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The Possibility to Use Bacterial Protease and Lipase as Biodetergent

[1]Amro A. Amara, [2]Soheir R. Salem and [3]Mohamed S.A. Shabeb

[1]Department of Protein Research, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt
[2]Department of Biological and Geological Science, Faculty of Education, Alexandria University, Alexandria, Egypt
[3]Department of Botany, Faculty of Science at Aswan, South Valley University, Aswan, Egypt

Abstract: Detergents are an undetectable source of pollution, which hidden in most of our daily activities. Detergents could cause harmful effects before they are completely degraded. It is wise to reduce the amount of detergents that usually used by invention new bio-friendly formula contains efficient enzymes such as lipase and protease. These two enzymes could be replaced detergents or their gradients in the detergents different formulas. Crude lipase and protease from two Bacillus strains are represent *Bacillus licheniformis* and *Geobacillus sp*. show the ability to produce thermophilic protease and lipase investigated in this study. The optimum protease activity at pH 9 was 34 Unit/ml at 70°C for *Geobacillus sp.* and 46 Unit/ml at 60°C for *Bacillus licheniformis*. The apparent lipase activity for *Geobacillus sp.* was 30.4 Unit/ml and 25.86 Unit/ml for *Bacillus licheniformis*. Lipase or protease that produced from these two Bacillus strains are tested on artificial fat and protein dirt clothes in presence and absence of commercial powder detergent to investigate their cleaning effect. The enzyme activity of each has been determined and the result proved the possibility to use the crude enzymes alone or in combination with the powder detergent in washing purposes. This study opens the way to formulate chemical free detergent.

Key words: Protease · Lipase · Powder detergent · Biodetergent

INTRODUCTION

Enzymes play a significant role in our life. Their existence had been associated with the history of ancient civilizations. Enzymes from plant and microorganisms had been used in brewing, baking, alcohol production, cheese, vinegar making etc. The uses of enzymes were variable ranging from just making wine or bread to producing complicated fermentations processes. Technical enzymes represent 1 billion USD in 1999 [1]. Part of these enzymes is the thermostable enzymes, which are better, suited for harsh industrial processes and constitute more than 65% of the global market [2-3].

Enzymes have many applications especially in paper industry, detergents, drugs, degradation of different wastes, textile, food, pharmaceutical, leather, degumming of silk goods, manufacture of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters etc [1, 4]. Meanwhile one of the most important and profitable applications for enzymes is in detergents, where the total global market size was ~ 0.6 billion USD in 2000 (Novozymes data) [5]. The first use of enzymes in detergents occurred in 1913 when Röhm and Haas introduced crude trypsin into their detergent. Burnus based on a German patent issued to Otto Röhm (1913) [1]. To provide desirable benefits, enzymes must be stable and function well in the presence of a variety of potentially unfriendly detergent ingredients (e.g., anionic/ nonionic/cationic surfactants, chelants, builders, polymers, bleaches) and in various forms of detergent products (i.e., liquids and powders) [1].

Thermostable enzymes are active and stable at temperature higher than optimal growth of their producer strains [6,7]. Relative ease of isolation of Bacilli from diverse sources has made these organisms the focus of attention in biotechnology [8]. Thermostable enzymes can be produced by both of thermophilic and mesophilic microbes [9]. Some reports are describing Psycrophiles as

Corresponding Author: Dr. Amro A. Amara, Mubarak City for Scientific Research and Technology Applications, Universities and Research Center District, New Borg Al-Arab, P.O., Box, 21934, Egypt

a source of some stable enzymes but using thermophils reduce the risk of contamination by common mesophiles. The use of high temperature has many significant applications due to solubility and reducing viscosity [10-11]. Microbial enzymes are involved in many applications. The enzymes production processes from microbes are in general more stable, convenient and safe than plants and animals enzymes [12].

Detergent is a general term for compounds that used for cleaning and removing wastes. The enzymes are used with detergent features must be suitable for the aim of their use. In general, enzymes have to be stable during their storage. Based on their catalytic mechanisms proteases can be classified into Ser, Cys, Asp and metalloproteases [13]. In nature, proteases have valuables biochemical and physiological functions. They are efficient specific by not only cleaving proteins into amino acids or short peptides but also cleaving them specifically to produce useful peptides. Thermostable enzymes are better suited for harsh industrial processes [14-16] and constitute more than 65% of the global market [17].

The researches on production of proteases are huge. Some scientific points have been need to be covered like those concerning with the overproduction of proteases by media optimization [17]. In general, synthetic media provides better control and allow the best conditions to understanding the relationships between nutrient and enzyme production [18-25]. Lipases are considered as the third largest industrial enzymes after proteases and carbohyderases with market represent about billion dollars/years [26].

