Optimum Conditions of Protease Production from *Bacillus licheniformis*(B1) and its Applications

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ABSTRACT

Forty isolates of Bacillus spp. were isolated from sixty samples including; soil, water and meat. Ability of these isolates to produce protease was evaluated. Bacillus B1 isolated that isolated from soil showed the highest protease production. it was identified as a strain of Bacillus licheniformis.The optimum culture medium and conditions for protease production were casein-yeast extract medium contained soluble casein (0.5g), yeast extract (0.5g), glucose (1g), KH2PO4 (0.02g), K2HPO4 (0.02g) and MgSO4.7H2O (0.01g), in 100ml distilled water, pH 8.0 and incubated at 37˚C. for 48 h. The crude protease exhibited ability to remove the blood color from cloth within 30 min and gelatin from X-ray film within 120 min.

Keywords: Protease, Production, *Bacillus licheniformis*, applications.

انتاج وبعض تطبيقات انزيم البروتييز من بكتريا *Bacillus licheniformis* B1

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الخلاصة:

تم الحصول على 40 عزلة عائدة لجنسBacillus والتي عزلت من 60 نموذج تضمنت عينات تربة و مياه ولحم. اختبرت قدرة هذه العزلات على انتاج انزيم البروتييز، وبينت نتائج الغربلة على ان العزلة Bacillus B1المعزولة من التربة هي الاغرز انتاجا والتي شخصت على انها احدى سلالات Bacillus licheniformis. درست الظروف المؤثرة في انتاج البروتييز، ولوحظ ان اعلى انتاجية تكون عند زرع البكتريا في وسط casein-yeast extract الحاوي على (0.5 g.) كازائين و (0.5 g.) مستخلص الخميرة و (1 g.) كلوكوز و (0.02g.) KH2PO4 و (0.02g.) K2HPO4 و MgSO4.7H2O (0.01g.) لكل 100مل ماء مقطر برقم هيدروجيني 8 وحضنها بدرجة حرارة 37م لمدة 48 ساعة. اظهر الانزيم قابيلة في ازالة بقع الدم من القماش عند الحضنه لـ30 دقيقة، وكذلك اظهرالانزيم امكانية أزالة طبقة الجلاتين من افلام اشعة اكس خلال فترة 120 دقيقة.

الكلمات المفتاحية: انزيم محلل البروتين، انتاج، *Bacillus licheniformis*، التطبيقات.

Introduction

Proteases are a group of enzymes that hydrolyze peptide bonds of proteins and break down into polypeptides or free amino acids [1]. Proteases constitute a class of industrial enzymes. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 billion by 2012 [2]. They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries [3]. One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity [1]. The preferred sources of proteases are microbes because of their rapid growth and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. However, many of the alkaline proteases applied to industrial purposes face some limitations such as low stability towards surfactants and production cost of the enzymes arisen from growth medium [4]. The genus “Bacillus” is an important source of industrial alkaline proteases and is probably the only genus being commercialized for alkaline protease production [5]. Screening of alkaline proteases producing Bacillus spp. from different ecological environments can result in isolation of new alkaline proteases with unique physio-chemical characteristics [6]. It is known that the amount of enzyme produced greatly depend on strain and growth conditions. Therefore, there is a need to the search of new strains of bacteria that produce proteolytic enzymes with novel properties and the development of low cost media .

Materials and Methods

Microorganism

Bacillus licheniformis isolated from soil and identified according to the morphological, microscopic examination and biochemical tests of Logan, and DeVos [7].

Determination of Proteases Production

Two methods were used for production of proteases.

Semi-quantitative method [8]

Skim milk-peptone agar consisted of Skim milk powder 10 g, Peptone 0.5 g, Agar powder 2 g and distilled water 100 ml. the mixture was inoculated for 24 h with old bacterial culture and incubated for 24 h at 40 °C. Clear zone around the spots and underneath the growth indicate protease production. The diameter of colonies and clear zones were measured. The ratio of clear zone diameter to colony diameter was calculated which represents a semi quantitative assay of protease.

2Quantitative method [9].

Ten ml of casein-peptone broth (Peptone 0.5 g, Soluble casein 0.2 g, NaCl 0.5 g and distilled water 100ml) was inoculated with 0.1 of activated bacterial suspension (optical density = 0.3 at 600 nm) and incubated at 40˚C for 24 h. The crude enzyme was extracted by cooling centrifugation for 15 min. Then the enzyme activity and protein concentration was measured in the supernatant.

