

Biotechnological Application of Bacterial Alkaline Thermostable Enzymes in Bio-Detergent Industry

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THIS is an investigation concerned on the production of alkaline thermostable microbial enzymes for application in biodetergent technology. *Bacillus licheniformis*- B42 and *Geobacillus stearothermophilus* – B78 were selected and identified among one hundred and fifty-three thermophilic bacterial isolates with respect to their ability to produce α -amylases, cellulases, proteases and lipases grown on some agro-industrial wastes at 55°C and at pH 9 for application in biodetergent technology. Productivity of four alkaline thermostable enzymes by both selected strains using slaughter house wastes (SHW) as best substrate for proteases and lipases and potato peel (PP) as best substrate for α -amylases and cellulases. The enzymatic level more affected by incubation temperature, pH, SHW and PP concentrations, inoculum size, incubation period, carbon, nitrogen, metal inducer and vitamins sources, under shaking conditions. Four alkaline thermostable enzymes were produced under all optimal nutritional and environmental conditions and purification by column chromatography on Sephadex G200 and G100, respectively were performed. Purification of four produced alkaline thermostable enzymes steps resulted in raising the purification fold to 17.04, 15.24, 411.9 and 27.33 times in comparable with crude enzymes for α -amylase, cellulase, protease and lipase, respectively. The wash performing analysis of the four enzymes revealed that, it could effectively remove a variety of stains such as blood, apple, chocolate, mango, strawberry, salad and pomegranate by treatment at 55°C for 15 min, when alkaliphilic-thermostable crude/purified enzymes were added separately or in combination with or without detergent (Rabso) as an Egyptian local detergent product. The crude enzymes of these two bacterial strains proved to be potentially candidates for the application in the detergent technology.

Keywords: Microbial enzymes, Alkaline thermostable enzymes, *Geobacillus*, Biotechnological application, Biodetergent.

The largest application of industrial enzymes is as additives in detergents (Kawada, 1998). Many detergent brands contain combination of protease, lipase, cellulases and other degrading enzymes. Generally proteases and lipases are added to detergent to

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digest proteins and lipids from human sweat, foods and soils in cloth fibers. These detergent enzymes (serine proteases) were produced by the fermentation of *B. licheniformis*, *B. amyloliquefaciens* and/or *Bacillus* sp. Lipases facilitate the removal of fatty stains such as lipsticks, frying fats, butter, salad oil, sauces and stains on collars and cuffs (Priest, 2000). Cellulases remove microfibrils and amorphous celluloses, which trap dirt particles and cause a loss of surface smoothness. Treatment with cellulase enzymes removes the small fibers without apparently damaging the major fibers and restore the fabric to its "as new" condition by improving the colour brightness, enhancing the softness and removing particulate soiling. Amylases remove the residues of starchy foods (Beguin & Aubert, 2000). A combination of amylases, protease and lipase is an important component of detergents for automatic dishwashing machines. Currently, the detergent industry occupies about 25-30% of the entire industrial enzymes market. Amylases constitute 25% of the enzyme market (Burhan *et al.*, 2003; Konsula & Liakopoulou-Kyriakides, 2004 and 2006). A growing new area of application of α -amylases is in the fields of laundry and dishwashing detergents (Rodriguez *et al.*, 2006). Detergents with α -amylases optimally working at moderate temperatures and alkaline pH can help to solve this problem (Van der Marrel *et al.*, 2002). *B. licheniformis* and *B. amyloliquefaciens* amylases (currently used in detergent) are only partially active under these conditions. Engineering or characterizing new amylases that are optimally active at high pH should make amylases' use in detergent more efficient. Application of thermostable cellulases that are produced by thermophilic microorganisms appear to be ideal in the enhancement of the efficiency of laundry detergents (Jang & Chen, 2003; Grgorivski De-Lima, 2005; Ekperigin, 2007 and Gusakov *et al.*, 2007). Cellulases are increasingly used in household washing powders, since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabric surfaces and improve the appearance and colour brightness (Bhat, 2000). One of the important parameters for selection of detergent proteases is the pH value. It is known that detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pH value for the enzyme. However, there are many more parameters involved in the selection of a good detergent protease such as compatibility with detergent components, *e.g.* surfactants, perfumes and bleaches (Venugopal & Saramma, 2006), good activity at relevant washing pH and temperature (Oberoi *et al.*, 2001), compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Showell, 1999). There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases which are effective over a wide temperature range (Oberoi *et al.*, 2001). Detergent lipases are especially selected to meet the following requirements: (1) A low substrate specificity, *i.e.* an ability to hydrolyzes fats of various composition; (2) Ability to withstand relatively harsh washing conditions (pH 10-11, 30-60°C); Ability to withstand damaging surfactants and enzymes [*e.g.* linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergents formulations (Sharma *et al.*, 2001). Lipases with the desired properties are obtained through a combination of continuous screening (Cardenas *et al.*, 2001) and

proteins engineering (Kazlauskas & Bornscheuer, 1998) This study aims to application of the four bacterial alkaline thermostable enzymes in biodetergent industry as safe technology.

Materials and Methods

Wastes

Slaughter houses (SH) wastes

The slaughter houses wastes applied in this study were collected from different slaughter houses (SH) of El-Hawamdia, Giza, Egypt. The SHW were composed of small intestine, large intestine, some particles from stomach and blood forms. The samples were mixed together, kept at 0°C in a refrigerator and then added to the production media.

Potato peels wastes (PPW)

The potato peels wastes were collected from different restaurants in El-Hawamdia, Giza, Egypt. These wastes were washed for clay removal, dried in open air and then grounded for application in the production media.

Production media

The basal medium (BM) was prepared according to Vincent (1970). It contained the following (g/l): Sucrose, 10; KNO₃, 0.6; KH₂PO₄, 1; MgSO₄, 0.25 and CaCl₂, 0.1 per liter. This medium was found most convenient for the production of different enzymes. It was modified to include the following constituents (g/l) NaCl, 6; (NH₄)₂SO₄, 1; yeast extract, 1; KH₂PO₄, 0.5; MgSO₄.7H₂O, 0.1; CaCl₂, 0.1 and distilled water up to one liter.

