samples under solid state fermentation (SSF) conditions. In this regard all bacterial isolates were isolated from different some crude oil pollutedsoil samples collected from different localities in Khormah governorate, Taif, KSA. These bacterial isolates were grown at 65^{o}C and at pH 10.5 to be able to produce a thermostable and alkaliphilic enzymes which favorable to be used as additive to biodetergent formulations.

A screening test of lipolytic productivities of all bacterial isolates resulted in the fact that, only three bacterial isolates gave good lipolytic productivities, whereas one bacterial isolate was found to be the best lipolytic enzymes producer. Screening for chosen as best thermoalkalostable lipase were observed as clear halos or opaque zones around the bacterial cultures in the alkaline medium under thermophilic conditions as recommended by **Nawani *et al*., (2006)**. In the present study, the maximum zones of hydrolysis and specific lipase activity noticed for crude thermoalkaliphilic lipase from ***Bacillus stearothermophilus*-KKSA12**.

In view of their commercial importance, cost effective production of the previously mentioned enzymes 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, because the contents of synthetic media such as nutrient broth, soluble starch as well as other components are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of the cost of the medium **(Haq *et al*., 2003)**.

From industrial point of view, in order to production of low cost of enzymes, these bacterial isolates under study were allowed to grow on natural substances such as palm fermented wastes, kitchen fermented wastes and slaughterhouse wastes under submerged fermentation (SmF) conditions. However, the selection of the previously mentioned substrates for the process of enzymes biosynthesis was based on the following factors viz (i) they represent the most cheapest agro-industrial wastes in Egypt; (ii) they are available at any time of the year, (iii) their storage represents no problem in comparison with other substrates and (iv) they resist any drastic effect due to the exposure to other environmental conditions e.g. temperature, variation in the weather from season to season and or from day to night.

Interestingly slaughterhouse wastes are an important pollutant factor for the environment, many pathogenic microorganisms can grow on it, and this may cause many diseases for man and animals, thus its use for enzymes production help, in prevention disease distribution **(Abdul-Raouf 1990)**.

Many workers dealt with the main different factors that effect on the enzymes production such as temperature, pH, aeration, addition of different carbon and nitrogen sources. Although, such factors were previously studied by many authors, e.g. **(Faoro *et al*., 2012; Royter *et al*., 2009; Shariff *et al*., 2011; Chalesand James; 2011; Cotarlet and Bahrim, 2011; Bhosale *et al*., 2016; Sreelatha *et al*., 2017; Abol Fotouh *et al*., 2016; Sharma *et al*., 2016)** still, we need for more investigation seems to be continuously required to give a change to isolate more enzymes producers is valid. Therefore, the purpose of the present work is to determine the best factors

controlling the lipase enzyme(s) productivities by the most potent bacterial isolate ***B. stearothermophilus*-KKSA12**.

On the other hand, the economic feasibility of the microbial enzymes production application generally depends on the cost of its production processes. In order to obtain high and commercially viable yields of thermoalkalinestable enzymes, it was essential to optimize the fermentation medium used for bacterial growth and enzymes production from both thermophilic ***Bacillus*** strains.

Optimal parameters of the thermoalkalostable enzymes biosynthesis from microbial origin varied greatly, with the variation of the producing strain, environmental and nutritional conditions **(Latip *et al*., 2016)**.

It is not clear from the literature whether the enzymes production pattern depends exclusively upon the producing strain, and different environmental conditions or both. Due to strain to strain variation, it is therefore necessary to determine the optimal conditions for the thermostable enzymes production by the most potent thermoalkaliphilic bacterial strains viz., **(***Bacillus stearothermophilus*-KKSA12**).**

Increase in the temperature of system result from increases in the kinetic energy of the system. Temperature control is very important for fermentation processes, since growth and production of the microbial enzymes are usually sensitive to high temperature **(Femi-Ola *et al*., 2018; Olusesan *et al*., 2011; Lima *et al*., 2004)**. It must be noted that, the optimum temperature for the production of an enzyme does not always concede with that for growth **(Femi-Ola *et al*., 2018)**.