Assay of Protease Activity

Protease Activity was determined spectrophotometrically according to previous method of Anson [10] with littel modification. Enzyme extracted solution (0.2 ml) was incubated with 1.8 ml of casein solution at 40 °C for 15 min. The blank consisted of 1.8 ml of reaction solution and 3.0 ml of 5 % TCA (trichloroacetic acid) and 0.2 enzyme solution. The reaction was stopped adding 3.0 ml. of 5 % trichloroacetic acid and incubated at 25 °C for 10 min. The mixture was centrifuged by cooling centrifuge (3000 g) for 10 min, then supernatant was separated. Quantity of 2.5 ml of 0.5M Na2CO3 solution was added to 1 ml of the supernatant and 1 ml of Folin–Ciocalteus reagent was added and incubated at 37°C for 20 min. The absorbance (O.D.) at 600 nm was measured. One unit of protease activity was defined as the amount of enzyme required to liberate one µg of tyrosine per minute per ml. under assay conditions.

Determination the optimum conditions of enzymes production.

Effect of different media in protease Production

The bacterial isolate was activated by culturing in nutrient broth and incubated at 37˚C for 24 h. Each 100 ml of different media casein–peptone medium [9], Horikoshii medium (11) and Casein-yeast extract medium [12] was inoculated with 2 ml of bacterial suspension (O.D = 0.3 at 600 nm) and incubated at 37˚C for 24 h. The cells were precipitated by cooling

centrifugation at 3000 rpm. The supernatants (crude enzyme) were assayed for enzyme activity, protein concentration and calculated specific activity, for select the best production medium.

Effect of Incubation Temperature on Enzyme Production

Quantity 100 ml of casein-yeast extract medium was inoculated with 2 ml of activated bacterial suspension (O.D = 0.3 at 600 nm) and incubated at different temperatures (37, 40, and 50 ˚C) for 24 h. The supernatant was assayed for enzyme activity, protein concentration and specific activity .

Effect of Initial pH on Enzyme Production

Hundred militer of casein-yeast extract medium was prepared at different pH values (7.0, 8.0, 9.0 and 10.0) adjusted with 1N HCl and 1N NaOH. The medium was inoculated with 2 ml of activated bacterial suspension (O.D = 0.3 at 600 nm) and incubated at 37 ˚C for 24 h. The supernatant was assayed for enzyme activity, protein concentration and specific activity.

Effect of Incubation Period on Enzyme Production

Hundred militer of casein-yeast extract medium at pH 8 was inoculated with 2 ml of activated bacterial suspension (O.D = 0.3 at 600 nm) and incubated at 37˚C for different time intervals (24, 48 and 72 h). The supernatant was assayed for enzyme activity, protein concentration, and specific activity. All experiments achieved as a duplicated.

Statistical Analysis

The Statistical Analysis System- SAS [13] was used to determine the significant difference between the different parameters. LSD test (Least Significant Difference) at probability level $P \leq 0.05$ was applied to be significant difference.

RESULTS AND DISCUSSION

Isolation of Bacillus

Sixty samples were collected from different sources; soil, water and meat. Forty bacterial isolates were identified as Bacillus spp. according to growth characteristics on nutrient agar and microscopic examination (Table 1). The growing isolates showed a very wide range of colonial morphologies, they varied from moist and glossy to wrinkled texture. Microscopic examination showed Gram positive rod cells, may occur singly, pairs, chains and filaments. Spore forming and spore shapes vary from cylindrical through ellipsoidal to spherical. Spores might be terminally, subterminal, or central position. However, a Gram-stain is sufficient to determine the presence of spores because the spore remains unstainable while the vegetative cells or the vegetative part of the spore will stain [14].

Screening for protease producing Bacillus

Semi-quantitative screening

Proteolytic activity was assayed using skim milk-peptone agar and expressed as diameter of clear zone to diameter of colony (Table 2). A clear zone of skim milk hydrolysis gave an indication of protease producing organisms [15]. Bacteria are the most dominant group of protease producers with the genus Bacillus being the most prominent and serve as an ideal source of this enzyme [9]. Due to their rapid growth and limited space required for their cultivation [16].

Quantitative screening

According to the previous results seven isolates were selected for quantitative screening of protease production (Table 3). The difference in the production of enzyme from isolates might be due to different source of the isolate or the variation in genes codes protease synthesis [17]. Assay of protease activity is depended on ability of casein hydrolysis thus casein containing medium is used to detect the protease producing microorganisms [12].