Potato peels basal medium (PPBM)

It contained the same constituents of BM supplemented with potato peels (5%, w/v). After incubation period 10 ml of distilled water was added for amylases and cellulases extraction.

Slaughter house basal medium (SHBM)

It contained the same constituents of BM plus slaughter houses wastes (5% w/v).

Identification of the most potent thermophilic bacterial isolates

The two most potent bacterial isolates (B-42 and B-78) were identified as *Bacillus licheniformis* and *Geobacillus stearothermophilus* by examination of their morphological, physiological and biochemical characteristics according to Schallmeyer *et al.* (2004), Collee *et al.* (1996) and Hensyl (1994).

Purification of four hydrolytic thermostable microbial enzymes

The following steps were performed during the course of production and purification of amylases and cellulases produced from potato peels wastes and proteases and lipases produced from SHW.

Amylases and cellulases production by G. stearothermophilus strain -B78

G.stearothermophilus-B78 was allowed to grow optimally under the natural static substrate- solid state fermentation conditions on potato peels for amylases and

cellulases production. In case of amylase production 1g of potato peels per flask of 1000 ml capacity was used and supplemented with 10 ml of the production medium, which contained of (g/l, w/v): NaCl, 6; K₂HPO₄, 0.5; MgSO₄.7H₂O, 0.1; CaCl₂, 0.1; yeast extract, 1; in addition to ammonium molybdate and L-histidine, pH was adjusted at 9, and inoculated with 2.5 ml of bacterial suspension, incubated for 72 hr at 55°C. While in case of cellulase production, 1.5 g of potato peels per flask of 500 ml capacity was used and supplemented with 10 ml of the production medium, which contained (g/l, w/v): NaCl, 6; KH₂PO₄, 0.5; MgSO₄.7H₂O, 0.1; CaCl₂, 0.1; yeast extract, 1 in addition to starch, DL-aspartic acid and ammonium sulphate, pH was adjusted at 9, inoculated with 2 ml bacterial suspension per flask and incubated at 55°C for 72 hr.

Proteases and lipases production by B. licheniformis strain B-42

Strain B-42 was allowed to grow optimally under the optimal semi solid fermentation conditions on slaughter house wastes for proteases and lipases production. In case of proteases production, 1.5 g of slaughter houses wastes per flask of 1000 ml capacity was used and supplemented with 20 ml of production medium contained the following ingredients(g/l, w/v): NaCl, 6; KH₂PO₄, 0.5; MgSO₄.7H₂O, 0.1; CaCl₂, 0.1; yeast extract, 1 in addition to Galactose, ammonium dihydrogen phosphate, EDTA (500ppm), thiamine and arginine, pH was adjusted at 9; inoculated with 0.5 ml of bacterial suspension and incubated at 55°C for 72 hr. While in case of lipases production, 1.5 g of slaughter house wastes per flask of 100 ml capacity was used, supplemented with 20 ml of production medium contained (g/l, w/v): NaCl, 6; KH₂PO₄, 0.5; MgSO₄.7H₂O, 0.1; CaCl₂, 0.1; yeast extract, 1.0 in addition to fructose, ammonium sulphate, EDTA(25 ppm), thiamin, pH was adjusted at 9, inoculated with 1.5 ml of bacterial suspension and incubated at 55°C for 72 hr.

Preparation of cell-free filtrate

At the end of incubation period, 10 ml of distilled water was added to each flask in case of potato peels, while in case of slaughter house wastes no water was added and then the media filtered through a gauze piece to remove solids. The bacterial growth was harvested by centrifugation at 5.000 rpm for 15 min and then was filtrated, to obtain the cell-free filtrate that was preserved in the refrigerator as a crude enzyme. This step was carried out in case of the four enzymes cell-free filtrate.

Ammonium sulphate fractionation

The chart of Gomori (1955) as mentioned by Dixon & 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. At first only 100 ml of the CFF of the crude enzymes under investigation were first brought to 20% saturation by gradual addition of solid ammonium sulphate and the precipitated protein was obtained by centrifugation for 15 min at 15.000 rpm under cooling (10°C). The obtained pellet was dissolved into 5 ml of Tris-buffer (0.2 M) at pH 9.0 in order to determine the enzyme activity and protein content. Whereas, the remaining supernatant was treated with ammonium sulphate, was added again to the left of supernatant in order to achieve 40, 60, 80 and 100% saturation.

For each one ammonium sulphate concentration, enzyme activity and protein content were measured, then the specific activity for each fraction was calculated.

Dialysis

The procedure was performed by introducing the obvious precipitate (Selected ammonium sulphate ppt. in solution after dissolving in Tris- buffer adjusted at pH 9) into dialysis bag for dialysis against distilled water for 12 hr. The obtained enzyme preparation was concentrated against sucrose before the application in column chromatographic technique.

Column chromatography on Sephadex G200 colum

The dialyzed-partially purified-enzymes preparation was applied onto a column packed with sephadex G 200 and equilibrated with Tris-buffer (0.2M) adjusted at pH 9, then eluted with the same buffer. Preparation of the gel column and the fractionation procedure was carried out as mentioned by (Soliman, 2003).

