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Full Length Research Paper

Thermo-alkaliphilic halotolerant detergent compatible protease(s) of *Bacillus tequilensis* MTCC 9585

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An extra-cellular thermo-alkaliphillic protease producing Bacillus was isolated from the soil and identified to be *Bacillus tequilensis* MTCC 9585 by microscopic, colony morphology, biochemical and 16S ribotyping. *B. tequilensis* MTCC 9585 produces protease up to 21 h of growth but interestingly 90% of the protease production occurred, just after 6 h of growth. The organism grew as well as and produced enzyme at wide pH (5 to 12) and temperature range (4, 25, 37 and 50°C), though optimum temperature and pH for the growth of the Bacillus were 37°C and pH 7.0. Optimum pH for enzyme activity coincided with optimum pH for enzyme production at pH 10. Optimum temperature for enzyme activity was 60°C and the enzyme stayed stable over the period of 270 days (9 months) at 10°C. Metal ions like Ca^{2+}, Mg^{2+}, K^{+} increased the enzyme activity whereas Cu^{2+}, Zn^{2+} inhibit the activity slightly. Wash performance and stain removal efficiency increased when partially purified enzyme was used in conjunction with selected detergent. *B. tequilensis* can be a potential candidate for use in detergent industry because: of couple of reasons such as (i) 90% of the protease is produced only after 6 h of growth (economically viable), (ii) it's activity in wide pH and temperature range (iii) it's stability over the period of 9 months at 10°C indicating good shelf life and (iv) detergent compatibility.

Key words: *Bacillus tequilensis*, alkaline protease, pH, temperature, detergent compatibility.

INTRODUCTION

Commercial proteases are produced by various bacteria and about 35% of the total microbial enzymes used in detergent are from bacterial sources (Huang et al., 2006). Bacillus in particular has been studied for the production of proteases in general and in special reference to detergent proteases. Various species of Bacillus produce a variety of extracellular and intracellular protease (Beison et al., 2000; Huang et al., 2003; Nascimento et al., 2006; Rao et al., 2007). Conventionally, detergents are used at elevated washing temperatures, but currently there is considerable interest in the identification of alkaline proteases, which are effective over a wide temperature and pH range. Bacillus is otherwise an attractive microbial producer for variety of reasons, including their high growth rates leading to short fermentation cycles, production of extracellular proteins and are regarded as GRAS (Generally regarded as safe) industrial tool. Subtilisin (Carlsberg) produced by *Bacillus licheniformis* and Subtilisin Novo produced by *Bacillus amyloliquefaciens* have been the enzymes of choice in detergent so far (Horikoshi, 1990). Complexity of the patent rules and development of new detergent formu-lations have led to renewed interest in search of novel enzymes from nature. Here we report isolation of thermostable, alkaliophilic protease produced by *Bacillus tequilensis* MTCC-9585 and its stability as well as the activity in the presence of commercial powered detergents.

MATERIALS AND METHODS

Bacterial strain and the media used

The *Bacillus* was isolated from the agriculture soil of R.S. Pura Jammu, J and K, India and deposited in culture repository at IMTech Chandigarh (India). Luria Bertani medium was used for routine growth and maintenance of the bacterium at 37°C in BOD incubator. Protease assay media was used to monitor the protease production qualitatively (Usharani et al., 2010). The diluted samples were plated on the production media plates and incubated in BOD at 37°C for 12 to 16 h for the formation of zone of clearance

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showing hydrolysis of skim milk.

Protease estimation assay

The protease activity was measured by method given by Sarath et al. (1989).

Bacterial identification

Identification of the bacteria was done by colony morphology, microscopic and biochemical analysis (Sneath, 1986). The results were confirmed by 16S ribotyping analysis done at culture identification centre at IMtech Chandigarh.

Protease production profile vis- a- vis growth

An overnight grown colony was inoculated in 300 ml broth and kept in incubator shaker at 37°C with constant shaking of 220 rpm. After every 3 h, 1 ml sample was collected under sterile conditions and stored on ice. The growth was measured by measuring optical density of sample (diluted 10 fold in LB broth) at 600 nm and protease produced was measured by protease estimation method. The generation time was calculated by the method given by Cappuccino and Sherman (2007).

Effect of pH and temperature on growth and production of enzyme

Effect of pH on the protease production and bacterial growth was determined by growing the bacterial isolate on protease assay media in the pH range of 5 to 12 prepared in appropriate buffer e.g. 50 mM Sodium acetate (pH range 4.5 to 5.5), 50 mM Potassium (pH range 6 to 7.5), 50 mM Tris Cl (pH range 7.5 to 9), 50 mM NaOH-Glycine buffer (pH range 9.5 to 10) and 50 mM Na_2HPO_4-NaOH pH range (10 to 12) for 21 h at 37°C in incubator shaker at 220 rpm. To study the effect of temperature on the production of enzyme and growth bacterial isolate was grown in protease production media at various temperatures 4, 25, 37 and 50°C for 21 h at pH 7 in incubator shaker with 220 rpm. The growth and protease production by the *Bacillus* was measured by the methods already described.

Effect of pH on the enzyme stability and activity

The effect of pH on the stability of isolated protease was determined incubating the 100 µg of 60% ammonium sulphate precipitate fraction of crude extract in 500 µl of buffers with different pH (from 5 to 12) for 30 min at 37°C. The stability profile of the enzyme under various pH was determined by estimating the residual protease activity (Sarath et al., 1989). The optimal pH for the enzyme activity was determined by carrying out enzyme assay at various pH in the range of 5 to 12 by using buffers mentioned above at 60°C (temperature optima).