Identification of Bacillus B1 isolate

Morphological and physiological properties of the selected isolate was investigated. Relying on the results it can be concluded that B1 isolate is belongs to B. lichenoformis depending on "Bergey's Manual of Systematic Bacteriology [7] (Table 4). *B. licheniformis* is spore former bacteria give positive results in VP-test and catalase [18]. Slepecky and Hemphill [14] mentioned that B. licheniformis has the ability to grow at 40˚C. and 50˚C. This species is used in a wide range of industrial processes, including production of enzymes such as protease [19].

Effect of culture conditions on protease production

Effect of medium compositions

B. licheniformis B1 was cultivated in different media then enzyme production was assayed Results in figure (1) indicated that the casein-yeast extract medium was the best for protease production. Glucose was found to be the optimum carbon source for protease activity by all Bacillus spp. [20]. From nutritional aspects glucose plays an essential role as enzyme inducer for B. subtilis strains [21]. Good protease activity was also observed with B. cereus isolates in media supplemented with glucose [22]. The organic nitrogen compounds support the growth and biosynthesis of protein, nucleic acid and other cell constituents [23]. Martins and Teodoro [24] found that the addition of yeast extract to the liquid medium shortened the lag period and increased the enzyme synthesis. The effect of various metal ions on protease production was reported, supplementation of Mg2+, Ca2+ and K+ salts to the culture medium exhibited slightly better production [25].

Effect of incubation temperature on enzyme production

Protease activity was assayed at various incubation temperatures (30, 37, 40 and

50 ˚C). The result showed that the best temperature for protease production by B. licheniformis B1was at 37˚C. (Figure 2). Temperature is one of the most important factors affecting the enzyme production [22]. Ray et al. [26] reported that temperature could regulate the synthesis and secretion of extracellular protease by microorganisms. The results of this experiment on Bacillus licheniformis B1 are in agreement with other literatures on alkaline Bacillus strains producing alkaline proteases [27] .

Effect of pH of medium on enzyme production

To investigate the effect of initial medium pH on protease production, B. licheniformis B1 was grown in casein-yeast extract medium with different pH values, the results showed that the enzyme was produced over pH ranged from 7.0 to 10.0 the a maximum value and of specific activity 5.5 U/mg protein, was observed at pH 8.0 (Figure 3). The most important characteristic of microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production [28]. Mona, [29] found that the maximum protease production could be achieved by controlled pH and temperature. Most of the proteases produced by genus Bacillus exhibit alkaline pH ranged from 8.0-10.0 [30] .

Effect of incubation period on enzyme production

The protease production by B. licheniformis B1 was observed after 24, 48 and 72 h of incubation period the results revealed that the maximum activity (6.1 U/mg) was obtained after 48 h. (Figure 4). It might be conclude that protease is produced during logarithmic phase and reaches its maximum value at stationary phase [31]. Enzyme production in culture medium did not change in the stationary phase and decreased after 32 h. of cultivation [32]. Other studies also suggested that incubation for 48 h was the best incubation time for extracellular protease production by Bacillus sp. [1], B. licheniformis Lbbl-11 (33), B. licheniformis SH-2 [20].

Some applications of protease.

Removing blood color.

The blood stain was removed from pieces of cloth cotton after incubating the cloth in crude protease from B. licheniformis B1 for 30 min (Figure 5) this illustrated showed a good efficiency as compared with control. This result is confirmed the potential application of this enzyme in the detergent industry as additives [34]. Nadeem et al., [35] studied the high capacity of blood stain removal by protease of B. licheniformisN-2. Gehan et al., [36] found that gradual removal of blood stain by increasing the contact time intervals from 10 to 50 min with the enzyme solution .

Vishalakshi et al., [37] reported that blood color is completely removed from the cloths after rinsing with a combination of detergent and partially

purified enzyme for a period of 20 min and it was removed after 25 min when rinsed with partially purified enzyme alone .