Microbes, which are able to produce lipases, are observed as early as in 1901 from *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens* which are nowadays known as *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*,respectively [23]. Lipases can be found in bacteria, fungi and yeasts but the most famous organisms are Bacillus, which are reported to be the main source of lipases [24-26]. Lipases are characterized by their ability to hydrolyze long chain triglycerides [27, 28]. The specificity of lipases causes tremendous interest among scientist and industrialists specially those dealing with the production of new chemical compounds from fats and oil degradation [29].

This study is concerned with the characterization of the ability of protease and lipase produced by thermophilic Bacillus strains to be able to use as biodetergent.

MATERIALS AND METHODS

Bacillus Strains and Growth Conditions: Out of eight thermophilic Bacillus strains, two were able to grow on skim milk, cottonseed oil and olive oil as the only carbon and nitrogen source at temperature higher than 50°C. The two strains were identified by standard criteria as *Bacillus licheniformis* and *Geobacillus sp*. They grow routinely in LB medium (Luria-Bertani) [30] at 50°C and maintained at -70°C by adding 300 µl glycerol to each 1 ml culture in suitable plastic container.

Detection of the Proteolytic Activity on Plates: The different Bacillus strains screened for their proteolytic activity using agar plates containing skim milk as substrate, where 3 gm skim milk suspended in 100 ml water and autoclaved for 15 min. After sterilization, the suspension allowed to be decanted and the soluble solution is added carefully to sterile water agar (16 gm agar/l) to avoid the disturbance of any coagulant. The suspension then stirred gently until complete homogenization and distributed in Petri dishes (25 ml/plate). One loop of each pure Bacillus strain strikes on the surface of the solid media and incubated overnight at 50°C. The clear zones around the growth of the Bacillus strains indicate the presence of proteolytic activity. This can be confirmed either by naked eye or after visualization using coomassie blue staining method.

Visualization of the Protease Clear Zone: Coomassie blue (0.25%, w/v) was dissolved in methanol-acetic acid-water 5:1:4 (v/v/v) [31]. 10 ml from the staining solution were added to each plate and incubated at room temperature for 15 min followed by removing the staining solution from the plate's surface and washing gently by distilled water. The plates de-stained using destining solution (66 ml methanol, 20 ml acetic acid and 114 ml H_2O_{bidest}) [31] for a suitable time [till appearing clear contrast between the plates background and the degradation zone around the bacterial growth].

Preparation of L-tyrosine Standard Curve: 1.1 mM L-tyrosine was dissolved in 100 ml deionized water by heating gently (without boiling). After complete dissolution of the L-tyrosine the standard curve generated by reading the absorbency for 0, 12.5, 25, 50, 100, 200, 250 and 500 µl from L-tyrosine solution which completed to 1 ml by adding deionized water at 280 nm using the spectrophotometer (PerkinElmer-UV/VIS

Spectrometer Lambda). The relationship between the absorbency and the mM L-tyrosine then plotted as y/x line plot.

Preparation of Casein-Universal Buffer for Different pH Enzyme Activity: Universal buffer was prepared according to Britton and Robinson [32] which consists of 40 mM H_3PO_4, 40 mM acetic acid and 40 mM H_3BO_3. The different pHs (5, 6, 7, 8 & 9) were adjusted by adding different volumes of 0.2 M NaOH. 0.325 mg casein are weighted and dissolved in 50 ml of Universal buffer at these different 5 pHs. The mixture is dissolved by heating gently to 80-90°C without boiling. The mixture is used immediately or is incubated for short time at 4°C [not longer than 12 hr].

***In vitro* Protease Activity:** 300 µl of each supernatant, which contains the crude enzyme, were added to the same volume of the Casein-Universal buffer at the different pH solutions (pHs 5, 6, 7, 8 and 9). The enzymes-substrate mixture for each pH is incubated at 50, 60, 70 and 80°C for 30 min. The reaction stopped by adding 600 µl of 110% trichloroacetic acid. The mixture were allowed to stay at room temperature for 15 min then centrifuged at 10000 rpm for 10 min (Biofuge 15 - Heraeus Sepatech). The absorbance of each sample was determined spectrophotometrically at 280 nm (PerkinElmer-UV/VIS Spectrometer Lambda), their tyrosine content was derived from the tyrosine standard curve and each enzyme activity was determined as Unit/ml.

Enzyme Stability: The enzyme stability was determined by incubating supernatants, which contain crude enzymes at time intervals represent 30, 90, 120, 180, 210 min and 24 hr. The incubation has been performed at two temperatures represent 37 and 70°C. After each time interval, 300 µl of the incubated enzymes added to 300 µl from the 1.1 mM Casein-Universal buffer solution (pH 9) and further incubated at 37 and 70°C. After 30 min, the reaction stopped by adding 600 µl 10% TCA followed by incubation on ice for 15 min. The mixture is then centrifuged at 10000 rpm for 15 min (Biofuge 15- Heraeus Sepatech). The absorbency of each sample was detected spectrophotometrically at 280 nm and the different enzyme activities were determined as Unit/ml.