Application of crude and purified enzymes in detergent trial experiments

Wash performance analysis

Wash performance analysis of enzymes on small square pieces (7cm X 7cm) of a new cotton fabric was performed in the similar way as described by Oberoi *et al.* (2001). Samples of human blood, chocolate, salad, pomegranate, apple, mango and strawberry were used to stain (spot) the cloth cotton fabric, while unstained cloth piece was used as a control. The stained cloth pieces were subjected to wash treatments at 28°C (room temperature) and 55°C with tap water, Tris-buffer(0.2 M) pH 9, local detergent 1%(w/v) (Rabso), purified amylase, cellulase, protease, lipase, collective group of four purified enzymes, collective group of four purified enzymes in addition to detergent, crude amylase and cellulase, crude protease and crude lipase, collective group of crude enzymes and collective group of crude enzymes in addition to detergent 1%. This experiment was carried out for each stain (spot) as following: (1) Unstained cloth piece as a control (1);(2) Stained cloth piece as a control (2); (3) Flask contain 100 ml Tris-buffer (0.2 M) at pH 9 and stained cloth without any treatment as a control; (4) Flask contains 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and + 0.7 g commercial detergent (Rabso) ; (5) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.1 ml of purified amylase solution; (6) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.1 ml of purified cellulase solution ; (7) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.1 ml of purified protease solution; (8) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.1 ml of purified lipase solution; (9) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.4 ml of collective groups from the purified enzymes (0.1 ml from each enzyme) solution without commercial detergent ; (10) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.4 ml of collective groups from the purified enzymes (0.1 ml from each enzyme) solution with commercial detergent ; (11) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9; stained cloth and 0.1 ml of crude enzymes from potato peels wastes ; (12) Flask contain 100 ml of used Tris-buffer (0.2 M) at pH 9;

stained cloth and 0.1 ml of crude enzymes from slaughter house wastes ; (13) Flask contain 100 ml of used Tris- buffer (0.2 M) at pH 9; stained cloth +0.2 ml of collective group of crude enzymes from potato peels and slaughter house wastes ; (14) Flask contains 100 ml of used 0.2 M. Tris- buffer (pH 9); stained cloth; 0.2 ml of collective groups from the crude enzymes solution and 0.7 g commercial detergent (Rabso).

Then all of these stained cloth pieces were incubated at two different temperatures (28 & 55°C) for 15 min. After incubation period, cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces showed the effect of enzyme in removal of stains in comparison with commercial detergent (Rabso). Also a colour measurement for each cloth piece was determined in Dyeing and Finishing Unit, Department of Cotton Chemistry and Textile Fibers, Cotton Technology Section, Cotton Research Institute, Agricultural Research Center. Results were measured by Perkin-Elmer double beam spectrophotometer, Model Lambda 35 equipped with integrated sphere. Each sample was scanned with D65 illuminant source in the wavelength range 400-700 nm using the standard white tile as the reference for all colour measurements.

Colour measurements

Colour measurement was determined by advanced spectroscopy in conjunction with Perkin Elmer UV-Vis Lab. Measurement of colour strength (K/S) was automatically calculated from reflectance (R %) by using Kubelka Munk equation. $K/S = (1-R)^2/2R$. From reflectance measurements on an automatic filter spectrophotometer, where K is the absorption coefficient, S is the scattering coefficient, and R is the reflectance of dyed samples at the wavelength of the maximum absorption.

Whiteness & yellowness index (ASTM, 1993)

ASTM E313-96 Standard practice for calculating Whiteness & Yellowness Indices from Instrumentally measured colour coordinates by using the CIE- system .

Results

Application of thermostable enzymes in detergent technology was carried out with or without commercial detergent such as an Egyptian product (Rabso) on different clean cloth samples blotched with different materials such as blood, apple, chocolate, pomegranate, mango, salad and strawberry. The blotched cloth samples treated at 55°C, then whiteness, yellowness, reflectance, colour strength value (K/M) for each cloth sample were recorded in comparison with unbleached control sample. Data presented graphically in Fig. 1&2 which showed that, the application of collective group of crude enzymes and commercial detergent (Rabso) on cloth piece blotched with blood and treated at 55°C gave the highest whiteness and reflectance values where it reached up to 59.88 and 60.4% , respectively. Also, treatment with collective group of crude enzymes without detergent gave a good result for blood removal from cloth piece. On the other hand, K/M value and yellowness exhibited the lowest values at these treatments.

Data presented graphically in Fig. 3&4 showed that, the application of crude enzymes from potato peels wastes on cloth piece blotched with apple and treated at 55°C gave the highest whiteness value where it reached up to 46.72%. Also, treatment with collective group of crude enzymes supplemented with detergent (Rabso), collective group of purified enzymes with detergent (Rabso) and pure amylase only gave a good removal of apple blotch which appeared by maximal value of whiteness and reflectance and lower value of yellowness and colour strength value (K/M).

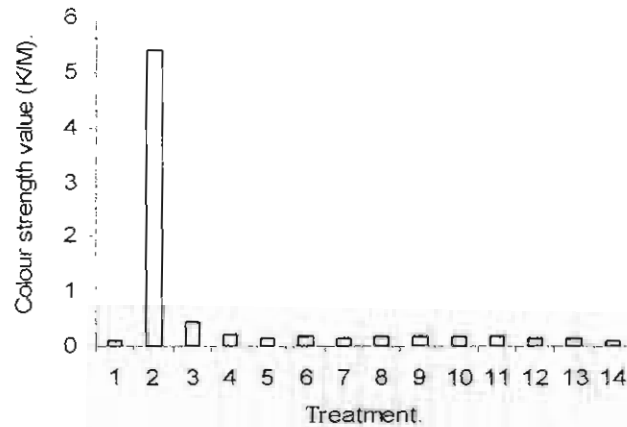


Fig . 1. The colour strength value (K/M) of cloth samples stained with blood treated at 55°C with thermostable enzyme(s) and/or commercial detergent(Rabso).

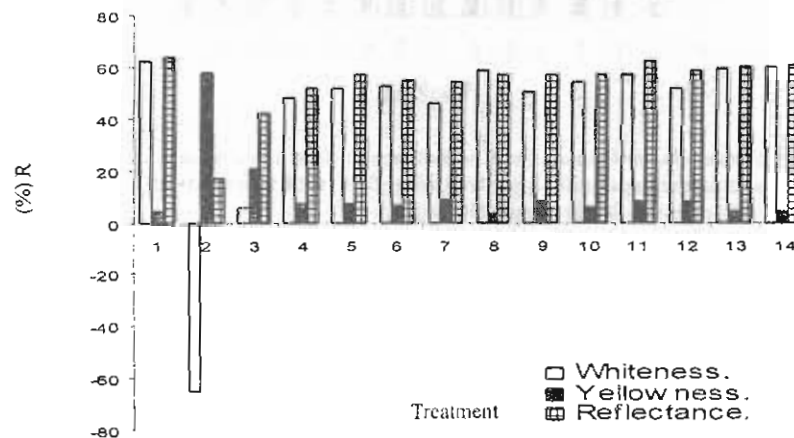


Fig . 2. Whiteness, yellowness and reflectance at their maximum wavelength of clean cloth samples stained with blood treated at 55°C with thermostable enzyme(s) and/or commercial detergent(Rabso).