Removing of gelatin from X-ray film

The gelatin coating X-ray film was removed by crude protease of B. licheniformis B1 after incubating at 30 °C for two hours (Figure 6). The enzyme when added to X-ray films removed the layer of gelatin and film became transparent [38]. In this study the hydrolysis of gelatin was relatively slow, probably duo to slow adsorption of protease on to the surface of films. Or maybe the concentration of protease as crude was low. Moreover, the incubation temperature (30 °C) may be below the optimum for activity of this enzyme for hydrolysis gelatin. Vijayalakshmi, et al., [39] experimented the efficiency of partially purified protease to hydrolyze the gelatinous coating on X-ray film, where incubated with X-ray films, the hydrolysis was completed within 30 min .

Conclusion

The local isolate of B. licheniformis B1 is an efficient protease producer. Casein-yeast extract medium was the best medium for protease production by B. licheniformis B1 in alkali environment (pH 8) after incubation at 37 C° for 48 h. Protease exhibited potential ability through removing blood stain from cloth and gelatin from X-ray film.

Table 1: *Bacillus* isolates obtained from different sources

Sources of Samples	No. of samples	No. of Isolates	*Bacillus* isolates
Soil	30	28	B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, B14, B15, B16, B17, B18, B19, B20, B21, B22, B23, B24, B25, B26, B27 and B28
Water	15	7	Bw2, Bw6, Bw7, Bw8, Bw9, Bw10 and Bw12
Meat	15	5	Bm2, Bm3, Bm4, Bm5 and Bm6
Total	60	40	

Table 2: Protein hydrolysis in Skim milk-peptone agar (at pH7.0 and pH8.0) cultured with *Bacillus* isolates and incubated for 24h. at 40°C.

Code number of isolates	Hydrolysis ratio(1)		Code number of isolates	Hydrolysis ratio		Code number of isolates	Hydrolysis ratio	
	pH 7	pH 8		pH 7	pH 8		pH 7	pH 8
B1	4	4	B5	1.3	1	B18	1.2	1
B20	3.5	3	B4	1.3	1	Bw7	1.2	1
B7	3	2.7	B12	1.3	1	Bw12	1.2	1
B3	2.4	2.7	Bm2	1.3	1	B2	1.2	1
B26	2	2.4	B21	1.2	1	B27	1.1	1
B15	2	2	B14	1.2	1	B10	1.1	1
B2	1.7	1.5	B13	1.2	1	Bw8	1.1	1
B11	1.7	1	Bm4	1.2	1	Bw9	1.1	1
B17	1.7	1	B28	1.2	1	Bm3	1.1	1
B16	1.6	1	B22	1.2	1	B6	1.1	1
B24	1.5	1	B23	1.2	1	Bw10	1.1	1
B8	1.5	1	Bm5	1.2	1			
B9	1.3	1	B25	1.2	1			
LSD Value:0.05	pH 7		0.729 *					
	pH 8		0.633 *					

(1) Diameter of clear zone/Colony diameter.

Table (3): Specific activities of protease produced by *Bacillus* isolates after 24h. incubation at 40°C in Peptone-casein broth (pH 8).

Code Number of isolates	Specific activity(U/mg protein)
B1	4.4
B20	3.7
B7	3.2
B3	3.0
B26	2.3
$B1^2$	2.0
B15	1.8
LSD Value : 0.05	0.702 *

Table 4: Morphological and Biochemical characteristics of *Bacillus* B1 isolate.

Characteristics	*Bacillus licheniformis*
Cell shape	Rod
Spore shape	Ellipsoidal
Spore site	Central
Gram stain	+
Catalase	+
Motility	+
Voges-Proskauer	+
Starch hydrolysis	+
Protease production	+
Egg-yolk reaction	-
Nitrate reduction	+
Sodium chloride tolerance 7%	+
Growth at 50°C	+
Citrate utilization	+
Anaerobic growth	+
Gelatin hydrolysis	+
Carbohydrates fermentation	
Galactose	+
Glucose	+
Maltose	+
Sucrose	+

+: positive result, -: negative result.