Preparation of Lipase Chromogenic Plates: A modified method from Singh *et al.* [33], was used where Chromogenic substrate plates were prepared by using phenol red (0.01%) along with 1% olive oil as substrate, 2 % Arabic gum, 10 mM $CaCl_2$ and 2% agar. The pH was adjusted to 7.3–7.4 by using 0.1 N NaOH, where 2% olive oil, 4% Arabic gum and 20 mM $CaCl_2$ were added to water (pH 7.3) and mixed using suitable mixture till complete homogenization was occurred then the mixture was added to the same volume of 4% melted agar (50°C). The phenol red was added in final concentration 0.01% to the mixture to give orange-reddish color. After mixing well, 25 ml were distributed in each agar plate. After complete solidification of the agar on plates, wells were punched out of the agar, by using a clean sterile cork borer (8 mm in diameter). The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H_2O) using sterile Pasteur pipette.

Detection of Lipase Activity on Plates: Fifty µl of the supernatant expected to contain lipases were impregnated to each well while sterile media (without any growth) was served as control. The plates were incubated at 37°C for 30 min [33]. The changes in the color around the wells indicate the presence of lipase activity.

Lipase Assays: Extracellular lipase was assayed according to the method adapted in this study. The substrate was prepared by adding 200 µl Triton-X-100 to 40 mg *p*-nitrophenyl palmitate dissolved in 10 ml DMSO. The crude enzyme activity determined by adding 500 µl from the substrate mixture to 500 µl from 50 mM Tris HCl, pH 8.0 and the enzyme reaction was started by adding 500 µl from the supernatant which contain the crude enzyme. The buffer and the supernatant each kept warm at 30°C during the experiment. The extracellular lipase activity was determined by the rate of *p*-nitrophenol production (*p*NP) which measured at 405 nm spectrophotometerically (PerkinElmer-UV/VIS Spectrometer Lambda). The increase in absorbance against times was measured against different time interval [Until constant absorbance]. The extinction coefficient under the conditions described was 14500 L mol / cm. Lipolytic activity was determined, using substrate free blanks as control. One unit (U) was defined as the amount of enzyme catalyzing the liberation of one *p*mol *p*-nitrophenol/min at 30°C under the given condition [34].

Determination of Washing Properties of Crude Enzymes: Artificial dirt represented in egg yolk and fat on white cotton tissue were used, where the weight of the tissue with or without each type of dirt were determined,

[The dirt is dried at 38°C for 12 hr]. The washing properties of crude enzymes were determined in presence or absence of detergent. 2 mg/ml of the detergent (contain 20% anionic surfactant and 15% phosphate) was dissolved in falcon tubes containing 50 ml tap water. 500 µl of the crude supernatant were added to each tube. Four different types of treatments were used: the first contained a tap water and 500 µl of the crud enzymes; the second contained tap water and 2 mg/ml detergent; the third contained 500 µl enzyme and 2 mg/ml detergent; the last contained only tap water and used as blank. One tissue of the white cloth which contain the artificial waste was added to each tube and allowed to be shacked in shaker incubators at 100 rpm (Innova 4230 – New Bruaswick Scientific) at 50°C for 50 min [38°C in case of fat]. After the process is completed, each tissue was dried overnight at 40°C and reweighted. The weight of each treatment, the control (without enzyme) and the blank (without enzymes and detergent) were determined. The lost in the weight due to each treatment has been calculated. The different changes in cleaning percentages regarding to the enzymes different activities without or with detergent were calculated.

The improvement in the dirt-removing percentage after mixing each crude enzyme with detergent was calculated using the following formula:

Improvement %= [removing % of enzyme-detergent mixture]-[removing% of detergent alone+removing% of crude enzyme alone]

RESULTS

Detection of the Thermophilic Proteolytic Activity on Plates: The thermophilic proteolytic activity of each strain was determined on skim milk agar plate. The clear zones around the bacterial growth appear clearly after visualization using coomassie blue staining method as in Figure 1. This technique gives preliminary fast idea about the ability of different Bacillus strains to produce thermophilic proteases. The proteolytic activities of the enzymes appear as a clear zoon around the bacterial growth, which can be used also to compare different samples.

Optimization of pH and Temperature of Proteases: The enzymes activities were optimized at different pHs (5, 6, 7, 8 and 9) and four temperatures (50, 60, 70, and 80°C). The results showed that the enzymes were thermostable and have activities in all cases (in different pHs and temperatures) as in Figures 2-5. The optimum activity at pH9 was 34 Unit/ml at 70°C for *Geobacillus sp.* and 46 Unit/ml at 60°C for *B. licheniformis* as in Figure 3 and 4.

