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Full Length Research Paper

Purification and Characterization of Bacterial Thermoalkalistable Lipase for Application in Bio-detergent Industry

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Lipases with unique substrate specificity are highly desired in biotechnological applications. Lipases from microbial sources have received heightened attention for an array of industrial applications, and these enzymes have been well exploited in the environmental sector as well. Thermostable lipases occupy a prominent position in aqueous and non-aqueous bio-catalysis. The primary goals of this research work are to isolate and identify a lipase producing thermoalkaliphilic *Bacillus stearothermophilus*- KKSA12 species from oil polluted soil samples collected from Khormah Governorate, KSA. The other purposes of this study are production, partial purification, characterization of lipase activity. The optimum conditions for *Bacillus stearothermophilus*-KKSA12hyperthermoalkalistable lipase with slaughter house wastes and tap water, pH value: 11.5; incubation period (hours): 24; incubation temperature (□C): 70 □C; substrate concentration (%) (slaughter house wastes):1% ; inoculum size(ml):2ml; incubation conditions: Shaking; best carbon source: Sucrose; Without investigated nitrogen source (Control);Yeast extract concentration(%):1(%); Surfactant: Tween 20; water content : 1 gm slaughter house wastes and 20 ml medium); 100 ml Erlenmeyer flask capacity and 1.25 gm sloid wastes and $ZnSO_4$ (100ml) The batch produced by New Brunswick USA Bioreactor 3000 by submerged fermentation method. The partially purified up to 35.66 % saturation using ammonium sulphate precipitation. One active peak for lipase obtained between 5 to 10 fractions and the fraction number 8 reached the highest specific activity up to 249.28 (U/mg. Protein/ml). Fifteen amino acids were detected. The purified lipase showed the maximum activity at 80□C, pH 9.5; at 1.5 % substrate concentration after 60 hours. Chlorine concentration resulted in decreasing the enzyme activity by different ratios depending on the time of enzyme exposure to chlorine. The purified lipase was stable towards strong anionic surfactants. Finally,*Bacillus stearothermophilus*- KKSA12hyperthermoalkalistable lipase candidate for widely used in food industry, detergent, paper, textile, leather and pharmaceutical industries because of their stability, selectively and substrate specificity for wider industrial applications.

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

INTRODUCTION

Lipase (triacylglycerol ester hydrolase, triacylglycerol acylhydrolase, EC 3.1.1.3) is a water –soluble enzyme that catalysis the hydrolysis of fats to produce mono glycosides, di-glycerides free fatty acids and glycerol. Lipase are triacylglycerol hydrolysis that catalyzes variety of conversion reactions, ranging from interestrification, esterification, alcoholysis, acidolysis and aminolysis (Bayoumi *et al*., 2012; Kaur *et al*.,2016; Gururaj *et al*.,2016; Singh *et al*.,2016).

Lipases are hydrolytic enzymes and catalyze the hydrolysis of long-chain triacylglycerols into glycerol and fatty acid. Lipases are ubiquitous enzymes widely present in many species of plants, animals, fungi, yeast and bacteria. However microbial lipases, especially from bacteria, are more useful than their plant and animal derivatives because of several important properties. (Dong *et al*., 1990; Prakash *et al*.,2013; Das *et al*.,2016; Kaur *et al*.,2016; Leisola *et al*.,2017; Sharma *et al*.,2017; Muthusamy and Beslin,2018).

Lipase have been used extensively in oleo chemical industry and dairy industry and to produce the structural triglycerides. Recently, lipases find a number of potential applications in detergent industry, oleo chemical industry, paper manufacturing industry, organic chemical processing, nutrition cosmetics, pharmaceuticals and agrochemical (Muthusamy and Beslin,2018).

Besides their ability to hydrolyze carboxylic ester bonds, lipases can catalyze esterification reactions in non-aqueous media. Lipases find applications in food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Hasan *et al*., 2006). Interestingly, lipases from different organisms provide different positional specificities, fatty acid specificities, thermal stabilities, and optimum pH values. Detergent formulations also include lipases, which are of great help for the removal of lipid stains, fatty food stains, and sebum from fabrics. Alkaline yeast lipases can work at lower temperatures than bacterial and mold lipases. Cold-active lipases are used as components of detergents for cold washing, with clear advantages in both energy consumption and textile durability (Houde *et al*.,2004; Haefner *et al*., 2005; Hasan *et al*.,2006).

Extracellular lipase produced by microorganisms is being investigated for its potential application in various industrial processes like detergents, oils, fatty acids and diary coupled with enormous therapeutic uses. Thermostable lipases have received much more attention, as they remain stable at high temperature. Reactions carried out at higher temperature lead to higher diffusion rate, increases solubility of lipids and other hydrophobic substrates in water, can reduce the risk of contamination. They display broader range of substrate specificity, tolerance towards extreme of acidic and alkaline *conditions*, and solvents**(**Park *et al*.,2005; Gupta *et al*.,2007; Grbavcic *et al*.,2007; Franken *et al*.,2009; Annamalai *et al*.,2011).

Lipase producing microorganisms including bacteria, yeast and fungi are found in various habitats for example coal tips, compost heaps, decaying food, dairies, industrial wastes, oil-processing factories, oil seeds, soil contaminated with oil and waste water (Qamsari *et al*., 2011). Among all these microorganisms several species of bacteria including mainly *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Pseudomonas*, *Staphylococcus* and *Chromobacterium* species are very efficient in producing extracellular lipases (Veerapagu *et al*.,2013; Sharma *et al*.,2017). While *Bacillus* and *Pseudomonas*, spp. are the most efficient (Rapp and Backhaus,1992). The catalytic ability of lipases can be further improved by molecular imprinting, solvent engineering as well as by molecular techniques for example directed evolution and protein engineering (Jaeger *et al*.,1999). Because of their higher activities at neutral and alkaline pH, bacterial enzymes are preferred than fungal enzymes (Gokbulut and Alper,2013). Most of the well-studied microbial lipases are inducible extracellular enzymes which upon their synthesis, are secreted to the extracellular environment (Zouaoui and Bouziane,2012). Mostly commercial enzymes are hydrolases including carbohydratase's, proteases, pectinases and lipases.

Lipases have broad variety of industrial applications such as food industry (improvement of flavor) (Veerapagu *et al*., 2013), detergent (hydrolysis of oil and fats) (Das *et al*., 2016), pharmaceutical (synthesis of chiral drugs), paper (control of pith), medicine (triglyceride measurement) , cosmetics (exclusion of lipids), wastewater(decomposition and removal oil) , leather (elimination of fat from animal skin) (Ferreira-Daias *et al*.,2015). Some important lipase producing bacterial genera includes *Bacillus*, *Pseudomonas* and *Acnetobacter*. Bacterial lipase is mostly extracellular and are produced by submerged fermentation (Gupta *et al*.,2003; Gupta *et al*.,2004; Patil *et al*.,2011; Gurung *et al*.,2013; Kumar and Dhar,2016).

The main purpose of this study an attempt has been made for the isolation of potential thermoalkalostable lipase producing bacteria, its purification and characterization and its application as detergent.