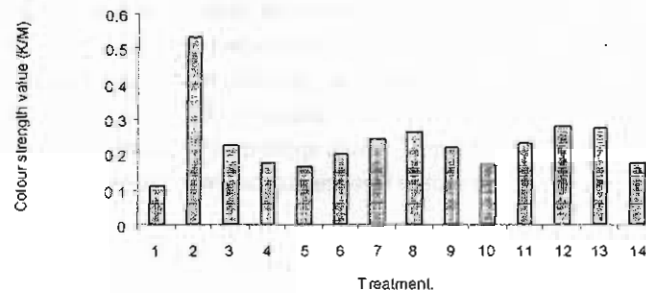


Fig. 3. The colour strength value (K/M) of clothes samples stained with apple treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

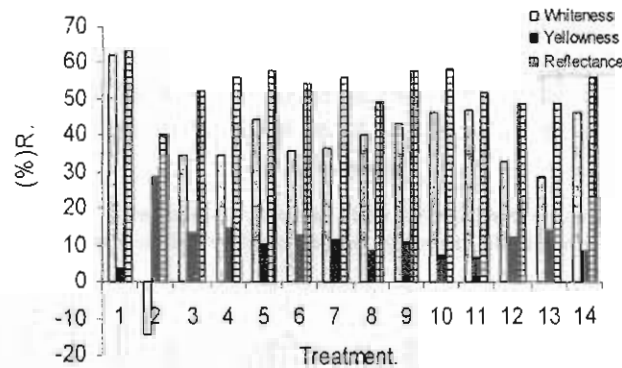


Fig. 4. Whiteness, yellowness, and reflectance at their maximum wavelength of cloth samples stained with apple treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

The treatment of chocolate blotched cloth piece at 55°C with collective group of thermostable crude enzymes mixed with commercial detergent (Rabso) exhibited a maximal whiteness and reflectance values more than control, where it reached up to 67.6 and 66.1%, respectively. Also, this treatment gave a minimum colour strength value (K/M). Interestingly, many other treatments not only exhibited a complete removal of chocolate blotches but also remove the non cellulosic materials and impurities of cloth piece such as treatments with collective group of the crude thermostable enzymes without detergent, collective group of the purified enzymes without detergent, pure lipase and detergent only, as shown in Fig. 5&6.

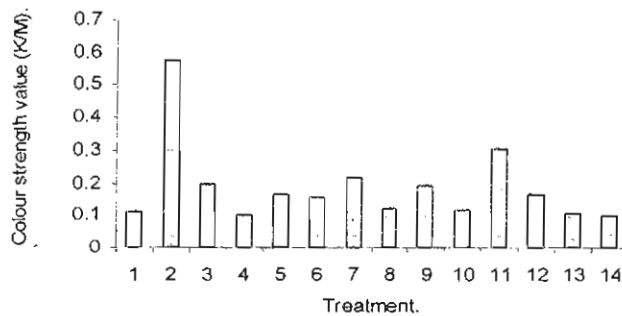


Fig. 5. The colour strength value (K/M) of cloth samples stained with chocolate treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

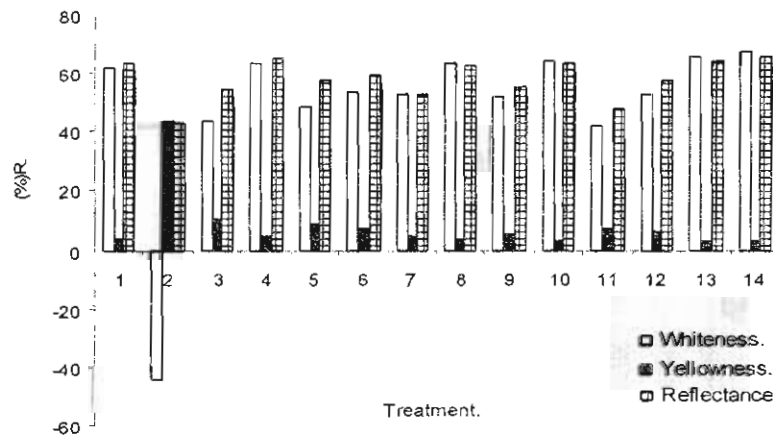


Fig.6. Whiteness, yellowness and reflectance at their maximum wavelength of cloth samples stained with chocolate treated at 55 °C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

Data recorded presented graphically in Fig. 7&8 showed that, the purified amylase exhibited the highest whiteness and lowest colour strength (K/M) value when applied to cloth samples blotched with pomegranate and treated at 55 °C.

Data presented graphically in Fig.7&8 showed that, the purified amylase, purified cellulase, purified protease, purified lipase and collective group of the purified enzymes exhibited a good removal of pomegranate blotch by treating at 55°C, where it exhibited the highest whiteness and reflectance value while lowest colour strength (K/M) value by application on cloth samples blotched with pomegranate.

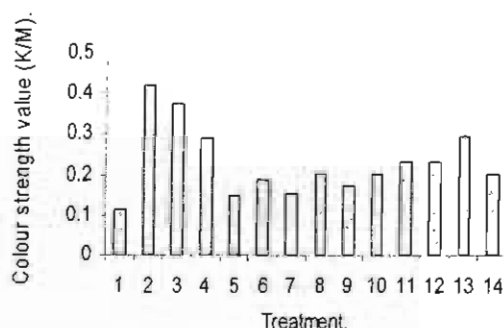


Fig. 7. The colour strength value (K/M) of cloth samples stained with pomegranate treated at 55°C with thermostable enzyme(s) with commercial and/or detergent (Rabso).