Enzyme Stability: The optimum activity of protease for *Geobacillis sp.* and *Bacillus licheniformis* that recorded at pH 9, which prove the alkaline properties of these enzymes was used for study their stability. Moreover, at pH 9, the enzyme stability at 37 and 70°C proved that

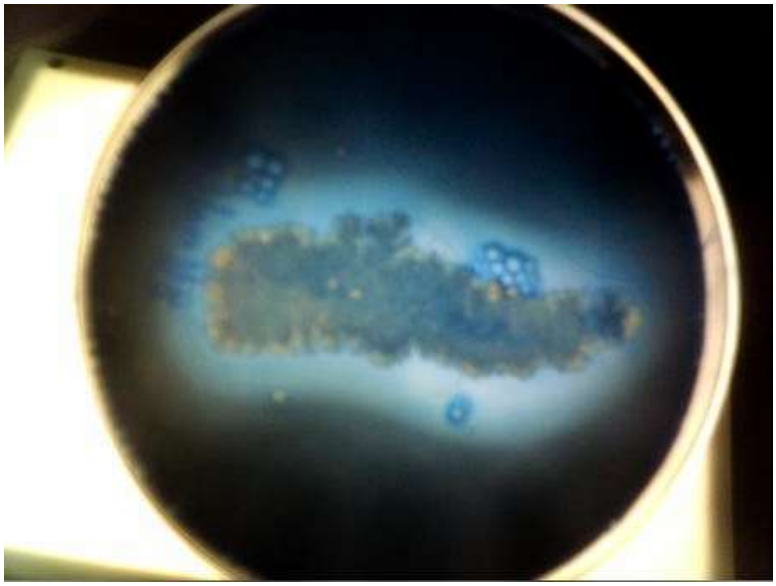

Fig. 1: Bacillus strain grown on skim milk plat and stained with coomassie blue; the clear zone indicate the presence of proteolytic activity

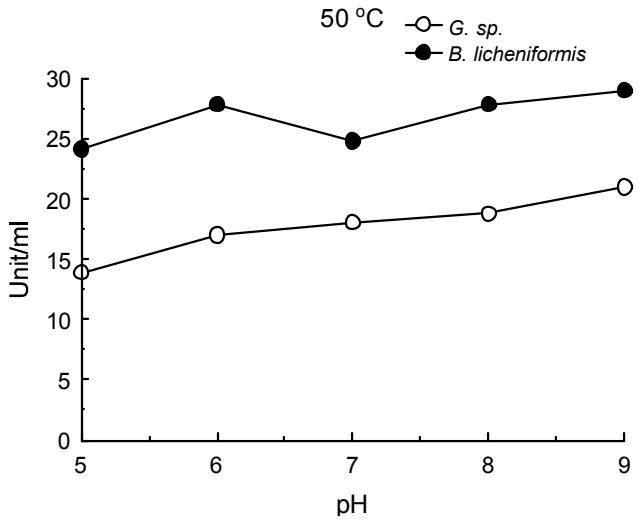

Fig. 2: Proteolytic enzyme activity of *B. licheniformis* and *Geobacillus sp*. calculated as Units/ml at 50°C at different pHs (from 5-9)

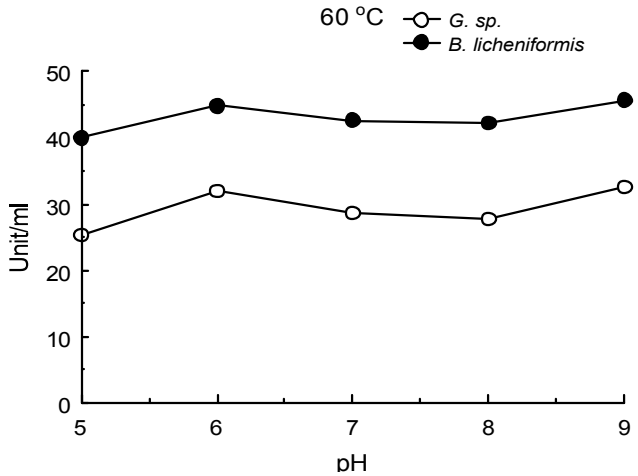

Fig. 3: Proteolytic enzyme activity of *B. licheniformis* and *Geobacillus sp*. calculated as Units/ml at 60°C at different pHs (from 5-9)

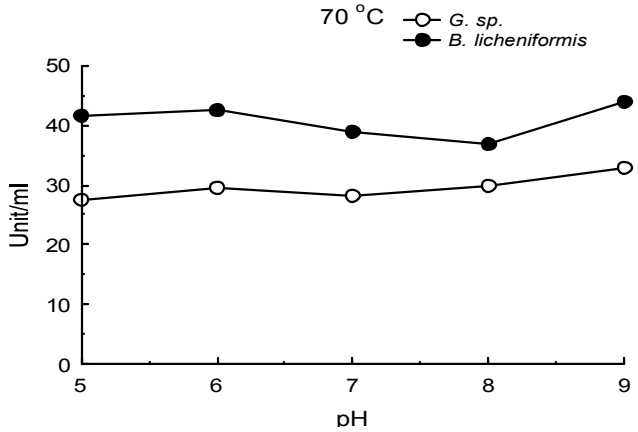

Fig. 4: Proteolytic enzyme activity of *B. licheniformis* and *Geobacillus sp*. calculated as Units/ml at 70°C at different pHs (from 5-9)