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MATERIALS AND METHODS

I. Culture media:

i) Isolation media: All isolatation media in the present study were prepared according to Lima *et al*.,
(2004) mentioned in details in Al-Domori and Bayoumi, (2019).

ii) Preparation of inoculum: Single colony of *Bacillusstearothermophilus*- KKSA12 was inoculated in 10 ml of olive oil broth in 250 ml conical flask incubated at 70°C with agitation on rotator shaker at 240 rpm for 24 hours

II. Production medium: All hyperthermoalkalistable lipase production media according to Aisaka and Terada (1980);Kademi *et al*., (2000);Cheng*et al*., (1998); Lee *et al*., (2001) were mentioned in details in Al-Domori and Bayoumi, (2019).

III. Isolation of thermoalkaliphilic lipolytic producing bacterial isolates: Thethermoalkaliphilic lipase producing bacterial were isolated from crude oil polluted soil samples collected from different localities in Khormah Governorate, Kingdom of Saudi Arabia. The soil samples were collected by sterile gloves, scalpel and placed in sterile container. Sterile dilution was carried out to isolate hyperthermoalkalistable lipase producing bacteria from these soil samples. The soil samples were suspended in 9 ml sterile water, serially diluted at 70□C for 7 days. The isolation methods were carried out according to Jaegar and Eggert (2002); Jaegar and Reetz (1996) and Jansen *et al*. (1996). pH was adjusted at 10.5 and all Petri- plates were incubated at 70°C for 7 days. The thermoalkaliphilic bacterial isolates were isolated from the seven crude oil polluted soil samples according to Lima *et al*., (2004). All the isolation media supplemented with tributyrin emulsified with Gum acacia as the carbon source and incubated for 1, 2, 3, 4 and 5 days at pH 10.5 and temperature 65-70^{o}C.

IV. Identification of the most potent bacterial isolates: The thermoalkaliphilic lipolytic bacterial isolates were subjected to characterization using the keys of Sneath (1986), Barrow and Feltham (1993) and Hensyl (1994). The morphological, physiological and biochemical characteristic of bacterial sample were carried out in Fermentation Biotechnology and Applied Microbiology Center (Al-Azhar University, Cairo, Egypt). The *B. stearothermophilus*- KKSA12 strain, which showed the highest activity, was selected for further study. This strain was maintained in glycerol stocks (50%, v/v) and stored at -20□C.

V.Extraction of lipase: After the submerged state fermentation, the enzyme was extracted from fermentation medium, 5 ml of production medium was withdrawn from the production flask and centrifuged at 10.000 rpm for 30 minutes at 4 °C. The clear supernatant served as a crude lipase source.

VI. Methods of lipase assay: Different thermoalkaliphilic bacterial strains were screened for lipase producing using Tributyrin hydrolysis test to screen hyper-lipolytic bacteria (Yamada *et al*.,1962). The zone forming bacterial strains were further screened for quantitative analysis of lipase using titrimetric method (Lawrence *et al*.,1967). Lipase activity as assayed by alkali titration using olive oil as substrate as described by Nahas (1988) with some modifications. The thermoalkalostable extracellular lipase released into the medium was assayed quantitatively by using p-nitrophenyl palmitate (PNPP) as the substrate according to (Winkler and Stuckmann,1979), with some modifications.One enzyme unit was defined as: mol of p-nitrophenol enzymatically released from the substrate ml/min. Each of the assayed was performed in triplicate unless otherwise stated and mean values were presented, as well as standard deviation (S.D.) were calculated and then taken as an indication of lipase activities. Lipase activities were estimated with the help of the standard curve pure lipase enzyme.

VII. Parameters controlling the hyperthermoalkalistable 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 carbon sources;8-Different nitrogen sources;9-Different yeast extract concentrations; 10-Different surfactants; 11-Different water contents; 12- Different flask capacities; 13-Different metallic ions; 14-Different vitamin requirements.

VIII. Production and purification of microbial hyperthermoalkalistable lipase: The following steps were performed during the course of production and purification of lipase produced by *B. stearothermophilus*- KKSA12, from crude oil polluted soil samples due to their growth on the optimal production medium described previously:

1) Large-Scale production of Hyperthermoalkalistable lipase by *B. stearothermophilus*- KKSA12: Applying the bacterial thermoalkalostable lipase in New-Brunswick Scientific, USA stirred tank bioreactor bioflo 3000 under the all optimal fermentation conditions:

i) Starting of batch: Slaughter house wastes waste grinded preparation 250 gm was introduced through the top of New Brunswick USA Bioreactor 3000. This followed by the addition of tap water 4.5 *l* adjusted to pH 11.5 with 5 gm $ZnSO_4$ as a concentration of 0.1 %. Only 0.0025 gm of folic acid was adding to reach a concentration of 500 ppm and yeast extract was added with a concentration of 10 gm/l while the stirrer rotor was operating the inoculum volume 500 ml was added.

ii) Batch performance: The present fermentation batch was performed under all specified optimal conditions previously determined under bench scale fermentation.

Thus, New Brunswick USA Bioreactor 3000 was adjusted according to the following optima: Temperature: 70°C; pH: 11.5; incubation time: 24 hours; Agitation speed: 100. One from the production medium will take every 2 hours to determine the enzyme productivity and protein content and then calculated specific activity during fermentation process, as well as temperature, pH, agitation and dissolved oxygen were recorded every two hours of the fermentation processes.

iii) Harvesting the yield: At the end of incubation period, extraction of the crude enzyme was performed. The whole fermented broth was collected from the bioreactor container and then filtered through a piece of cotton using a special big plastic funnel. The filtration process was repeated many times to obtain more clarified crude enzyme preparation**.** The bacterial growth was harvested by centrifugation at 5000rpm. The supernatant was filtrated, and the obtained cell-free filtrate was preserved in the refrigerator as a crude enzyme according to Ammar *et al*., (1999).

2) Ammonium sulphate fractionation: The stability of protein is markedly affected by the, solubility of the protein also increases this is referred to as "Salting in". However, beyond a certain point, the solubility begins to decrease, and this is known as salting out. The chart of Gomori (1955) as mentioned by Dixon and Webb (1964) was applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell free filtrate under investigation. Ammonium sulphate was used for fractional precipitation of proteins. It was available in highly purified form, has great solubility allowing for significant changes in the ionic strength. The changes are the ammonium sulphate concertation of a solution by adding a solution of known saturation to crude enzyme extract. Ammonium sulphate was added very slowly with continuous stirring of the solution on a magnetic stirrer in cold conditions. The solution was centrifuged at 10.000 rpm for 10 minutes at 4°C. These pellets were collected and dissolved in 10 ml of 50 mM Tris- buffer solution.

3) Dialysis: The precipitate was collected by centrifugation the extract at 10.000 rpm for 10 minutes at 4°C. The precipitate was dissolved in 10 ml of 50 mm Tris- HCl and subjected to dialysis. About 10 cm size of dialysis bag was successively boiled in 100 ml of distilled water, 2% sodium bicarbonate and 1MM EDTA solution and again 100 ml of distilled water for 10 min at 100°C. Then the dialysis bag was cooled to room temperature and kept in refrigerator for 30 min, the dissolved enzymes were transferred to dialysis bag of one end. The bag was tightly tied, and dialysis bag was suspended in a beaker containing distilled water the help of glass rod. This set up was kept in refrigerator overnight.