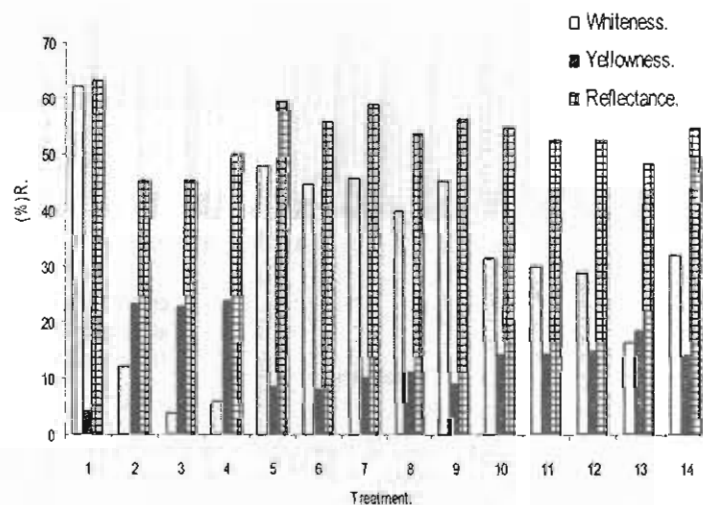


Fig. 8. Whiteness, yellowness and reflectance at their maximum wavelength of cloth samples stained with pomegranate treated at 55 °C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

Treatment with collective group of the purified thermostable enzymes without detergent gave the highest whiteness and reflectance value by treating the cloth sample blotched with mango at 55°C. Needless to say that, this treatment not only removed the blotch but also removed any colour and impurities occurred on cloth sample, where the whiteness and reflectance reached up to 62.12 and 62.995, respectively as presented graphically in Fig. 9&10.

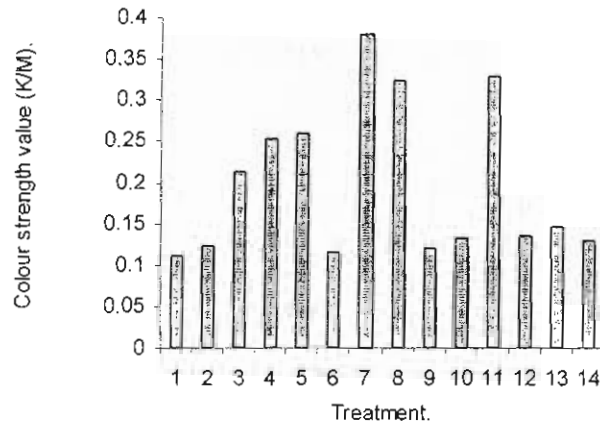


Fig. 9. The colour strength value (K/M) of cloth samples stained with mango treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

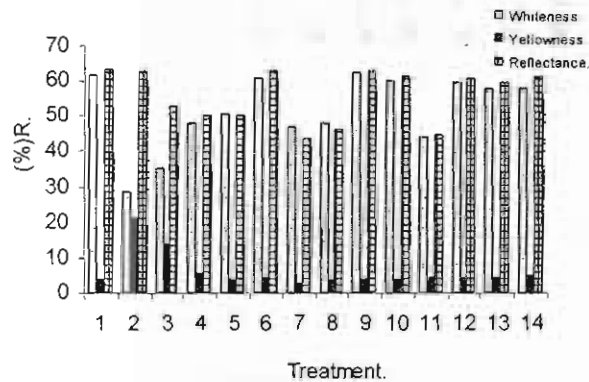


Fig. 10. Whiteness, yellowness and reflectance at their maximum wavelength of cloth samples stained with mango treated at 55 °C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

Data presented graphically in Fig. 11&12 showed that, the cloth piece blotched with salad and treated at 55°C recorded its maximum washing performance when treated with collective group of the crude thermostable enzymes.

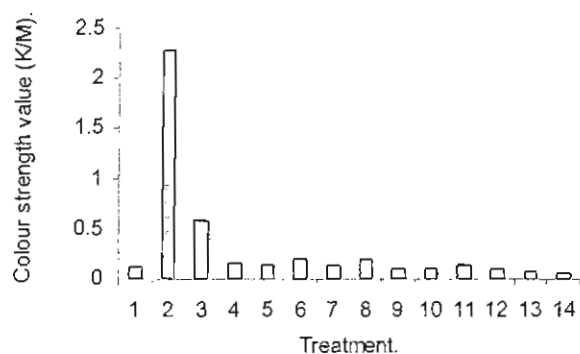


Fig. 11. The colour strength value (K/M) of cloth samples stained with salad treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

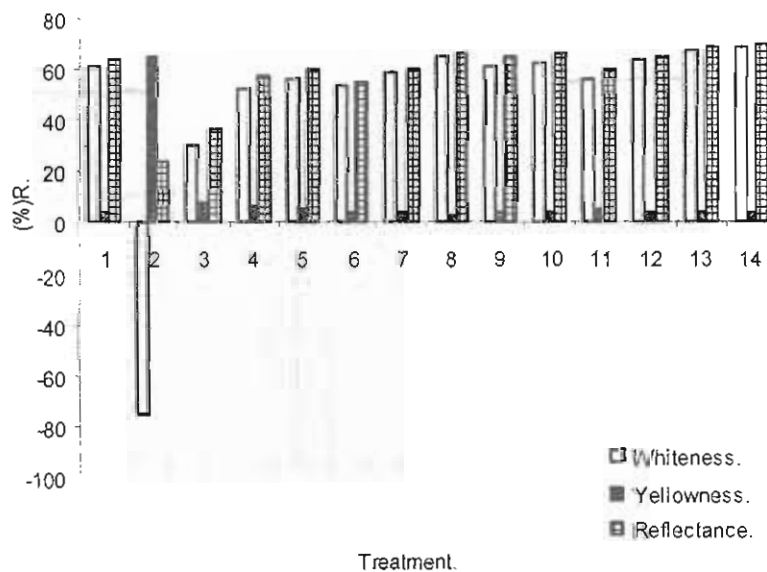


Fig. 12. Whiteness, yellowness and reflectance, at their maximum wavelength of cloth samples stained with salad treated at 55 °C with thermostable enzyme(s) and/or with commercial detergent(Rabso).