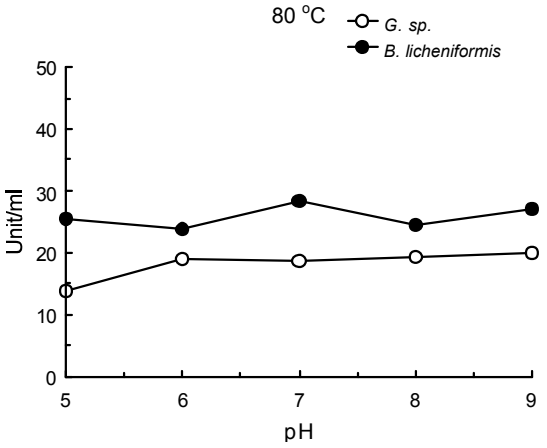

Fig. 5: Proteolytic enzyme activity of *B. licheniformis* and *Geobacillus sp*. calculated as Units/ml at 80°C at different pHs (from 5-9)

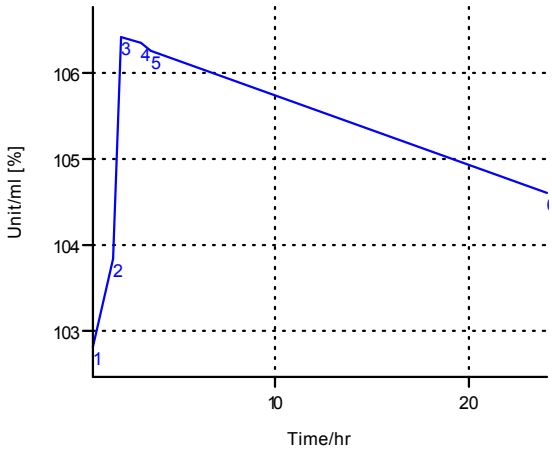

Fig. 6: Enzyme stability of *Geobacillus sp*. crud protease calculated as Units/ml at pH 9 during 24 hr incubation at 70°C.

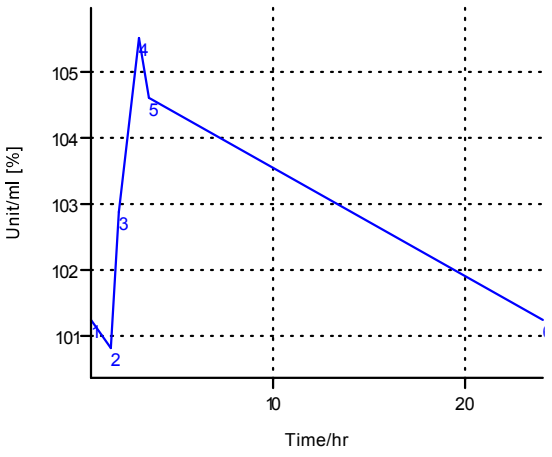

Fig. 7: Enzyme stability of *B. licheniformis* crud protease calculated as Units/ml at pH 9 during 24 hr incubation at 70°C.

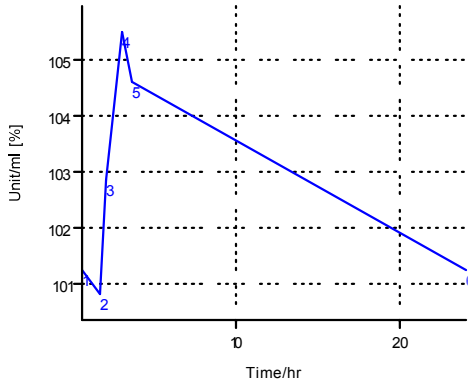

Fig. 8: Enzyme stability of *Geobacillus sp*. crud protease calculated as Units/ml at pH 9 during 24 hr incubation at 37°C.

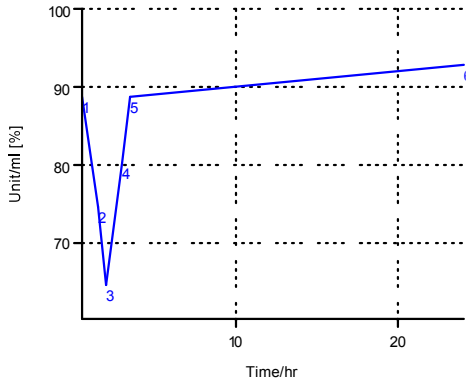

Fig. 9: Enzyme stability of *B. licheniformis* crud protease calculated as Units/ml at pH 9 during 24 hr incubation at 37°C.