4) Applying columnchromatographic technique: Applying on Sephadex G-200: The dialyzed-partially purified enzyme preparation was applied onto a column packed with Sephadex G-200. For this purpose, 4 gm of Sephadex G-200 were suspended and allowed to swell in 200 ml of the suitable buffer applying before bacterial partially purified enzyme (phosphate buffer at pH 8). Sodium azide (0.02%) was added to prevent any microbial contamination, the gel washed several times with the same buffer until all fine particles were removed. A column (2x20 cm) fixed in a vertical position was washed several times with the buffer solution, then the column was packed carefully by pouring of a previously degassed thin slurry of gel in the buffer solution into a vertical column partially filled with the same buffer. The addition of gel was continued until a bed height of 2 cm in the column was attained. The column was connected to the buffer reservoir and the flow of the buffer was maintained at a rate of approximately 10 ml/hour for two hours to allow the settlement of the bed. The gel height was adjusted at 20 cm by the precise addition or removal of the extra gel (Wilson and Walker, 1995).Two ml of the dialyzed partially purified enzyme preparation sample was applied carefully onto the top of the gel. It was allowed to pass into the gel by running the column buffer, then elution was continued by adding the buffer without disturbing the gel surface and then the column was connected to the reservoir. Fifty fractions were collected (each of 5 ml). Both enzyme activity and protein content were determined for each separate fraction, (Wilson & Walker, 1995). Specific activities were also determined to plot the relationship between the fifty fractions and its specific activities.

5) Amino acid analytical data of the purified enzyme: The hydrolyzed proteinaceous amino acids have been determined in the HPLC & Amino acid analyzer Lab. National Research Center according to the methods described by Pellet and Young (1980).Eppendorf-Germany LC 3000 Amino acid analyzer was used for this purpose under the following conditions: Flow rate: 0.2 ml/min. Pressure of buffer from 0 to 50 bar, pressure of reagent to 0 – 150 bar and Reaction temperature 123°C. Data analysis of chromatogram was done by Ezchrom TM Chromatography Data System Tutorial and User's Guide-Version 6.7.

6) SDS-polyacrylamide gel electrophoresis: The SDS-PAGE was performed on 10% poly-acrylamide gel (with 4% stacking gel) by a standard technique (Laemmli, 1970) using reference molecular weight marker (SDS-6H, Sigma Chemical, and USA). Proteins in the gel were visualized with Coomassie brilliant blue R-250. This experiment was performed in National Research Center.

XI. Factors affecting the purified hyperthermoalkalistable lipase activity:

1-Substrate concentration: This experiment was carried out by incubating different substrate concentration (DNPP) solution viz., 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 ml for 30 minutes at 60°C, then lipase activity was assayed according to the method of (Winker & Stuckmann 1979).

2-Enzyme concentration: This experiment was carried out by incubating enzyme concentrations viz., 50, 100, 200, 300, 400, 500 and 1000 µl with same substrate concentration for 30 min. at 60°C then lipase activity was assayed.

3-Incubation period: This experiment was carried out to determine the optimum incubation period at which the purified enzyme reach to their optimum activity. The reaction mixtures were incubated for 15, 30, 45, 60 and 90 minutes respectively. At the end of incubation period in all cases, the activity of the purified enzyme was assayed as usual.

4-Effect of temperature: The effect of temperature on the purified lipase of *B. brevis* B_2 was studied by separately incubating the enzyme reaction mixture at the selected temperature (40, 50, 60, 70, 80, 90 and 100°C). Therefore, lipase activity was determined as usual.

5-Thermostability: The sensitivity of lipase to heat was evaluated by incubating lipase (100 µl with 100 µl phosphate buffer pH 8 (v/v) taken in an Eppendorf tube at 40, 50, 60, 70, 80, 90 and 100°C in a water bath for a period of 2 hours then, 100 µl of enzyme was sampled and assayed for residual lipase activity.

6-Effect of pH: The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pHs ranging from 7 to 12 at 80°C for 30 min. The pH of the 0.05M Tris buffer (a part of the reaction mixture) was adjusted separately to 7, 8, 9, 9.5, 10, 11 and pH 12 using 2N NaOH and pH meter for adjusting. To the reaction mixture containing PNPP and Tris buffer at each the above pH, 100 µl of purified enzyme was added and the reaction was carried out at 80°C for 30 min.

7-pH stability: To study the effect of prolonged exposure of lipase to a particular pH buffer, in a separate experiment the purified enzyme (100 µl) was incubated with 100 µl buffer of pH 7, 8, 8.5, 9, 9.5, 10, 11 and pH 12 (0.05 M Tris buffer) separately in Eppendorf tubes at 80°C for 2 hours. Thereafter, lipase activity was assayed at interval of 30 min. as usual.

8-Effect of metal-ions: To evaluate the effect of various metal-ions on lipase activity, an attempt was made to study the effect of calcium chloride, sodium azide, cupric sulphate, zinc sulphate, mercuric chloride, cadmium chloride and EDTA respectively on lipase activity. Each of the salt-ions was separately included in the reaction mixture at a final concentration of 250, 500 and 1000 ppm. The lipase activity was assayed after 30 min. incubation at 80°C.

9-Stability of the purified thermoalkalostable enzyme with chlorine: This experiment was carried out according to Singh *et al*., (1999). The purified enzyme was incubated with different concentrations of chlorine viz., 5, 7, 10 and 15 µl/ml. For 0, 15, 30, 45 and 60 min. Then the enzyme activity was assayed as previously mentioned.

10-Effect of oxidizing agents and surfactants: This experiment was carried out according to Moreira *et al*., (2002). The purified lipase enzyme was incubated with different concentrations of some oxidizing agents and surfactants viz., sodium dodecyl sulphate (SDS), hydrogen peroxide, tween 20, tween 80, triton X-100 and sodium deoxycholate at 3, 5, 7 and 10 (% v/v or w/v) for 2 hours. Enzyme activity was determined as previously mentioned and the stability was determined in terms of remaining hydrolytic activity.

RESULTS

I. Isolation, characterization, screening and selection of the most potent thermoalkaliphilic lipase producers isolates: In the present study, the thirteen positive isolates were screened for a maximum secretion of extracellular lipases by assaying the lipase activity in liquid culture using p-nitrophenyl palmitate (PNPPas a substrate at 70□C. The bacterial colonies that formed a zone of clearance were lipolytic positive strains. The *Bacillusstearothermophilus*-KKSA12 was used for the production of extracellular lipase by submerged state fermentation by using various substrates. The *Bacillusstearothermophilus*-KKSA12 were identified from oil polluted soil samples collected from Khormah governorate, Taif, KSA**.**

1.1. Screening of thermoalkaliphilic lipase producers' bacterial isolates in relation to lipid clearing zoon and temperature: Only three bacterial isolates from thirteen 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. Screening of all lipase-producing strain resulted in isolation of bacterial strain that showed a clear zone of hydrolysis around the colonies.

1.2. Screening of lipolytic thermoalkaliphilic bacterial isolates with best different wastes:

The most potent lipolytic thermoalkaliphilic bacterial isolate which exhibited highest clear zones were allowed to grow on all the previously mentioned as 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.