Data presented graphically in Fig. 13&14 showed that, purified amylase, cellulase, protease and lipase exhibited a good removal of strawberry blotch by treating at 55°C, where it exhibited the highest whiteness and reflectance values and lowest yellowness and colour strength (K/M) value of the cloth piece blotched with strawberry.

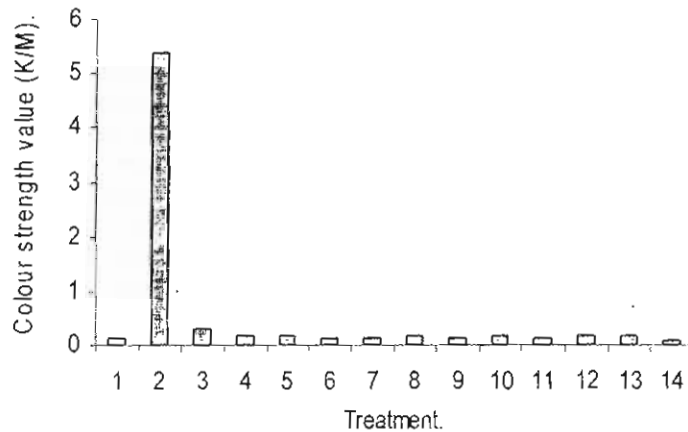


Fig.13. The colour strength value (K/M) of cloth samples stained with strawberry treated at 55°C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

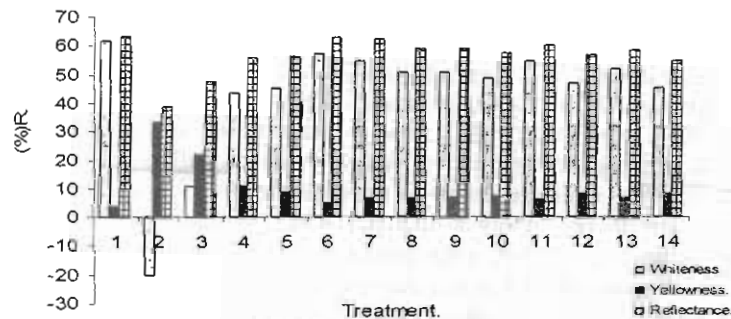


Fig.14. Whiteness, yellowness and reflectance, at their maximum wavelength of cloth samples stained with strawberry treated at 55 °C with thermostable enzyme(s) and/or with commercial detergent (Rabso).

Discussion

Limited information are 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 enzymes revealed that, it could effectively remove a variety of stains such as blood, apple, chocolate, mango, strawberry, salad and pomegranate while treated at 55°C for 15 min by adding thermostable crude/purified enzymes separately or in combination with or without detergent (Rabso) as an Egyptian product. Noteworthy was the effective removal of chocolate stain at 55°C by treating with collective group of the thermostable crude enzymes with and without detergent, purified lipase and collective group of purified enzymes with detergent, also removal of mango stain at 55°C by treating with collective group of the purified enzymes without detergent and removal of salad stain at 30°C by treating with collective group of the thermostable crude enzymes without

detergent and also removal of salad stain at 55°C by treating with collective group of crude enzymes with and without detergent, with the thermostable crude enzymes produced from slaughter house wastes, purified lipase and collective group of the purified enzymes with detergent. The previously mentioned treatments not only resulted in complete removal of stains but also removed the impurities and non cellulosic materials holed in cloth samples, indicated by whiteness degree, comparing with unbleached control. The best removal of most stains were 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. The present enzymes preparations offer applicable value in detergents industries, where it act actively over a broad temperature range. Needless to say, stains can be caused by a variety of substances and appropriate enzymes may not be available, or if available may not be incorporated into detergents or presoak compounds used in launders. But, thus it is not surprising that all stains are not removed by commercial products (Wistreich & Lechtman, 1984). In the present study, treatments at two temperatures were carried out because the detergent market is very segmented due to widely differing consumer practices which determine the use of detergents and their formulations. For instance, Americans use a short washing cycle, the water is added at 50°C and allowed to cool during a 10-15 min wash at 20-30°C (Layman, 1986 and Kalisz, 1988). Even in Europe, the boiling wash (95°C) is largely a thing of past, but there are a significant national differences. In general, European washing conditions involve warming the water to 40°C or 60°C in a 30-60 min cycle with or without a cool pre-wash (Winkhaus, 1987 and Kalisz, 1988). In the findings of other investigators, wash performance analysis revealed that, alkaline protease from *Bacillus clausii* I-52 exhibited high efficiency for the removal of blood, milk and ink stains in the presence of commercial detergent (Joo & Chang, 2005 and 2006). Also Grebeshova *et al.* (1999) found that, the addition of alkaline protease to detergents considerably increases (30-40%) the cleaning effect (particularly in removing stains containing proteins, e.g., blood, cocoa, milk, egg and sauces) and increases the consumption of surface-active substances, thereby improving the ecological situation. Banerjee *et al.* (1999) found that, thermostable alkaline protease produced by *B. brevis* improved the cleaning power of various detergents, and remove blood stains completely when used with detergents (Surf Excell[®] and Rin[®]) in the presence of Ca⁺² and glycine. Gerhartz (1990 a&b) reported that, alkaline proteases were extensively used in laundry detergents for stain removal. Tovey *et al.* (2001) studied the effect of four laundering agents (water alone, soap, detergent with enzymes and detergent without enzymes) on the removal of allergens at four temperatures (15, 25, 45 and 60°C) and three extraction times (5, 20 and 60 min) and found that, detergent extracted allergens more than either soap or water alone. Beg & Gupta (2003) found that, combination of detergent and alkaline protease produced by *B. mojavensis* resulted in complete stain removal and enzyme alone could effectively remove a variety of stains such as blood, grass and beetle. Mahmoud (2004) found that, application of BBW-protease in laundry detergent gave a promising result (using blood and chocolate blotches) compared with commercial detergent (Ariol).