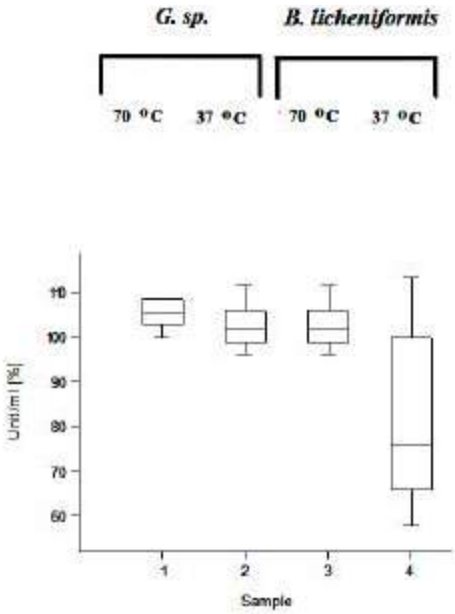

Fig. 10: Box-Plot showing *Geobacillus sp.,* and *B. licheniformis* proteolytic stability at 37 and 70°C; the residual activity calculated as Unit/ml [%]

Fig. 11: Phenol-red plate: the clear zone indicate the presence of lipolytic activity

Table 1: Different percentage of dirt lost (% of cleaning) result from the washing experiment using protease and lipase alone or in combination with detergent

	% of the lost in dirt weight#			
Treatment	Protein waste			Fat waste
Detergent only	25			17
	Enzymes alone			
	Proteases			Lipases
Geobacillus sp. protease	10.2			8
Bacillus licheniformis protease	36			14.5
	Enzymes + Detergent			
	% of cleaning	% of + change*	% of cleaning	% of + change*
Detergent + *Geobacillus sp*. protease	39.6	4.4		
Detergent + *Bacillus licheniformis* protease	72.7	11.7		
Detergent + *Geobacillus sp*. lipase			58	33
Detergent + *Bacillus licheniformis* lipase			21	- 10.5

* The change based on the total + improvement duo to mixing detergent with enzymes

The dirt were ? 200 mg/tissue

Bacillus protease was stable. Slight decrease in the enzymes stability was shown by increasing time in all cases as in Figures 6, 7 and 8; except *B. licheniformis* at 37°C, where the activity showing decrease in the first 3 hr followed by increase as in Figure 9. For comparison between the performance of proteases of both strains at 37°C and 70°C, the activity plotted using Box plot as in Figure 10 which showing that enzymes in all conditions were stable in spite of *B. licheniformis* protease at 37°C that shows variable activity against time.

Detection of the Extracellular Lipase Activity on Plates: The supernatants, which contain activities, give a clear zone on olive oil-phenol red plates, which was an indicator about the degradation of fatty acids as shown in Figure 11. The methods was simple, fast, cheap and give a preliminarily indication about the presence of extracellular lipase activity. Both of *Geobacillus sp.* and *B. licheniformis* were revealed positive lipase activity on the olive-oil-phenol red plates as shown in Figure 11.

***In vitro* Lipase Activity:** The increase in the absorbance at 405 nm which was a result of liberation of *p*NP was determined spectrophotometrically (PerkinElmer-UV/VIS Spectrometer Lambda) and the Unit/ml activity of each treatment was calculated. The calculated lipase activity was 30.4 Unit/ml for *Geobacillus sp.* and 25.86 Unit/ml for *B. licheniformis*.

Enzymes and Enzymes-Detergent Washing Experiment: Using the detergent alone results in removing 25% of yolk dirt and 17% of fat dirt in conditions as described above.

For possible commercial applications, the protease and lipase were examined with and without detergent. The *Geobacillus sp*. and *B. licheniformis* protease were showed an ability to remove 10.2% and 36% of the egg yolk dirt respectively in one hr incubation period at 50°C alone without detergent. On the other hand, *Geobacillus sp*. and *B. licheniformis* lipase were showed an ability to remove 8% and 14.5% of fat dirt respectively after one hr incubation period at 38°C alone without detergent. By mixing both detergents and enzymes, the *Geobacillus sp*. protease was showed 4.4% improvement, while *B. licheniformis* protease showed 11.7% improvement. Moreover, *Geobacillus sp*. lipase was showed an improvement of 33%, while *B. licheniformis* lipase was showed a reduction with -10.5% when mixed with powder detergent. The experiments data are summarized and represented as dirt removing percentage in Table 1.

DISCUSSION

Enzymes were used widely in our life in many applications including powder detergents [1, 13-15]. It is seldom nowadays to found market did not seal any kind of detergent products. Their wide applications cause accumulation for their chemical components. Detergents were considered as a hidden constant source of pollutions.

It was not enough to depend on the fact that microbes could degrade detergents in various polluted areas. Detergents could cause harmful effects before they were completely degraded. It was wise to reduce the amount of detergents, which we were used by invention new bio-friendly formula contains efficient enzymes such as lipase and protease enzymes. Lipase and protease could be replaced detergents or their gradients in the detergents different formulas.