1.3. Different oils in relation to the most potent bacterial isolates: The purpose of this experiment was to investigate the best oil used in medium A that induced the highest thermoalkalostable lipase productivity by the most potent bacterial isolate.

Table (1): A summary of the optimal nutritional and environmental parameters controlling thermoalkalostable lipase productivity by *B.stearothermophilus*-KKSA12.

No.	Parameter	Optimal conditions	No.	Parameter	Optimal conditions
1	Selective medium and most + potent bacterial isolate.	Slaughter house wastes & tap H_2O.	8	Carbon source.	Sucrose
2	pH value.	11.5	9	Nitrogen source	Control
3	Incubation period (hours).	24	10	Yeast extract concentration (%).	1
4	Incubation temperature (^oC).	70°C	11	Surfactants.	Tween 20
5	Substrate concentration (%).	1	12	Water content.	(1 gm slaughter house wastes& 20 ml media)
6	Inoculum size (ml).	2	13	Erlenmeyer flask capacity (ml).	100 ml Erlenmeyer flask capacity &1.25gm solid wastes.
7	Incubation conditions.	Shaking	14	Metallic ions.	Zn SO_4 (100 ppm)

1.4. Selection of the most potent thermoalkaliphilic lipolytic bacterial isolates when allowed to grow their best substrate: While when allowed the most potent bacterial isolate KKSA12 to grow on slaughter house 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. It is means that the bacterial isolate has the ability to use the agricultural wastes as the sole carbon and nitrogen source 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 wastes only and tap water at pH 11.5 and temperature 60^oC.

1.5. Morphological, physiological and biochemical characteristic of bacterial sample: The bacterial isolate (KKSA12) suggested to be *Bacillusstearothermophilus*-KKSA12 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. Data recorded in table (1) exhibited that, the ability of the most potent bacterial isolate to produce the four enzymes previously mentioned above as well as lipase for application in detergent technology.

1.6. Production, purification and properties of hyperthermoalkalistable lipase produced by the most potent bacterial isolate *B. stearothermophilus*-KKSA12:The optimum conditions for hyperthermoalkalistable lipase with slaughter house wastes and tap water, pH value: 11.5; incubation period (hours): 24; incubation temperature (□C): 70 □C; substrate concentration (%) (slaughter house wastes):1% ; inoculum size(ml):2ml; incubation conditions: Shaking; best carbon source: Sucrose; Without investigated nitrogen source (Control);Yeast extract concentration(%):1(%); Surfactant: Tween 20; water content : 1 gm slaughter house wastes and 20 ml medium); 100 ml Erlenmeyer flask capacity and 1.25 gm sloid wastes and $ZnSO_4$ (100ml).(Table1). These previously data published previously byAl-Domori and Bayoumi, (2019). Large scale fermentation of *B. stearothermophilus*- KKSA12 thermoalkalistable lipase in New Brunswick, USA, Bioreactor Bioflo 3000-5L.

Step 1: Production of hyperthermoalkalistable lipase in New Brunswick, USA, Bioreactor Bioflo 3000-5L: The present fermentation batch was performed under all specified optimal conditions as previously mentioned in table (2) under bench scale fermentation (BSF). Thus, New Brunswick, USA, Bioreactor Bioflo 3000-5L, was adjusted according to the following optimal: Initial pH = 10.31 Initial temperature=70^oCDissolved oxygen = 168.
Agitation = 100Incubation period = 24 hours.
Sample of one ml was taken from the fermentation batch every two hours in order to determine lipase productivity and protein content during fermentation process. On the other hand, temperature, agitation, pH and dissolved oxygen were recorded every two hours as shown in table (2). Lipase productivity were increased from 11.82 (U/ml) after 2 hours to 45.89 (U/ml) after 24 hours. The protein content was also increased from 1.20 (mg/ml) after 4 hours to 2.125 (mg/ml) after 24 hours while the specific activity also increased from 11.82 (U/ml protein /ml) after 2 hours to 21.59 (U/ml protein /ml).

Step 2. Fractional precipitation by ammonium sulphate: Results recorded in table (3) indicated that, the

Table (2): Fermentation process in relation to temperature, agitation speed, dissolved oxygen, pH value; lipase productivity and protein content were calculated.

Sample No.	Time (hours)	Temp. (^{o}C)	Agitation speed	pH value	D.O.	Lipase productivity (U/ml)	Protein content (mg/ml)	Specific activity (U/ml protein)
0	0	70	100	10.30	169.1	0.00	0.00	0.00
1	2	70	100	9.88	144.1	11.82±0.03	0.00	11.82
2	4	70	100	9.65	140.6	13.58± 0.02	1.200	11.31
3	6	70	100	9.62	158.3	14.05±0.05	0.773	18.17
4	8	70	100	9.58	116.2	14.06±0.01	1.000	14.06
5	10	70	100	9.56	118.2	28.71±0.01	1.200	23.92
6	12	70	100	9.53	121.1	33.55±0.02	1.661	20.19
7	14	70	100	9.52	99.8	35.53±0.02	1.561	22.76
8	16	70	100	9.52	116.5	38.02±0.01	1.561	24.35
9	18	70	100	9.48	105.2	39.44±0.04	1.562	25.26
10	20	70	100	9.48	115.2	41.66±0.00	1.625	25.63
11	22	70	100	9.47	114.3	45.77±0.03	2.125	21.53
12	24	70	100	9.47	114.0	45.89± 0.00	2.125	21.59

Table (3): Ammonium sulphate fractionation for thermoalkalostable lipase produced by *B. stearothermophilus*- KKSA12 allowed growing on slaughter house wastes substrate at 70^{o}C.

$(NH_4)_2SO_4$ saturation level, (%)	Volume (ml)	Total activity (U/ml)	Total protein content (mg/ml)	Specific activity (U/ml, protein)	Purification fold	Recovery (%)
Crud extracts	100	4677.0	215.4	21.71	1.0	100.0
0 – 20	15	823.5	248.4	3.315	0.152	17.60
20 – 40	15	978.5	148.4	6.593	0.303	20.92
40 – 60	15	1011.2	118.5	8.533	0.393	21.62
60 – 80	15	1045.5	98.2	10.64	0.490	22.35
80 – 100	15	1668.2	64.3	25.944	1.195	35.66

- Total activity = volume (ml) X Activity (Unit/ml).
- Total protein content = volume (ml) X protein content (mg/ml).
- Recovery (%) = the total activity of purified enzyme/total activity of crude enzyme x100.
- Purification fold = specific activity of purified/specific activity of crude enzyme.

most active enzyme protein preparation was obtained at an ammonium sulphate level at 100% where the activity was reached up to 1668.2 Unit/ml and protein content 64.3 mg/ml corresponding to a specific activity 25.944 Unit/mg protein with purification fold 1,195 and recovery (%) 35.66. Only 100 ml were obtained at the end of the process of dialysation against tap water and sucrose. The recovery (%) of the ammonium sulphate fractionation increased from 17.60; 20.92; 21.62; 22.35 and 35.66 with 20;40;60;80 and 100 saturation levels (%) respectively.