Conclusion

Results of the present study suggested the potentially value of using *B. licheniformis* B 42 and *G. stearothermophilus* B78 to produce enzymes by SSF and SmF using a cheapest substrates for enzyme production. These enzymes were stable over a wide range of pH and temperature and also showed compatibility with various commercial detergents tested. These enzymes were used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent (Rabso) could significantly improve the cleaning performance towards different stains. Considering the overall properties of different alkaline enzymes of microbial origin and the thermostable alkaline enzymes of the present strains, *B. licheniformis* B 42 and *G. stearothermophilus* B78 are better as regards to pH and temperature stability, stability in the presence of surfactants, detergents compatibility and above all bleach stability for a potential application not only in heavy-duty detergents for fabrics but also in bleaches and detergents for automatic dishwashing machines.

References

- American Society for Testing and Materials (ASTM) (1993) D:2288 and D:1448 "Calculation of colour differences for instrumentally measured colour co-ordination" and micron air reading.
- Banerjee, U., Sani, R., Azmi, W. and Sani, R. (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem.* **35**, 213-219.
- Beg, Q. and Gupta, R. (2003) Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technol.* **32**, 294-304.
- Beguin, P. and Aubert, J.P. (2000) Cellulases. In: "Encyclopedia of Microbiology". 2nd edition. Volume 1 A-C. Lederberg, J. (Ed.) pp.744-758. Academic Press.
- Bhat, M.K. (2000) Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, **18**, 355-383.
- Burhan, A., Nisa, U., Cokhan, C., Omera, C., Ashabil, A. and Osman, G. (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. Isolate ANT- 6. *Process Biochemistry*, **38**, 1397 – 1403.
- Cardenas, J., Alvarez, E., De Castro-Alvarez, M.S., Sanchez-Montero, J.M., Valmaseda, M., Elson, S.W. and Sinisterr, J.V.(2001) Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. *J. Mol. Catal. B: Enzyme.* **14**, 111-23.
- Collee, J.G., Marmion, B.P., Fraser, A.G. and Simmons, A. (1996) "Medical Microbiology" (Mackie and Mac Carteny). 14th edition. Churchill Livingstone.
- Dixon, M. and Webb, E. (1964) "Enzymes". 2nd edition. Academic Press Inc. New York.
- Egypt. J. Microbiol.* **44** (2009)

- Ekperigin, M. M. (2007)** Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. *African Journal of Biotechnology*, **6** (1), 28-33.
- Gerhartz, W. (1990a)** General production methods. In: "*Enzymes in Industry, Production and Applications*". pp.36. VCH Publishers.
- Gerhartz, W. (1990b)** Industrial uses of enzymes. In : "*Enzymes in Industry, Production and Applications*". pp. 110-112. VCH Publishers.
- Gomori, G. (1955)** Preparation of buffers for use in enzyme active studies. In: "*Methods in Enzymology*". Vol I. Colwick, S. P. and Kaplan, N. O. (Ed.). Academic Press Inc. Pub. New York.
- Grbeshova, R.N., Saldeco-Torres, L.E. and Hidalgo, M.A. (1999)** Serine protease of *Bacillus subtilis* R. *Applied Biochemistry and Microbiology*, **35**, 131-134.
- Grgoravski de Lima, A.L., Nasemento, R.P., Bon, E.P. and Coelho, R.R. (2005)** *Streptomyces drozdowiczii* cellulose production using agroindustrial by-products and its potential use in the detergent and textile industries. *Enzyme and Microbial Technology*, **37**, 272-277.
- Gusakov, A.V., Salanovich, T. N., Antonov, A. I., Ustinov, B.B., Okunev, O.N., Burlingame, R., Emalfarb, M., Baez, M. and Sinitsyn, A. P. (2007)** Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. *Biofuels and Environmental Biotechnology and Bioengineering*. 2007 Wiley Periodicals, Inc.
- Hensyl, W. R. (Ed.) (1994)** "*Bergey's Manual of Determinative Bacteriology*", 9th edition, Williams & Wilkins, Baltimore.
- Jang, H.O. and Chen, K.S. (2003)** Production and characterization of thermostable cellulases from *Streptomyces transformant* T3-1. *World J. Microbiol. Biotechnol.* **19**, 263-268.
- Joo, H.S. and Chang, C.S. (2005)** Production of protease from a new alkaliphilic *Bacillus* sp. 1-312 grown on soybean meal: optimization and some properties. *Process Biochemistry*, **40**, 1263-1270.
- Joo, H.S. and Chang, C.S. (2006)** Production of oxidant and SDS-stable alkaline protease from an alkaliphilic *Bacillus clausii* 1-52 by submerged fermentation: Feasibility as a laundry additive. *Enzyme and Microbial Technology*, **38**, 176-183.
- Kalisz, H.M. (1988)** Microbial proteinase (s). In: "*Advances in Biochemical Engineering/ Biotechnology*", Vol. 36. Fiechter, A. (Ed.). pp. 1-65. Springer, Berlin.
- Kawada, T. (Ed.) (1998)** Industrial Enzymes Nikkei Biotechnology. *Annual Report*. Tokyo: Nikkei BP, 1997: pp. 611-626 (in Japanese).
- Kazlauskas, R.J. and Bortischeuer, U.T. (1998)** Biotransformations with lipases. In: "*Biotechnology*". Vol. (8). Rehm, H.; Pihler, G.; Stadler, A. and Kelly, P. (Ed.). pp. 137 - 192. New York, VCH.
- Konsula, Z. and Liakopoulou-Kyriakides, M. (2004)** Hydrolysis of starch by the action of an α -amylase from *Bacillus subtilis*. *Process Biochemistry*, **39**, 1745-1749. *Egypt. J. Microbiol.* **44** (2009)