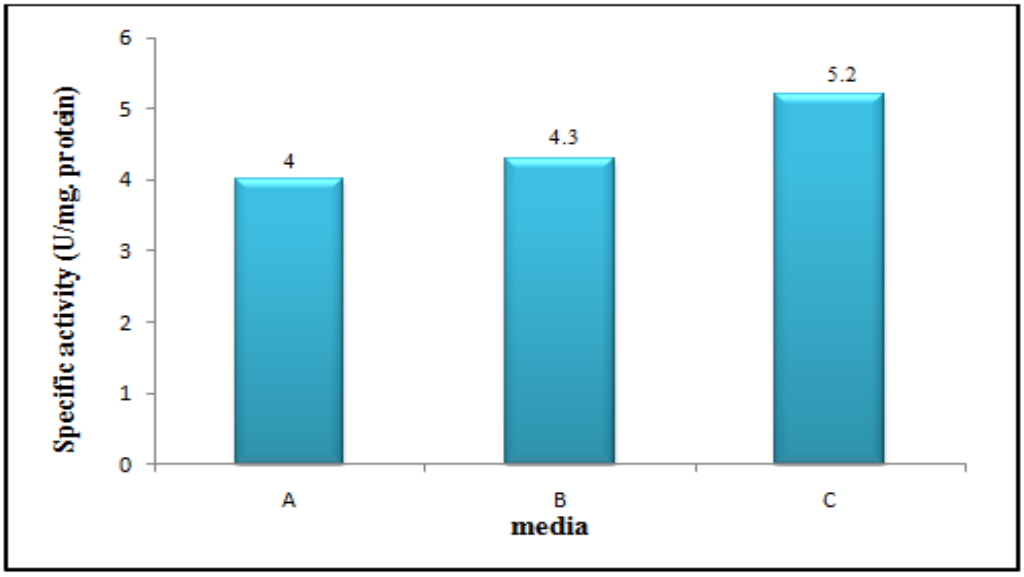

Figure 1: Protease production by *B. licheniformis* B1 cultured in different media incubated at 37˚C. for 24 hrs. at pH 8.0; A: Casein–peptone medium. B: Horikoshi medium. C: Casein-yeast extract medium. [LSD Value: 0.05 = 0.493]

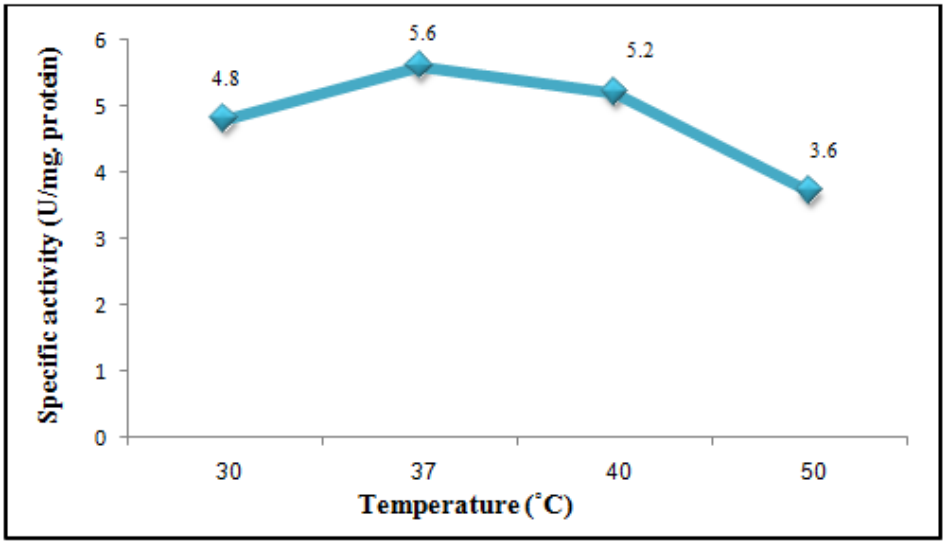

Figure 2: Protease production by *B. licheniformis* B1 cultured in casein-yeast extract medium pH 8.0 and incubated at different temperatures for 24 h.
[LSD Value: 0.05 = 1.02]