Step 3. Concentration by dialysation against sucrose: The most active ammonium sulphate fractions previously obtained at the best saturation, (100 ml) was dialyzed against distilled followed by dialysis against sucrose crystals until a volume of 30 ml was obtained and specific activity was determined as 119.85 U/mg proteins. The specific activity for the purified enzyme fraction between (5-

Table (4):A summary of the purification steps of thermoalkalostable lipase produced by *B. stearothermophilus*- KKSA12, allowed to grow on fish wastes at 70°C.

Purification Step	Volume (ml)	Protein content (mg/ml)	Total protein content (mg/ml)	Lipase activity (U/ml)	Total activity (U/ml)	Specific activity (U/mg protein)	Recovery (%)
CFF	800	3.211	2568.8	46.73	37384	14.55	100.0
$(NH4)_2SO_4$ precipitation	100	5.02	502	113	11300	22.50	30.22
Dialysis against sucrose	30	0.82	24.6	98.28	2948.4	119.85	7.88
Sephadex G-200 F (5-10)	30	0.52	15.6	30.8	924	59.23	2.47

CFF: Cell free filtrate.

Table(5): Fractionation pattern of thermoalkalostable lipase produced by *B. stearothermophilus*- KKSA12 using Sephadex G-200 column chromatographic technique.

Fraction No.	Lipase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg. protein/ml)	Fraction No.	Lipase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg. protein/ml)
1 – 4	UD	UD	UD	11	1.45	0.071	20.42
5	24.6	0.435	56.55	12	4.91	0.083	59.15
6	28.8	0.28	102.85	13	4.88	0.088	55.45
7	9.25	0.07	132.14	14	5.77	0.23	25.08
8	6.98	0.028	249.28	15	4.55	0.24	18.95
9	3.97	0.026	152.69	16	3.97	0.29	13.68
10	3.85	0.028	148.07	(17-50)	UD	UD	UD

10) using Sephadex G200 59.23 (U/mg protein /ml) with the recovery (%) 2.47 (Table 4).

Step 4. Preparation of Sephadex G-200 gel filtrate column and applying the enzyme sample: The results showed that there was one active peak for lipase enzyme obtained in fractions (5 – 10) (56.55-148.07(U/mg) proteinand the fraction number 8 reached the highest specific activity up to 249.28(U/mg) protein (Table 5).

Step 6. Amino acids analysis of the purified thermoalkalostable lipase enzyme produced by *B. stearothermophilus*- KKSA12: -Data recorded in table (6) showed that 15 amino acids were detected in addition to ammonia. The amin acids detected in the purified hyperthermoalkalistable lipase were aspartic acid, thereonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine found 75.77; 23.45; 24.22; 135.98;33.80;55.33;18.88;12.33;18.87;45.66;5.16;18.28;39 .93;29.10 and 15.23 (µg/ml) respectively. Glutamic and aspartic acids represented the highest value i.e. 135.98 and 75.77µg/ml respectively. Methionine and tyrosine acids represented the lowest value i.e. 12.33 and 5.16 (µg/ml) respectively.

II. Characterization of the purified hyperthermoalkalistable lipase enzyme produced by *B. stearothermophilus*- KKSA12: The aim of the present series of experiments was to investigate some properties of the partially purified lipase produced by *B. stearothermophilus*- KKSA12 allowed to grow on slaughter house wastes as best substrate and incubated under all optimal nutritional and environmental fermentation conditions.

1) Effect of different substrate concentration on the purified enzyme activity: Data recorded in table (7) showed that, an increase of substrate (PNPP) concentrations was corresponding by lipase activity decrease.

2)Different incubation periods, different concentrations; incubation temperature; pH values and thermal stability of purified enzyme in relation to their activities: Data recorded in table (7) showed that, the highest lipase activity was obtained after60minutes.

Table (6): A summary of amino acids analytical data of *B. stearothermophilus*- KKSA12 purified lipase.

No.	Time (min)	Amino acid	Concentration (µg/ml)	No.	Time (min)	Amino acid	Concentration (µg/ml)
1	12.56	Aspartic acid	75.77	9	38.75	Isoleucine	18.87
2	14.88	Threonine	23.45	10	39.77	Leucine	45.66
3	16.16	Serine	24.22	11	42.77	Tyrosine	5.16
4	17.94	Glutamic acid	135.98	12	44.88	phenylalanine	18.28
5	25.79	Glycine	33.80	13	52.35	Histidine	39.93
6	26.77	Alanine	55.33	14	55.56	Lysine	29.10
7	31.55	Valine	18.88	15	64.22	Arginine	15.23
8	37.79	Methionine	12.33				

Table (7): Different substrate (PNPP) concentrations, incubation periods and enzyme concentrations in relation to the activity of purified enzyme.

Substrate concentration (ml)	Lipase activity (Unit/ml)	Incubation period (min.)	Lipase activity (Unit/ml)	Enzyme concentration (µl)	Lipase activity (Unit/ml)
1	40.98 ± 0.04	10	9.22 ± 0.02	50	26.77 ± 0.03
1.5	42.99 ± 0.02	15	25.66 ±0.02	100	30.89 ± 0.01
2	35.66 ± 0.05	30	37.55 ± 0.05	200	38.55 ± 0.02
2.5	33.85 ± 0.20	45	45.44 ± 0.02	300	70.58 ± 0.02
3	30.89 ± 0.04	60	58.54 ± 0.04	400	76.75 ± 0.04
3.5	27.89 ± 0.03	70	55.33 ± 0.04	500	76.44 ± 0.02
4	25.44 ± 0.02			1000	88.44 ± 0.03
5	12.41 ± 0.01				

58.54 (Unit/ml). Above or below this time lipase activity with gradually decreased. From the obtained results for lipase enzyme under study generally it was found that there is a continuous increasing of enzyme activity units due to increase of enzyme concentration, as shown in table (7). Data recorded in table (8) showed that the maximum activity of lipase was obtained at 80^{o}C viz.95.77 (Unit/ml). Below or above this particular degree lipase activity decreased gradually. Also, data recorded in table (8) showed that the purified lipase exhibited the maximum activity at pH 9.5 of tris-buffer viz. 110.51 (Unit/ml), while lipase activity reached its maximal value at (80-90^{o}C) viz. 22.76(Unit/ml). Although, the enzyme exhibited its ability to act up to 100^{o}C.

3. pH stability of the purified hyperthermoalkalistable lipase enzyme activities: Data recorded in table (8), showed that, the purified lipase still very active at alkaline pH and exhibited its maximal value 30.22 U/ml at pH 9.5 of Tris-buffer. The Hyperthermostable lipase was exhibited stability with pH values between 7 and 12.

4. Effect of different metallic ions (activators and/or inhibitors) on then purifiedthermoalkalistable lipase activity: Results recorded in table (9) showed that, mercuric chloride and cupric sulphate exhibited a drastic inhibitory effect against lipolytic activity at all concentration applied in this experiment. On the other hand, the best activator for lipase was cadmium chloride at 100 ppm.

5. Stability of the purified lipase in the presence of chlorine (Na-hypochloride): The aim of this experiment is to study the compatibility of the purified enzyme with chlorine. This is an important observation because of the fact that enzyme incompatibility with chlorine is the reason for its failure to use as a part of cleaning agent in some detergent formulations which contain chlorine as bleaching agent. As shown in table (10) all chlorine concentration resulted in decreasing the enzyme activity by different

Table (8): Different incubation temperatures, pH, temperatures and pH stability in relation to purified lipase produced by *B. stearothermophilus*- KKSA12 on their activities.