Researcher focused on the possibility of mixing protease and lipase especially thermophilic ones with detergent based on their unique properties rather than substituting the chemical detergents completely with biological based formula [1]. This study highlights the possibility to use lipase and protease alone as biodetergent and to improve detergents. More studies should be done to optimize efficient biodetergent formula. Biodetergents could be used as a safe alternative to chemical detergent. Chemical free detergents can be used in many important applications.

Crude lipase and protease from the two Bacillus strains that isolated from Egyptian ecosystem and identified by standard criteria as *Geobacillus sp*. and *Bacillus licheniformis* were investigated for their ability to use in dirt cleaning purposes. Agar plates contain the skim milk as substrate was used preliminarily to detect the presence of protease, while olive oil or cottonseed oil-phenol red plates were used preliminarily to detect the presence of lipase. Both strains were showed the ability to degrade and grown on skim milk and crude cottonseed oil or olive oil in media containing only tap water in thermophilic conditions.

For production of lipase, cottonseed oil was used instead of olive oil based on the different in their costs. The enzymatic activity of both of lipase and protease were studied. pH and temperature as two efficient factors in washing processes were optimized. The activity of crude enzymes determined as Unit/ml.

The optimum protease activity at pH 9 was 34 Unit/ml for *Geobacillus sp.* at 70°C and 46 Unit/ml for *B. licheniformis* at 60°C. Moreover, the enzyme was showed a significant activity in all treatments, the Box-plot proves that protease for both *Geobacillus sp.* and *B. licheniformis* were stable at 70°C than at 37°C, which prove the thermostability properties of this enzyme.

The apparent lipase activity was 30.4 Unit/ml for *Geobacillus sp.* and 25.86 Unit/ml for *B. licheniformis*, which indicate suitable rang of activity and confirmed by their ability to clean dirt.

Simple washing method was conducted to evaluate the ability of both enzymes to clean artificial fat and protein dirt. The enzymes analyzed for their ability to degrade of both fat and proteins in presence or absence of powder detergent. Comparison study on the activities of the enzymes performed in presence or absence of detergent, which result in mapping their mode of action. In case of protease, there was slight improvement in the washing behavior in presence of detergent, which proves that protease from both strains able to function alone or in combination with detergent.

B. licheniformis lipase lost about 10.5% of its activity after mixing with powder detergent. A significant increase of *Geobacillus sp.* lipase has been appeared when mixing it with powder detergent, where it was increased with 33%, which might be due to the alkaline behavior of the enzyme or due to other factors. A result should be investigated in future study. In general, the protease and lipase that were investigated in this study proved to be able to work with and to substitute powder detergent.

Practically, there were many applications require the use of enzymes as the main active ingredients in chemical free formula like the presence of allergy from detergent or in some medicinal applications, where there were needed for removing dirt or chemical based detergents can affect wastes from instruments made of material. From all the above data, it was clear that protease and lipase could play crucial roles in substituting chemical detergent. We recommended for using thermophilic lipase and protease alone without detergent in cleaning dirt and wastes and to substitute powder detergent.

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REFERENCES

1. Schäfer, T., O. Kirk, T.V. Borchert, C.C. Fuglsang, S. Pedersen, S. Salmon, H.S. Olsen, R. Deinhammer and H. Lund, 2005. Enzymes for Technical Applications. In: Biopolymers,eds Fahnestock, S.R. and A. Steinbüchel, Chapter 13, pp: 377-437, Wiley VCH Editor.
2. Leuschner, C. and G. Antranikan, 1995. Heat stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J. Microbiol. Biotechnol., 11: 95-114.
3. Rao, M., A. Tankasale, M. Ghatge and V. Desphande, 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev., 62: 597-634.
4. Cowan, D., 1996. Industrial enzyme technology. Trends Biotechnol., 14(6): 177-178.
5. Fan, Z., Q. Zhu and J. Dai, 2001. Enzymatic treatment of wool. J. Dong Hua University (English edition), 18: 112-115.
6. Mozersky, S., W. Marmer and A.O. Dale, 2002. Vigorous proteolysis: Relining in the presence of an alkaline protease and bating (Post- Liming) with an extremophile protease. JALCA 97: 150-155.
7. Saboto, D., R. Nucci, M. Rossi, I. Gryczynski, Z. Gryczyniski and J. Lakowicz, 1999. The b-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: enzyme activity and conformational dynamics at temperatures above 100 °C. Biophys. Chem., 81: 23-31.
8. Johnevelsy, B. and G. Naik, 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus sp.* JB-99 in a chemically defined medium. Proc. Biochem., 37: 139-144.
9. Lee, J.K., Y.O. Kim, H.K. Kim, Y.S. Park and T.K. Oh, 1996. Purification and characterization of a thermostable alkaline protease from Thermoactinomyces sp. E79 and the DNA sequence of the encoding gene. Biosci. Biotechnol. Biochem., 60: 840-6.
10. Mozhaev, V., 1993. Mechanism-based strategies for protein thermostabilization. Trends Biotechnol., 11: 88-95.
11. Krahe, M., G. Antranikian and H. Markel, 1996. Fermentation of extremophilic microorganisms. FEMS Microbiol. Rev., 18: 271-285.
12. Haki, D., G. Antranikan and S.K. Rakshit, 2003. Developments in industrially important thermostable enzymes: a review Bioresource Technology, 89: 17-34.
13. Saboto, D., R. Nucci, M. Rossi, I. Gryczynski, Z. Gryczyniski and J. Lakowicz, 1999. The b-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: enzyme activity and conformational dynamics at temperatures above 100°C. Biophys. Chem., 81: 23-31.
14. Leuschner, C. and G. Antranikan, 1995. Heat stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J. Microbiol. Biotechnol., 11: 95-114.
15. Fredrich, A. and G. Antrakian, 1996. Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilican aerobic species of the order Thermotogales. Appl. Environ. Microbiol., 62: 2875- 2882.
16. Zeikus, J., C. Vielle and A. Savachenko, 1998. Thermozymes: biotechnology and structure-function relationships. Extremophiles, 2: 179-183.
17. Salleh, A.B., M. Basri and C. Razak, 1977. The effect of temperature on the protease from *Bacillus stearothermophilus* strain F1. Mal. J. Biochem. Mol. Biol., 2: 37-41.