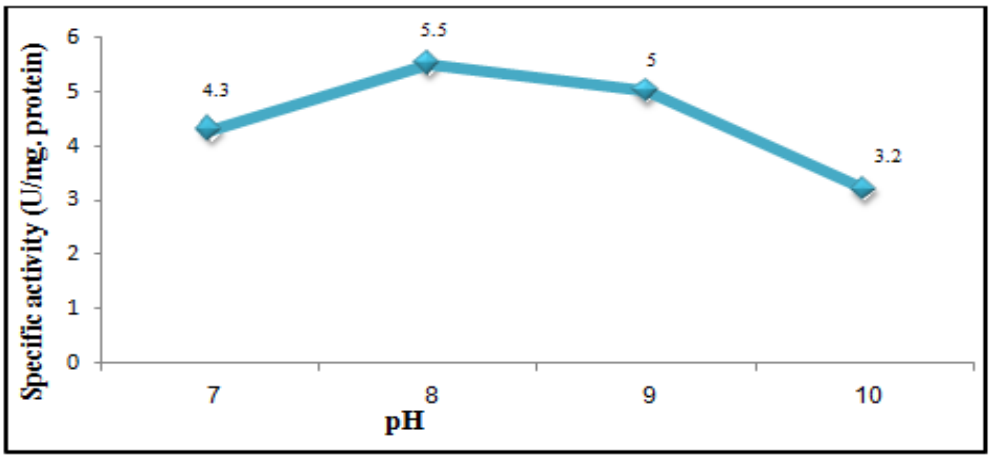

Figure 3: Protease production by *B. licheniformis* B1 cultured in casein-yeast extract medium prepared at different pHs and incubated at 37˚C. for 24h.
[LSD Value: 0.05 = 1.16]

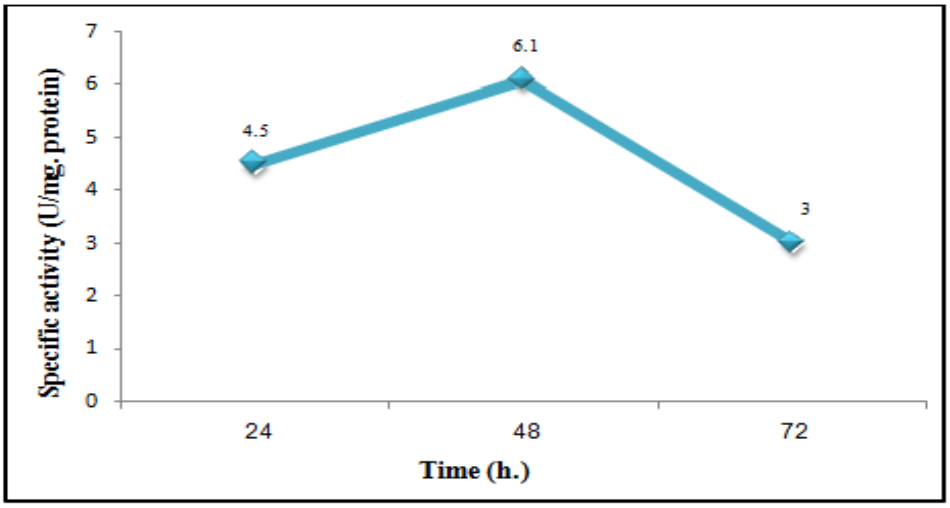

Figure 4: Protease production by *B. licheniformis* B1 cultured in casein-yeast extract medium prepared at pH 8.0 and incubated at 37°C for different times. [LSD Value: 0.05 = 1.55]

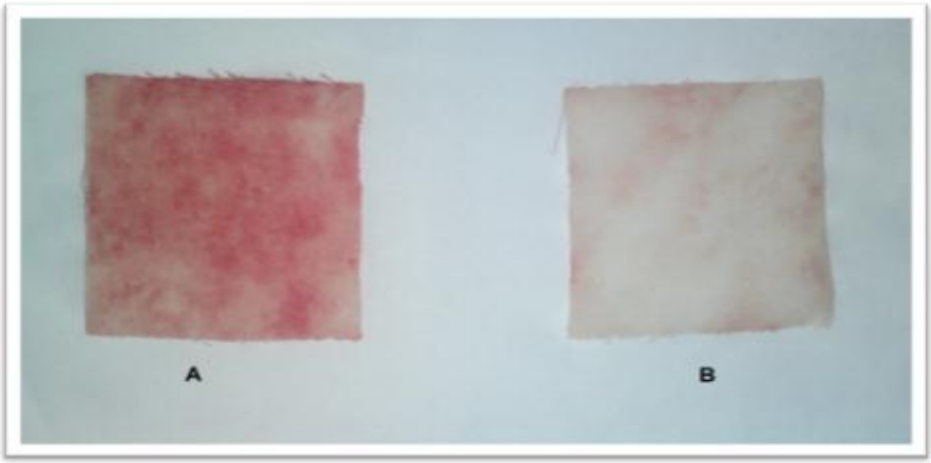

Figure (5): Removal of blood colors from cloth piece by crude protease of *B. licheniformis* B1. The blood-cotaining cloth piece was incubated with crude protease at 30°C for 30min. A, control (without enzyme); B, Sample treated with enzyme.