Incubation temperature (°C)	Lipase activity (Unit/ml)	pH value	Lipase activity (Unit/ml)	Temperature (°C)	Lipase activity (Unit/ml)	pH value	Lipase activity (Unit/ml)
40	5.66 ± 0.02	7	15.35±0.02	40	13.38±0.00	7	15.62±0.02
50	8.51 ± 0.00	8	16.88±0.04	50	14.22±0.02	8	19.77±0.02
60	60.51 ± 0.04	9	20.33±0.10	60	15.72±0.01	8.5	20.38±0.00
70	67.15 ± 0.05	9.5	110.51±0.01	70	16.82±0.02	9	29.02±0.01
80	95.77 ± 0.01	10	79.77±0.00	80	22.76±0.01	9.5	30.22±0.02
90	33.91 ± 0.00	11	42.21±0.01	90	20.99±0.02	10	19.77±0.01
100	34.67 ± 0.02	12	0.32±0.02	100	22.25±0.01	11	20.22±0.02
						12	14.88±0.04

Table (9): Effect of different metallic ions (activators and/or inhibitors) on the purified hyperthermoalkalistable lipase activity.

Activator and/or inhibitor	Concent-ration (ppm)	Lipase activity (U/ml)	Inhibition (%)	Activation (%)
Control		115.5±0.02	0.0	0.00
Zinc sulphate	250	7.94±0.02	93.12	0.00
	500	39.88±0.00	65.47	0.00
	1000	120.5±0.01	0.0	4.32
Ethylene diamine tetra-acetic acid (EDTA)	250	18.55±0.00	83.93	0.00
	500	49.42±0.02	57.21	0.00
	1000	120.5±0.01	0.0	4.32
Sodium azide	250	75.55±0.01	34.58	0.00
	500	99.02±0.02	14.26	0.00
	1000	125.88±0.02	0.0	8.98
Mercuric chloride	250	0.88±0.01	99.23	0.00
	500	9.22±0.04	92.01	0.00
	1000	5.92±0.02	94.87	0.00
Cadmium chloride	250	5.755±0.02	95.01	0.00
	500	98.66±0.00	14.58	0.00
	1000	129.44±0.02	12.06	0.00
Cupric sulphate	250	0.530±0.2	99.54	0.00
	500	0.333±0.1	99.71	0.00
	1000	0.355±0.1	99.69	0.00
Calcium chloride	250	28.99±0.02	74.90	0.00
	500	120.41±0.02	0.00	4.25
	1000	128.77±0.01	0.00	11.48

Table (10): Different chlorine concentration in relation to the activity of the purified lipase produced by *B. stearothermophilus*- KKSA12.

Exposure time (min.)	Chlorine concentration (µl/ml)							
	5		7		10		15	
	Enzyme activity (U/ml)	Remai-ning activity (%)	Enzyme activity (U/ml)	Remai-ning activity (%)	Enzyme activity (U/ml)	Rema-ining activity (%)	Enzyme activity (U/ml)	Remaini-ng activity (%)
0	115.8±0.02	100	115.8±0.02	100	115.8±0.02	100	115.8±0.02	100
15	38.2±0.02	32.98	9.35±0.04	8.07	4.8±0.03	4.14	0.7985±0.03	0.689
30	30.8±0.02	26.59	4.7±0.003	4.05	2.88±0.03	2.48	0.7475±0.01	0.645
45	8.5±0.000	7.34	2.88±0.1	2.48	0.797±0.04	0.688	0.7475±0.03	0.645
60	2.05±0.05	1.77	2.81±0.2	2.42	0.758±0.03	0.647	0.7475±0.004	0.645

Table (11): Effect oxidizing agent surfactant in relation to the activity of the purified lipase produced by *B. stearothermophilus*- KKSA12.

Oxidizing agent/surfactant	Concentration (% w/v or v/v)	Lipase activity (U/ml)	Final activity (%)
Control	0.00	105.8±0.03	100
Tween 80	3	4.22±0.01	3.9888
	5	28.91±0.02	27.325
	7	31.31±0.03	29.593
	10	35.77±0.01	33.809
Tween 200	3	65.7±0.02	62.098
	5	105.5±0.01	99.710
	7	125.1±0.02	118.24
	10	135.2±0.02	127.78
Triton X-100	3	33.42±0.03	31.587
	5	53.20±0.01	50.283
	7	75.33±0.02	71.200
	10	220.55±0.02	208.45
H_2O_2	3	315.8±0.01	298.48
	5	316.8±0.01	299.43
	7	309.1±0.02	292.15
	10	302.0±0.01	285.44
Sodium dodecyl sulphate (SDS).	3	114.0±0.03	107.75
	5	116.4±0.02	110.01
	7	119.4±0.01	112.85
	10	119.5±0.01	112.94
Sodium deoxycholate ($C_{24}H_{39}NaO_4$)	3	94.66±0.01	89.470
	5	105.8±0.01	100.0
	7	160.35±0.01	151.55
	10	160.2±0.02	151.31

ratios depending on the time of enzyme exposure to chlorine.

6. Effect of oxidizing agent and surfactants on the purified lipase enzyme activity: The aim of this experiment was to study the stability of the purified enzymes in the presence of oxidizing agents and surfactants because of the fact that a good enzyme detergent should be stable in the presence of oxidants and surfactants. Results recorded in table (11) showed that, the purified lipase wasstable towards strong anionic

surfactants like sodium dodecyl sulphate (SDS) and sodium deoxycholate at all concentration used. On the other hand, it was shown that the purified lipase not only stable toward H_2O_2, but also the enzyme activity was enhanced many times. However, maximum stimulation was carried out with (3 and 5%) concentration of H_2O_2.

DISCUSSION

Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Applications of detergent enzymes have grown substantially and the largest application is in house hold laundry detergent formulations. The increased reliance of detergent manufactures on enzyme technology is because of consumer-recognizable cleaning benefits. The addition of completely new performance benefits, fabric restoration and an increased performance/cost ratio because of the availability of more efficient enzymes and the industry trend towards reducing pricing. Current market trend and consumer needs are influencing the development of enzymes for detergent applications, with the emphasis on enzymes that have improved performance/cost ratios, increased activity and improved compatibility with other detergent ingredients. In addition, enzyme supplier and detergent manufactures are activity pursuing the development of new enzyme activities that address the consumer-expressed need for improved cleaning, fabric care and antimicrobial benefits. However, apart from their use in laundry detergent, they are also popular in the formulation of household dishwashing detergents, and both industrial and institutional cleaning detergents (Godfrey and West 1996; Showell 1999; Abol Fotouh *et al*., 2016; El-Kasaby *et al*., 2018).