- Konsula, Z. and Liakopoulou-Kyriakides, M. (2006)** Starch hydrolysis by the action of an entrapped in alginate capsules α -amylase from *Bacillus subtilis*. *Process Biochemistry*, **41**, 343-349.
- Layman, P.L. (1986)** Industrial enzyme; battling to remain specialties. *Chem. Eng. News*, **15**, 11-14.
- Mahmoud, A.I. (2004)** Biotechnological recycling of infectious hospital wastes for the sake of safe environment. *M.Sc. Thesis*. Bot.& Microbiol.Dept., Fac. of Sci. Al- Azhar University, Cairo, Egypt .
- Oberoi, R., Beg, Q., Puri, S., Saxena, R. and Gupta, R. (2001)** Characterization and wash performance of an SDS-stable alkaline protease from *Bacillus* sp. *World J. Microbiol. Biotechnol.* **17**, 493-497.
- Priest, F.G. (2000)** Enzymes. Extracellular. In: *"Encyclopedia of Microbiology"*. 2nd edition. Volume 2 D-K. Lederberg, J.(Ed.), pp.210-221. Academic Press.
- Rodriguez, V. P., Alameda, E.J., Gallegos, J.F., Requena, A.R. and Lopez, A.I. (2006)** Thermal deactivation of a commercial α - amylase from *Bacillus licheniformis* used in detergents. *Biochemical Engineering Journal*, **27**, 299-304.
- Schallmey, M., Singh, A. and Ward, W.P. (2004)** Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **50**, 1-17.
- Sharma, R., Chisti, Y. and Banerjee, U.C. (2001)** Production, purification, characterization and applications of lipases. *Biotechnol. Advances*, **19**, 627-662.
- Showell, M.S. (1999)** Enzymes, detergent. In: *"Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation"*, Vol 2. Flickinger, M.C.; Drew, S.W. (Ed.). pp. 958-971. Wiley, New York.
- Tovey, E.R., Taylor, D.J., Mitakakis, T.Z. and De Lucca S.D. (2001)** Effectiveness of laundry washing agents and conditions in the removal of cat and dust mite allergen from bedding dust. *Journal of Allergy and Clinical Immunology*. **108**(3), 369-374.
- Van der Maarel, M., van der Veen, B., Uitdehaag, J., Leemhuis, H. and Dijkhuizen, L. (2002)** Properties and applications of starch-converting enzymes of the α - amylase family. *Journal of Biotechnology*, **94**, 137-155.
- Venugopal, M. and Saramma, A. (2006)** Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. *Process Biochemistry*. Accepted in 20 December 2005.
- Vincent, J.M. (1970)** A manual for the practical study of the root nodule bacteria. *International Biological Program 7* Marylebone Road, London. NW1, Blackwell Scientific Publications, Oxford & Edinburgh. p. 75.
- Winkhaus, H.D. (1987)** Primary influencing factors and major trends in the European detergent and cleanser market. *J. Am. Oil Chem. Soc.* **64**, 244.

Wistreich, G.A. and Lechtman, M.D. (1984) "Microbiology". 4th edition. pp. 865-868. Collier Macmillan Publishers, London.

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التطبيق البيوتكنولوجي لإنزيمات بكتيرية قلوية صامدة للحرارة في المنظفات الحيوية الصناعية

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تهدف الدراسة إلى محاولة إنتاج إنزيمات صامدة للحرارة من الكائنات الدقيقة وذلك لاستخدامها في تكنولوجيا المنظفات الصناعية. تم اختيار وتعريف أقوى عزلتان بكتيريتان على أنهما *باسيلس ليكينوفورمس* و *جيوباسيلس ستياروثيرموفيلس* من أصل 135 عزلة محبة للحرارة من حيث قدرتهم على إنتاج إنزيمات الأميلاز والسيلولاز والبروتياز والليباز بعد تمييزهم على بعض المخلفات الزراعية- الصناعية عند 55°م وأس هيدروجيني 9 وذلك لتطبيقها في تكنولوجيا المنظفات الصناعية. تأثرت إنتاجية الإنزيمات القلوية الصامدة للحرارة الأربعة من السلالتان المختارتان وذلك باستخدام مخلفات المجازر كأفضل وسط لإنتاج البروتياز والليباز وقشر البطاطس كأفضل وسط لإنتاج الأميلاز والسيلولاز بالآتي: درجة حرارة التحضين والأس الهيدروجيني وتركيزات كلا من مخلفات المجازر وقشر البطاطس وحجم اللاقحة وفترة التحضين ومصادر الكربون والنيتروجين والأملاح المعدنية والفيتامينات والتحصين تحت ظروف هزارة . تم إنتاج الإنزيمات الأربعة تحت جميع الظروف البيئية والغذائية المثلى كما تمت تثقيبتهم بواسطة عمود الفصل الكروماتوجرافي على سيفادكس ج.200، ج.100 على التوالي . ازدادت درجة نقاوة الإنزيمات إلى 17,04 ، 15,24 ، 11,9 ، 43,27 مره لكل من الأميلاز والسيلولاز والبروتياز والليباز على التوالي . تم استخدام الإنزيمات الخام والمنقاة منفصلة أو كإضافات على المنظف الصناعي (رايسو) كمنتج مصري كتطبيق في مجال المنظفات البيولوجية لإزالة العديد من البقع مثل (الدم والتفاح والشيكولاته والمانجو والفراولة والصلصة والرمال) وقد أظهرت الدراسة أن هذه الإنزيمات تعطى درجة عالية من النظافة عند استخدامها بدون منظف كما أنها أظهرت قدره فائقة ليس فقط على التنظيف وإنما على إزالة بعض الشوائب من الأقمشة في بعض المعاملات باستخدام أو بدون استخدام المنظف الصناعي (رايسو) . وتتلخص نتائج الدراسة إلى أنه تم الحصول على أربعة إنزيمات قلوية صامدة للحرارة من سلالتين من البكتريا المحبة للحرارة من المصادر التي قد تلوث البيئة والرخيصة الثمن وتطبيقها في تكنولوجيا المنظفات الصناعية الحيوية وذلك للحد من استخدام الكيماويات الملوثة للبيئة.