Figure (6): Removal of gelatin from X-ray films by crude protease of *B. licheniformis* B1. The X-ray films pieces were incubated with crude protease at 30°C. for 2h. A, control (without enzyme); B, Sample treated with enzyme.

References

1. Khan, M.A., Ahmad, N., Zafar, A.U., Nasir, I.A., Qadir, M.A. (2011) Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme. Afr. J. Biotechnol. 10(33): 6203-6212.
2. Jon, S. (2008) Global Industry Analysts. www.theprofitdepot.com.
3. Deng, A., Wu, J., Zhang, Y., Zhang, G., Wen, T. (2010) Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. Bioresource Technol. 101(18): 7100-7106.
4. Joo, H.S., Chang, C.S. (2005) Production of protease from a new alkalophilic*Bacillus sp.* I-312 grown on soybean meal: optimization and some properties. Proc. Biochem. 40: 1263-1270.
5. Kocher,G.S., Mishra, S. (2009) Immobilization of *Bacillus circulans* MTCC 7906 for enhanced production of alkaline protease under batch and packed bed fermentation conditions. Int. J. Microbiol. 7: 359-378.
6. Shumi, W.M., Hossain, T., Anwar, M.N. (2004) Proteolytic activity of a Bacterial isolate *Bacillus fastidiosus* den Dooren de Jong .JBS. 4: 370-374.
7. Logan, N. A., De Vos, P. (2009) Genus I. *Bacillus* Cohn 1872. p. 21-128. In: De Vos, P. (eds) Bergey's Manual of Systematic Bacteriology, 2nd edn, Vol 3. Springer, New York.
8. Sharma, J., Singh, A., Kumar, R., Mittal, A. (2006) Partial purification of an alkaline protease from a new strain of *AspergillusOryzae* AWT 20 and its enhanced stabilization in entrapped Ca-alginate beads. Int. J. Microbiol.2 (2).
9. Ramakrishna, D.P.N., Gopi, N.R., Rajagopal, S.V. (2010) Purification and properties of an extra cellular alkaline protease produced py*Bacillus Subtilis* (MTTC N0-10110). Int. J. Biotechnol. Biochem. 6(4): 493-504.
10. Anson, M.L. (1938) The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. J. Gen. Physiol. 20: 79-89.
11. Horikoshii, K. (1999) Extracellular enzymes isolation procedures for alkaline enzymes. In: Alkaliphiles.Kodensha Ltd. Tokyo.147.

12. Rao, K., Narasu, M.L. (2007) Alkaline protease from *Bacillus firmus* 7728. Afr. J. Biotechnol. 6 (21):2493-2496.
13. SAS .2004. SAS User's Guide :Statistics Version .7.0 , SAS Institute . Inc. Cary, NC. USA.
14. Slepecky, R.A., Hemphill, H.E. (2006) The genus Bacillus-nonmedical, P.530–562,.in: Dworkin, M. (eds) The Prokaryotes, A Handbook on the Biology of Bacteria:, Third Edition, Volume 4. Springer, New York.
15. Nunes, A.S., Martins, M.L.L. (2001) Isolation, properties, and kinetics of growth of a thermophilic*Bacillus*. Braz. J. Microbiol. 32:271–275.
16. Arulmani, M., Aparanjini, K., Vasanthi, K., Arumugam, P., Arivuchelvi, M., Kalaichelvan, P. T., (2007) Purification and partial characterization of serine protease from thermostablealkalophilic*Bacillus laterosporus*-AK1. World J. Microbiol. Biotechnol. 23:475–481.
17. Genckal, H., Tarib, C. (2006) Alkaline protease production from alkalophilic*Bacillus* sp. isolated from natural habitats. Enzyme and Microbial Technology 39:703–710.
18. Sakai, K., Yamanami, T. (2006) Thermotolerant*Bacillus licheniformis*TY7 produces optically active L-lactic acid from kitchen refuse under open condition. J. Biosci. Bioen. 102 (2): 132–134.
19. He, L., Chen, W., Liu, Y. (2006) Production and partial characterization of bacteriocin-like pepitdes by *Bacillus licheniformis* ZJU12. Microbiol. Res. 161: 321-326.
20. Boominadhan, U., Rajakumar, R., Sivakumaar, P. K. V., Joe, M. M. (2009). Optimization of protease enzyme production using *Bacillus* Sp. isolated from different wastes. Botany Res. Int. 2(2): 83-87.
21. Ghafoor, A., Hasnain, S. (2009) Production dynamics of *Bacillus subtilis* strain AG-1 and EAG-2, producing moderately alkaline proteases. Afr. J. Microbiol. Res. 3 (5): 258-263.
22. Uyar, F., Porsuk, I., Kizil, G., Yilmaz, E.I. (2011) Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15. Eur-Asia J. BioSci. 5: 1-9.
23. **Prescott**, L. M., Harley, J., Klein, D.A. (2005) Microbiology.(6th Ed.).Published by McGraw Hill. New York.
24. Martins, M. L., Teodoro, C.E. (2000) Culture condition for the production of thermostable amylase by *Bacillus* sp. Braz. J. Microbiol. 31(4): 982-302.
25. Nadeem, M., Qazi, J.I., Baig, S., Syed, Q. (2007) Studies on commercially important alkaline protease from *Bacillus licheniformis*N-2 isolated from decaying organic soil. Tur. J. Biochem. 32: 171-177.
26. Ray, M.K., Devi, K.U., kumar, G.S., Shivaji, S. (1992) Extracellular protease from yeast *Candida humicola*. Appl. Environ. Microbiol. 58:1918–1923.
27. Puri, S., Khalil, O., Gupta, R. (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr. Microbiol. 44:286–90.
28. Ashgher, M., Javaid-Asad, M., Rahman, S.U., Legge, R.L. (2007) A thermostable α-amylase from a moderately thermophilic*Bacillus subtilis* strain for starch processing. J. Food Eng. 79: 950–955.