Some important lipase producing bacterial genera includes *Bacillus*, *Pseudomonas* and *Acinetobacter*. Bacterial lipase is mostly extracellular and are produced by submerged fermentation (Haalck *et al*., 1991; 1992; Gupta *et al*., 2004). Under controlled conditions, lipases are able to catalyze a large number of reactions (Hasan *et al*., 2014). Lipases of microbial origin are of considerable commercial importance, because of the high versatility and high stability, moreover, the advantage of being readily produced in high yields (Shaini and Jayasree, 2016). Many microbial lipases have been commercially available in free or immobilized form. numerous species of bacteria (*Bacillus*, *Pseudomonas*, and *Burkhoderi*), yeast (*Candidarougosa*, *Yarrowialipolytica* and *Candidaantarctica*) and molds (*Aspergillus*, *Trichodermaviride*) produce lipases with different enzymological properties and specificities but microbes (Choo *et al*.,1998; Veerapagu *et al*.,2013; Das *et al*., 2016; Ferreira-Das *et al*., 2015).

The main object of the present work was an investigation of screening, production, purification and characterization of thermoalkalostable lipase enzyme for application in detergent technology has been undertaken. In this regard thirteen bacterial isolates were isolated from different crude oil polluted soil 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 bio-detergent formulations. Similar to that reported for lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, and decaying food, compost heaps, coal tips, and hot springs. 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. 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 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 KSA; (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.

Needless to say, that, most of the enzyme purification schemes described in the literature focused on purifying small amounts of the enzyme to homogeneity to characterize it. Little information has been published on large-scale processes for commercial purification. Most commercial applications of enzymes do not require highly pure enzyme. Excessive purification is expensive and reduces over all recovery of the enzyme (Chisti, 1998).

In the present study, the purification procedure included preparation of cell free filtrate; applying precipitation technique, dialysis and then passing the enzyme preparation through Sephadex G-200 column chromatography techniques.

The obtained purified enzyme(s) were further investigated for some factors affecting their activities. Similar purification scheme was used by (Roushdy, 2001; Mahmoud, 2004).

Fractional precipitation of enzymes was carried out firstly by ammonium sulphate since it is highly soluble in water, cheap and has no deleterious effect on structure of protein, so for all these reasons, precipitation by ammonium sulphate was selected as a first step of purification program. Many investigators used ammonium sulphate precipitation processes.

In a trial to precipitate enzymes by ammonium sulphate, results revealed that, increasing the concentration of ammonium sulphate resulted in an increase in specific activity of lipase up to 100% saturation; a decrease in specific activity was recorded above this value. In the findings of other investigators, Lee *et al*., (2001) used 80% saturation ammonium sulphate for lipase purification from *B. thermooleovarans* ID-I and resulted in 27% yield increase.

On the other hand, Saxena *et al*., (2003) used, 85% ammonium sulphate fractionation for alkaline lipase purification. Sharma *et al*., (2002) used ammonium sulphate saturation for alkaline lipase purification by *Bacillus* sp. RSJ-1.

The most active protein preparations, after ammonium sulphate precipitation were dissolved in a least amount of buffer (pH 8) then it was dialyzed against distilled water to exclude sulphate ions then it was concentrated by dialysis against sucrose crystal to reach a minimum volume and this resulted in raising the purification fold for lipase enzyme many times from the origin. The dialyzed consequently applied to the Sephadex G-200 column chromatography. Gel filtration of the thermoalkalostable lipase Sephadex G-200 showed that, the enzyme activity was detected, infractions (5-10) where the highest activity 1184 U/mg^{-1} protein was recorded in fraction 8.

In view of lipase purification by many authors, the extracellular lipase by *B. stearothermophilus* MC7 was purified to 19.25-folds with 10.2% recovery and a specific activity of about 12 $U(mg\ protein)^{-1}$ (Kambourova *et al*., 2003).

Hiol *et al*., (2000) purified extracellular lipase by ammonium sulphate precipitation, sulfopropyl sepharose chromatography, Sephadex G-75 gel filtration and a second step sulfopropyl sepharose chromatography with 1200 folds.

A thermostable lipase produced by a thermophilic *Bacillus* sp. J33 was purified to 175-folds by ammonium sulphate and phenyl sepharose columnchromatography (Nawani and Kaur 2000). Lee *et al*., (2001) purified a lipase from *B. thermoleovorans* ID-A (BTIDA) and *B.thermoleovoran*-ID-B (BTIDB) with a purification fold of 300 and 108 respectively, while the overall yield was 16 and 3.2% respectively.

Amino acids analysis of the purified enzyme was carried out, data showed that, glutamic acid and aspartic acid gave the highest concentrations among the amino acid detected in case of *B. brevis* B_2 thermoalkalostable lipase in the findings of other investigators, (Mahmoud ,2004) reported that, amino acid analytical data of purified lipase produced by *B.licheniformis* B-42 revealed that, aspartic acid and therionine exhibited the highest concentrations. Similar aspartic acid gave the highest value for purified lipase from *Rh. delemar* (Hass *et al*., 1992).

While comparing the hydrophobic amino acid contents in the lipase from different *Bacillus* sp., the hydrophobic amino acid content was found to be quite high (60.2) for Lip2. High content of alanine (50%) in this enzyme might be playing a role in its thermostability as more alanine could lend more hydrophobic bonds to the lipase with increasing heat stability. Arg/(Arg + Lys) molar ratio for Lip2 (0.54) is higher than the standard value for thermophiles (0.48). Therefore, this study further suggests that even all the information necessary for protein thermostability is encoded by amino acids of proteins (cloned thermostable protein has same properties when expressed in mesophilic host), no single traffic rule for correlation of thermostability with particular amino acids could be generalized.

In order to assess the utility and compatibility of enzymes with the commonly used detergents, its properties such as pH and temperature stabilities…. etc. should be determined.

Results recorded in the present work showed that, temperature and pH optima for the purified lipase were 80°C and 9.5 respectively. This enzyme was thermally stable up to 80-100°C with maximal activity and stable over a wide pH in alkaline range with optimal activity at pH 9.5. Enzyme activity below and above these particular degrees was decreased. In accordance to the present results, Castro-ochoa *et al*., (2005) reported that, *Bacillus thermoleovorans* CCR11 lipase was found to be most active at a pH between 9 and 10 and stable in a broad range of pH values (5-11), retaining more than 80% of activity after 26 h at 30°C. Similar to that reported for lipases from *B. thermoleovorans* ID-1 (pH 9). Lee *et al*., (2001) and Bacillus A30-1 (pH 9.5) Wang *et al*., (1995) and higher to other reported lipases from thermophilic *Bacillus* which lie in the range of pH 7.2 – 8.5 (Schnidt-Dannert *et al*., 1994; Kim *et al*., 1995; Lee *et al*.,2001;Dharmsthiti and Luchai, 1999; Handelsman and Shoham, 1994).

At elevated temperature the enzymatic protein denaturation occurs and decreases the lipase activity. Thus, the elevated temperature decreases the lipase

activity. The relative activity remains almost stable at 80°C. The thermostablity of the lipase at high temperature indicates its industrial applications, because of unique nature of protein and its thermostable nature. Enzyme being protein is sensitive to changes in the environment in which the y work, affecting the activity of enzyme. Purified lipase from *Bacillusmethylotrophicus* PS3 was most stable at pH 7.0 and activity remains stable from pH 7 to 9, the activity start losing at above the 9 and at lower acidic range of pH indicating the neutral behavior of the purified lipase. Change in pH has a varied effect on the enzyme activity, as altering the structure of enzyme and substrate and inhibiting the catalysis of reaction.