In the present study the optimum incubation temperature for maximum thermoalkalostable lipase production by *Bacillus stearothermophilus*-KKSA12was 70^{o}C. These results were in complete accordance with **Malhotra *et al*., (2000)** found that the optimal temperature in shake flasks for growth and enzyme production by the ***B. thermoaleovorans*** was 70^{o}C. Also, production of a thermostable lipase from thermophilic ***Bacillus sp***. strain Wai 28A 45, in the presence of tripalmitin at 70^{o}C, was described by **Janssen *et al*. (1994). (Deive *et al*., 2009)** detected that extracellular lipolytic activity was lower than intracellular enzyme activity and that the highest final values were obtained after 30 h of growth of ***T. thermophilus*** at 70^{o}C.

In this study, the most adequate culture conditions for maximum extracellular lipolytic enzyme production by *Bacillus stearothermophilus*-KKSA12were initially studied namely the operational conditions and inducing effects of several lipid compounds and surfactants. Finally, the optimized conditions and operational strategies designed in shake flask scale were scaled-up to a stirred tank bioreactor. Thus, this enzyme seems to have considerably higher thermostability and is therefore appropriate for industrial use because bacterial lipases generally have temperature optima in the range 30-60^{o}C **(Haki and Rakshit, 2003; Charoenpanich *et al*., 2011; Bhosale *et al*., 2016).**

pH is among the other most important factors for any fermentation process and dependent on the type of the moistening agent used in the medium. Each microorganism possesses a pH range for its growth and activity of metabolite production with an optimal value in between the range**.** The pH the culture medium plays a critical role for the optimal physiological performance of the microbial cells and the transport of various nutrient components across the cell membrane aiming at maximizing the alkaline enzymes yields. Thus, the pH of the fermentation medium has a marked effect on the cell growth and enzyme production **(Kumar *et al*., 2004).**

One of the most fascinating data, the optimum pH value for the growth of the most potent thermoalkaliphilic bacterial isolate under study viz., ***B. stearothermophilus*-KKSA12** was at 11.5.

Femi-Ola *et al*., (2018) found that, pH range between 7–9 were the optimal value for lipase production by thermophilic ***Bacillus subtilis*** isolated from oil contaminated soil with an optimum activity at pH 8.0. The optimum pH values for lipase production by ***B. stearothermophilus*** were recorded between 9-10 **(Kim *et al*., 1998).**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. Also, these results are in agreement with **Kambourova *et al*., (2003)** who reported the production of a lipase by *B.stearothermophilus* MC 7 that had a maximum activity at pH 8.0 but showed inhibition at pH 6.0 and was stable over the range of 6.5 to 8.0. **Singh *et al*.,**

(2010) also reported the effect of pH on the activity of lipase enzyme from *Bacillus subtilis* showed that pH is an important factor in enzyme activity and the maximum pH was 8.0.

In the present work, it was found that, the optimum substrate concentration for *Bacillus stearothermophilus*-KKSA12 was (1%) from cheapest natural solid substrate slaughterhouse wastes stimulated the highest yield of thermoalkalostable lipase production. This means simply that, this solid natural substrate fulfilled both the energy and nutritional requirements for *Bacillus stearothermophilus*-KKSA12 to be able to biosynthesize the highest yield of both thermoalkalostable lipases under chemical fermentation (SmF) conditions.

Microbial lipases are produced mostly by submerged culture, but solid-state fermentation methods can be used also. Immobilized cell culture has been used in a few cases**.** The highest enzymes productivity was followed by a decline at higher concentrations. It may be due to the substrate thickening of the production fermentation medium that result in bad mixing of air which is essential for the growth of organism, and subsequently the production of enzyme **(Haq *et al*., 1998),** and interfere with O_2 leading to limitation of dissolving oxygen for the growth of bacteria **(Rukhaiyar and Srivastava, 1995).Femi-Ola *et al*., (2018)** study the effects of different carbon (lipid) sources used for lipase production by *Bacillus subtilis.* They found that, the highest lipase activity (45 u mol/min/ml) was observed when olive oil served as the carbon source. The substrate specificity of lipase is important for their application in analytical and industrial purposes. The profile of the different substrates (Palm oil, olive oil, soybean oil and groundnut oil) was tested by **Femi-Ola *et al*., (2018)**. The result of the study showed that Bacillus subtilis showed most preference for olive oil as the best substrate. **Praveen and Sharmishtha (2011)** reported *Bacillus* lipase with most preference to olive oil as substrate (100%) and then groundnut oil (95%). Similar hydrolytic activity in groundnut oil and olive oil substrates was reported by **Kojima *et al*., (2003)** and **Dahiya *et al*., (2010).**

Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration **(Femi-Ola *et al*., 2018; Sharma *et al*., 2014; Charoenpanich *et al*., 2011; Bhosale *et al*., 2016)**. Lipdic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils.