29. Mona, A., Esawy, W., Helmy, A., Samia, A., Yannick, C. (2007) Natural material role in production, activation and stabilization of alkaline protease produced from a new isolated *Geobacillus caldoxylosilyticus*. J. App. Sci. Res. 3(10): 106–109.
30. Sayem, S.M.A., Alam, M.J., Hoq, M.M. (2006) Effect of temperature, pH and metal ions on the activity and stability of alkaline protease from novel *Bacillus licheniformis* MZK03. Proc. Pakistan Acad. Sci. 43(4):257-262.
31. Mona, K., Goud, A. (2006) Optimization and purification of alkaline proteases produced by marine *Bacillus* sp. MIG newly isolated from eastern harbour of Alexandria. Polish J. Microbiol. 55(2):119–126.
32. Nguyen, T.T, Quyen, D. T. (2011) Overproduction of an extracellular protease from *Serratia* Sp. DT3 Just Using Soybean Powder. World J. Agric. Sci. 7 (1): 29-36.
33. Olajuyigbe, F. M, Ajele, J.O. (2011) Some properties of extracellular protease from *Bacillus licheniformis*Lbbl-11 isolatedfrom "iru", a traditionally fermented African Locust Bean Condiment. Global J. Biotechnol Biochem. 3 (1): 42-46.
34. Dodia, M.S, Bhimani, H.G., Rawal, C.M., Joshi, R.H., Singh, S.P. (2008) Salt dependent resistance against chemical denaturation of alkaline protease from a newly isolated halophilic Bacillus sp. Bioresour. Technol. 99: 6233-6227.
35. Nadeem, M., Qazi, J.I., Baig, S., Syed, Q. (2008) Effect of medium composition on commercially important alkaline protease production by *Bacillus licheniformis*N-2.Food Technol. Biotechnol. 46(4): 388- 394.
36. Gehan, M., Abou-Elela, G.M., Ibrahim, H.A.H., Hassan, S.W., Abd-Elnaby, H., El-Toukhy, N.M.K. (2011) Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa-Matrouh(Egypt) with special emphasis on *Bacillus cereus* purified protease. Afr. J. Biotechnol. 10(22): 4631-4642.
37. Vishalakshi. N., Lingappa, K., Amena, S., Prabhakar, M., Dayanand, A. (2009) Production of alkaline protease from *Streptomicesgulbargensis* and its application in removal of blood stains. Indian J. Biotechnol. 8: 280-285.
38. Shankar, S. (2010) Stability of protease in organic solvents, detergents and denaturants.Ph.D. Thesis, University of Pune.
39. Vijayalakshmi, S., Venkatkumar, S. Thankamani, V. (2011) Screening of alkalophilicthermophilic protease isolated from *Bacillus* RV.B2.90 for Industrial applications. Res. Biotechnol. 2(3): 32-41.