Mahmoud (2004) found that, the optimal temperature and pH were 50-60°C and 9-9.5 respectively also the enzyme was found to be thermostable up to 60oC and it had a wide pH range (8-10.5) for its maximal activity at pH 9.5.One of the more notable thermostable lipases was isolated by Wang *et al*., (1995) from *Bacillus* strain. This enzyme had an activity maximum at 60°C and retained 100% of the original activity after being held at 75°C for 30 min. Lipase from Bacillus subtilis MTCC6824 was optimally active at pH ranging from 6 to 10 (Chakroborty and Raj,2008). The *Bacillussubtilis* PCSIR NL-39 lipase at active in pH range of 3.5 -9.0.

The activity of various enzymes is influenced by the presence of metal ions either by directly involving in their catalysis or by structural modifications. Thermal stability of lipase is obviously related to its configuration and subsequently, the melting point. Thermostability is influenced by environmental factors such as the presence of metal ions. At least in some cases, thermal denaturation appears to occur through intermediate states of unfolding of the polypeptide (Zhu *et al*., 2001) mutation in the 'lid' region of the enzyme can significantly affect heat stability.

Lee *et al*., (2001) purified two thermostable lipases (A&B) from *Bacillus thermoleovorans* ID-1 with an optimal temperature of 60-65°C and 60°C for lipase A and B respectively, while the pH optima were 9 and 8-9 respectively. Lipase A retained 75% of its activity when incubated for 30 min. at 60°C.

Attempt are being made to protein-concerning the properties of purified lipase enzyme, it seems that, the enzyme in the present study, concentration affects the enzyme activity. It appeared that, extent of catalytic action of purified enzyme is a true response of the enzyme concentration.

Concerning the incubation time, it appeared that, increasing incubation period resulted in a corresponding increase in enzyme activity up to 60 min. Two possible mechanisms of ion action were suggested by Lee and Rhee (1993); (1) direct inhibition of the catalytic site, like many other enzymes; (2) specific for lipases formation of complexes between metal ions and ionized fatty acids, changing their solubility and behavior at the interface.

Metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme. In the present study results suggested that Ca^{2+} and Mg^{2+} both stimulates were required for the stability of enzyme. Lee *et al*., (2001) isolated two lipases from *B. thermooleovorans* ID-1 lipase A was inhibited by divalent ions including Cu^{2+}, Hg^{2+} and Co^{2+}. In contrast lipase B was slightly activated by Ca^{2+}, Na^{+}, Co^{2+} and Mn^{2+} ions. EDTA treatment strongly inhibited both enzymes. *B. stearohermophilus* MC7 lipase was inhibited by divalent ions of heavy metals, entirely by Cu^{2+} and strongly by Fe^{2+} and Zn^{2+} (Kambourova *et al*., 2003).

Sharma *et al*., (2001) detergent may contain chlorine, which degrades proteins into smaller peptide chains, thereby lowering binding energies and affecting desertion from the surface (Singh *et al*., 1999). Hence enzymes used as cleaning agents should be stable in the presence of chlorine. In the present study, data showed that, lipase activity not stable with chlorine at all applied concentrations and time intervals. In the findings of other investigators, chlorine stimulate lipase activity where it enhanced up to 149.6% of its activity with 10 µl chlorine /ml for 60 min.

Substances that reduce the activity of an enzyme catalyzed reaction are known as inhibitors. They act by either directly or indirectly influencing the catalyzed are known as inhibitors. They act by either directly or indirectly influencing the catalytic properties of the active site. Inhibitors can foreign to the cell of natural components of it. In the latter instance, they can represent an important element of the regulation of cell metabolism. It was confirmed that the higher lipase yields obtained with these additives were not due to increased lipase transcription, but to enhanced secretion.

In a trial to investigate the effect of oxidizing agent and surfactant on lipase activity, it was found that, lipase activity not only stable but also enhanced by H_2O_2 at all applied concentration Triton X-100 (10%), sodium deoxycholate (7-10%), Tween 20 (7-10%) and SDS at all applied concentrations. The previous resistance which is essential requirements, suggest that, the enzyme may be used as an effective additive in detergents.

The partially purified lipase preparation had an optimal activity temperature of 60°C and the optimum pH was 9.5. This enzyme was stable to both hydrogen peroxide and alkaline protease (Wang *et al*., 1995). Lin *et al*., (1996) reported an extracellular alkaline lipase produced by *P.alcaligenes* F-111 in a medium that contained soybean meal (1%), peptone (1.5%) and yeast extract (0.5%). The lipase produced was unaffected by various detergents. The cationic surface-active agents such as SDS, sodium tripolyphosphate, sodium dodecyl benzene sulfonate and sodium alkyl benzene sulfonate did not affect the enzyme activity, suggesting that this enzyme is a good candidate for detergent applications. Because of their ability to

hydrolyze fats, lipases find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10-11, 30-60^{o}C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations.

Regarding computability with commercial detergent, lipase activity not only stable but also enhanced with various commercial detergent applied in this study. In view of other findings, Sharma *et al*., (2002) found that, an alkaline lipase from thermophilic *Bacillus* sp. RSJ-1 was highly stable in the presence of some commercial detergent formulations, whereas the presence of various oxidizing agent, reducing agents and surfactants, reduce the enzyme activity.

Limited information is available about the removal of stains by application of the thermostable crude and purified enzymes (separately or in combinations) with or without commercial detergent. The wash performing analysis of the present enzyme revealed that, it could effectively to remove a variety of stains such as blood, chocolate, mango, tomato sauce and oil, while treated at 30^{o}C for 15 min. by adding thermostable crude/purified enzymes separately or in combination with or without detergent (Fol) as an chemical product.

Noteworthy was the effective removal of blood stain at 80^{o}C when treated with crude lipase only as well as crude lipase and/or purified lipase with commercial detergent (Fol). Also, removal of chocolate stain at 80^{o}C by treating with crude lipase with commercial detergent (Fol) and also removal of mango stain at 80^{o}C by treating with commercial detergent (Fol) only, purified lipase only, crude and purified lipase with commercial detergent (Fol). Also, removal of tomato sauce stain at 80^{o}C by treating with purified lipase with commercial detergent (Fol), also removal of oil stain at 80^{o}C by treating with crude lipase only, crude lipase and purified lipase with commercial detergent. The previously mentioned treatments not only resulted in complete removal of stains but also remove the impurities and non-cellulosic materials holded in cloth samples, indicated by whiteness degree, comparing with unblotched control.

The best removal of most stains was observed by adding the combination of both thermostable enzymes and detergent to work together. Hence, the supplementation of the enzyme preparations to Rabso detergent could significantly improve the cleaning performance towards many stains. Thus, the present thermoalkalostable lipase offers applicable value in detergent industries.

CONCLUSION

Athermoalkalostable lipase produced by *B. stearothermophilus*- KKSA12 was investigated in this investigation. Of particular note, the lipase was observed to be active at wide range of temperature and pH and displayed activity in presence of organic solvent, metal ions and detergents. By virtue of all these properties, this thermo-alkali-tolerant lipase can be explored for its industrial applications.

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