18. Zhang, J. and R. Greasham, 1999. Chemically defined media for commercial fermentations. Appl. Microbiol. Biotechnol., 51: 407- 421.
19. Mao, W., R. Pan and D. Freedman, 1992. High production of alkaline protease by *Bacillus licheniformis* in fed-batch fermentation using synthetic medium. J. Ind. Microbiol., 11: 1-6.
20. Ferrero, M., G. Castro, M. Abate, M. Baigori and F. Sinerz, 1996. Thermostable alkaline protease of *Bacilus licheniformis* MIR 29: isolation, production and characterization. Appl. Microbiol. Technol., 45: 327-332.
21. Johnevelsy, B. and G. Naik, 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus sp.* JB-99 in a chemically defined medium. Proc. Biochem., 37: 139-144.
22. Razak, C., M. Samad, M. Basri, W. Yunus, K. Ampon and A. Salleh, 1993. Thermostable extracellular protease by *B. stearothermophilus*. World J. Microbiol. Biotechnol., 10: 260-263.
23. Van Der Walle N., 1927. Über synthetische Wirkung bakterieller Lipasen. Cbl Bakt Parasitenk Infektionskr, 70: 369-373.
24. Wu, X., S. Jaaskelainen and Y. Linko, 1996. An investigation of crude lipase for hydrolysis, esterification and transesterification. Enzyme Microb. Technol., 19: 226-231.
25. Kim, H., M. Sung, M. Kim and T. Oh, 1994. Occurrence of thermostable lipase in thermophilic *Bacillus sp.* Strain 398. Biosci. Biotech. Biochem., 58: 961-962.
26. Jaeger, K.E., B.W. Dijkstra and M.T. Reetz, 1999. Bacterial biocatalysts: molecular biology, three-dimensional structures and biotechnological applications of lipases. Annu. Rev. Microbiol., 53: 315-351.
27. Schmidt, C., H. Sztajer, W. Stocklein, U. Menge and R. Schimid, 1994. Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatelnulatus*. Biochem. Biophys. Acta, 1214: 43-53.
28. Van Dyke M.I., H. Lee and J.T. Trevors, 1991. Applications of microbial surfactants. Biotechnol Adv, 9: 241-52.
29. Sih, C.J. and S.H. Wu, 1989. Resolution of enantiomers via biocatalysts. Topics Stereochem, 19: 63-125.
30. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular cloning: a laboratory manual, 2nd, edn,: Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. Weber, K. and M. Osborn, 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis, J. Biol. Chem., 244: 4406-4412.
32. Britton, H.T.S. and R.A. Robinson, 1931. Universal buffer solutions and the dissociation constant of veronal. J. Chem. Soc., pp: 1456-1462.
33. Singh, R., N. Gupta, V. Kumar and Geobacillus R. Gupta, 2006. A simple activity staining protocol for lipases and esterases. Appl. Microbiol. Biotechnol., 70: 679-682.
33. Sullivan, E.R., Leahy, J. Geobacillus and R.R. Colwell, 1999. Cloning and sequence analysis of the lipase and lipase chaperone- encoding genes from *Acinetobacter calcoaceticus* RAG-1 and redefinition of a proteobacterial lipase family and ananalogous lipase chaperone family. Gene, 230: 277-286.