In a trial to study the effect of introducing some carbon sources on the thermoalkalostable lipase production, it was found that, all the disaccharide carbon sources were the best carbon source for highest lipase production by *Bacillus stearothermophilus*-KKSA12. Bacterial lipases are typically extracellular and are influenced greatly by nutritional and physico-chemical factors, such as temperature, pH and dissolved oxygen concentration. Since lipases are inducible enzymes that are normally produced in the presence of a lipid source, the major requirement for the expression of lipase activity has always been for the production of carbon.

In accordance to the present results, in view of other workers, **Femi-ola (2018)** reported that, maximum lipase production from *Bacillus subtilis* was attained in the presence of 1% olive oil as the carbon source in the culture medium. **Kim *et al*., (1998)** 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, ***Bacillus stearothermophilus*** 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*., 1999). Charoenpanich *et al*., (2011)** produced extracellular thermostable lipase by *Aeromonas* sp. EBB-1 isolated from marine sludge. They found that, maximum lipase activity of the culture medium was obtained after 15 h at 25°C and pH 8.0 with 0.5% (v/v) olive oil as a carbon source.

Concerning the effect of the addition of different nitrogen sources for the purpose of biosynthesis and production of thermoalkalostable lipase by *Bacillus stearothermophilus*-KKSA12, it was found that, all the tested nitrogen sources failed to induce lipase enzyme production.

These results indicated the independent nature of the temperature, pH, and carbon source and nitrogen source effect irrespective to the type of solid substrate used. Also, effect of different yeast extract concentrations was studied in this work. Addition of

1% yeast extract concentration without any other additions to fish wastes gave the highest lipase productivity.

Wang *et al*., (1995) reported production of a highly thermostable alkaline lipase by ***Bacillus*** strain A 30-I (ATCC 53841) in a medium that contained yeast extract (0.1%) and ammonium chloride (1%) as nitrogen sources. These results were in complete accordance with **Charoenpanich *et al*., (2011).** They found that, organic nitrogen medium (nutrient medium) alone was not sufficient to stimulate the bacterial lipase production. Lipase activity in oil-supplemented medium is limited by the exposure of the lipid substrate to attack by the bacterium which increases dispersion of the substrate and enhances growth and enzyme production. Among natural oils, olive oil dominantly enhanced lipase production. This might concerning the effect of inoculum size, the obtained results revealed that, the optimal inoculum size needed to produce the highest yield of lipase production was 2 ml of 0.477 O.D./100ml media by ***B. stearothermophilus*- KKSA12.**

In the present study, incubation period has an obvious effect on thermoalkalostable lipase by *Bacillus stearothermophilus*-KKSA12, it seems from the results that, maximal lipase productivity on fish wastes was observed at the end of 24 hours. **Bhosale *et al*., (2016)** produced Hyperthermostable alkaline lipase from *Bacillussonorensis* 4R at 80°C and 9.0 pH in glucose-tween inorganic salt broth under static conditions for 96h.

Concerning lipase production, it was found that, addition Tween 20, Tween 80 and Triton X-100 separately as surfactants to the production medium. Tween 20 exhibited slightly increase in lipase production by *Bacillus stearothermophilus*- KKSA12. In contrast to our results **(Lima *et al*., 2004)** found that, the addition of tween 80 led to a total inhibition of cell growth, while triton X-100 originated a decrease of 50%. Likewise, **(Lin *et al*., 1995)** observed no enzymatic activity after addition of Tween 80 and triton X-45 in cultures of ***Pseudomonas pseudoalcaligenes*** F-111.

On the other hand, when Tween 80 was added, a clear change of the enzyme location could be detected. A large increase in extracellular activity and a decrease in membrane activity indicated that, when this compound was present, the solubilization of the lipolytic enzymes bound to the membrane was increased and that those enzymes were released.

To check the influence of moisture on thermoalkalostable lipase productivity by *Bacillus stearothermophilus*-KKSA12during SmF, the selected substrate was moistened with different amounts of liquid media (0 – 100%) prior to fermentation. The level of moisture content in the fermentation medium is a crucial factor in fermentation process which often determines the success of a process. As increase or decrease in the moisture content will significantly affect the biosynthesis of enzymes **(Virupakshi *et al*., 2005)**. Result of the present indicated that, 20% of initial moisture content gave the highest yield of lipase productivity lower (0-10%) or higher (75, 100%) moisture level obviously inhibited the biosynthesis of lipase by *Bacillus stearothermophilus*-KKSA12. In view of other workers **(Sun & Xu, 2008)** found that, 70% of initial moisture content gave the highest yield of WCSL. Lower (50%) or higher (100%) moisture level obviously inhibited the biosynthesis of WCSL.
In view of the present data, it could be observed that, ammonium sulfate and zinc sulfate were the best inducer for thermoalkalostable lipase production by *Bacillus stearothermophilus*-KKSA12. In view of the findings of other workers, **Ammar *et al*., (1985)** observed that, Ni^{++} and Co^{++} at certain concentration exhibited a good yield of lipase by ***B. stearothermophilus***. Lipase production by a thermophilic ***Bacillus*** sp. was increased several folds when magnesium, iron, and calcium ions were added to the production medium **(Janssen *et al*., 1994)**.

K^+, Fe^{3+}, Hg^{2+} and Mg^{2+} ions were found to enhance the enzyme activity while Al^{3+}, Co^{2+}, Mn^{2+} and Zn^{2+} ions inhibited the enzyme activity; Na^+ ions had no effect on the enzyme activity. Activity of a lipase from ***Geotrichummarinum*** was enhanced by K^+ and Mg^{2+} ions **(Huang *et al*., 2004)**. **Kumar *et al*., (2005)** reported that, an approximately 358% increase in lipase activity of ***B. coagulans*** BTS-3 due to the Hg^{2+} ions is noticeable since nowhere in the literature Hg^{2+} ions were found to modulate the lipase activity. In one of the previous studies, exposure of Ca^{2+} ions to an extracellular lipase of ***P. fluorescens*** 2D brought about approcimately a 360% increase in the lipase activity but the presence of Hg^{2+} and Co^{2+} strongly inhibited the enzyme activity **(Makhzoum *et al*., 1995)**. **Charoenpanich *et al*., (2011)** found that, the lipase activity was inhibited by most of the salt ions (except Ca^{2+}ions) as well as by Tween 80, DDT, PMSF, SDS and EDTA.

Among all tested vitamins, folic acid was found to stimulate thermoalkalostable lipase production by *Bacillus stearothermophilus*-KKSA12. While none of the test vitamins exerted inductive effect on lipase productivity. As a support to the obtained results, **Omar (2000)** found that, folic acid was the best vitamin for maximal cellulases production by ***B. brevis & B. brevis***var ***xyloamylolyticus.*** **Bayoumi *et al*. (2003)** found that, vitamins B6 at 100 ppm was the best vitamin for maximal cellulose production by ***B. brevis*** TAC-1.

In the present work, it was found that, shaking conditions exerted drastic effect against the biosynthesis of thermoalkalostable lipase under SmF conditions by *Bacillus stearothermophilus*-KKSA12on slaughterhouse wastes as cheapest substrates.

Different Erlenmeyer flask capacities were employed under SmF conditions. In the present work, it was found that, flasks of 100 ml with 1.25 gm of fish wastes were the best volume for maximal lipase productivity by *Bacillus stearothermophilus*-KKSA12.

In accordance to our results, **Al-Mousallamy (1995)** found that, lipase(s) productivity was markedly enhanced by increasing the atmosphere of oxygen over the production medium since it reached the maximum upon use of flask volume 2000 ml/25 ml production medium under static condition.

Conclusion

Lipases are enzymes which are used in a variety of biotechnological applications being able to catalyze a broad-spectrum reaction. This fact has to an increased interest in the isolation of novel microbial lipases with particular properties. A more economic process is required for producing lipase enzyme with the use of very cheap and easily accessible raw material. This study has shown that lipase producing bacteria can be easily isolated from fermented crude oil polluted soil samples collected from Khormah Governorate, Taif, KSA. The thermoalkalostable lipase produced under all presented optimal conditions. The thermoalkalostable lipase produced in the present study characterized by alkaliphilic, thermostable and lacks the requirement for interfacial activation, when small substrate is used. These properties make the lipase produced a potential advantageous biocatalyst in industry and biotechnology.

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