Effect of temperature on the enzyme stability and activity

Effect of temperature on protease activity was determined by incubating the reaction mixture containing substrate and enzyme for 30 min in Tris-HCl buffer (pH 10) at different temperatures ranging from 4 to 80°C and the optimal temperature for enzyme activity was measured by performing the enzyme assay at various temperatures in Tris-HCl buffer (pH 10). Enzyme activity was measured quantitatively by hydrolysis of azo-casein (Sarath et al., 1989).

Effect of metal ions on the enzyme activity

The effect of metal ion the protease activity was determined by incubating 10mM solution of different salts ($CuSO_4$, $ZnSO_4$, $CaSO_4$, MgCl2, KCl) at 60°C for 30 min with reaction mixture containing substrate and enzyme in Tris-HCl buffer (pH 10) and the activity was measured subsequently.

Compatibility with detergents and enhancement of their de-staining properties

Three detergent brands namely Surf excel, Ariel, Tide were used for studying compatibility of alkaline protease under buffered and normal conditions. Detergent solutions were prepared as per directions given on their respective sache. Casein solution (0.2% w/v) was used as substrate, prepared either in buffer (carbonate-bicarbonate buffer, 0.1 M, pH 9.5) that is for buffered conditions or in distilled water that is for normal conditions of assay. Both buffered and non-buffered solutions were used in reaction mixture comprising of 2 ml of casein solution, 5 mg/ml of detergent solution and 0.040 mg/ml of alkaline protease. The reaction mixture was incubated at optimized conditions for 60 min followed by protease assay. Controls were taken which comprised of assay mixture without detergent under similar conditions. The de-staining property was studied by dipping pieces of cloth artificially stained with blood either in detergent solution or detergent solution supplemented with enzyme followed by incubation for 10 min at 60°C.

RESULTS AND DISCUSSION

Commercial detergents available anywhere in the world are mixture of detergent and stain digesters, to which hydrolases in general and proteases in particular contribute a lot. Mesophilic bacterium, *B. tequilensis* MTCC 9585 producing thermophillic alkaliphillic proteases was isolated from the agricultutral soil of R.S. Pura Jammu, J and K, India.

Screening, characterization and growth properties

The *Bacillus* is a fast growing bacterium, as it reaches its stationary phase with in 24 h with log phase extending from 6 to 21 h. The generation time calculated using method given by Cappuccino and Sherman (2007) is 3 h. The *Bacillus* was identified morphological, microscopically and on the basis of 16S ribotyping as *B. tequilensis* and was given accession number 9585 by IMtech, Chandigarh, India (Table 1).

B. tequilensis MTCC 9585 was characterised as best protease producer out of 150 microbes screened on basis of the size of the clearing halo formed on agar diffusion assay plate containing milk casesin (0.5%) as substrate. *Bacillus species* is a common producers of proteases that is proteases are isolated from *Bacillus pumilus* (Huang et al., 2003), *Bacillus firmus* 7728 (Rao et al., 2007), *Bacillus* sp (Oberoi et al., 2001),

Table 1. Comparative account of Bacillus proteases.

No	Name	Growth in h	Enzyme Production (h)	Optimum Temperature (°C)	Optimum pH	Temperature stability (°C)	pH stability	References
1	*B. pumilus*	36	16-28	55	10	30-60	6-11	Huang et al., 2003
2	*B. licheniformis* LBBL-1	48	48	60	8	60	5-11	Olajuyigbe and Ajele., 2008
3	*B. circulans*	96	96	60	9	40-65	8-11	Jaswal et al 2007
4	*B. firmus*7728	72	48	40	9	20-55	4-12	Rao and Narasu., 2007
5	*B. clausi* GMBAE42	72	72	60	11.3	30-40	9 - 12.2	Kazan et al, 2005
6	*B. sp.po2*	24	24	37	8	37	7 - 9	Patel et al., 2006
7	*B. subtilus* PE-11	48	-	60	10	60	8-10	Kunaminini et al., 2003
8	*B. halodurans*	48	48	70	10	65-75	8-11	Ibrahahim et al., 2007
9	*B. tequelensis* MTCC 9585	21	6	60	10	16-80	7-11	This study
10	*Bacillus strain* HSO8	16	16	65	7.5	40-65	7 - 9	Huang et al., 2006
11	*Bacillus sps*	16	-	45	10.7	-	-	Khorsan et al., 2008
12	*B. stearothermo*philus AP4	36	-	55	9	-	-	Dhandapani et al., 1993
13	*B. sp B-21*	-	-	55–60	-	-	-	Rahman et al., 1994
14	*B. sp* RGR-14	-	36	45-70	11	20-80	-	Oberoi et al., 2001
15	*B. cereus* MCMB-326	-	-	55	9	25-65	6-12	Nilegaonkar et al., 2007

B. Licheniformis AP-1 (Tang et al., 2004), *Bacillus clausii (*Kazan et al., 2005), *Bacillus Circulans* (Jaswal et al., 2007), *B. licheniformi* (Olajuyigbe et al., 2005) and have been already characterized and the list of *Bacillus* producing the protease and the variety of proteases produced by them is quite vast. Emphasis is laid on the high temperature or pH proteases producing *Bacillus* as this is primary requirement of washing procedure but now because of energy concerns detergent enzymes should also remain active in wide temperature and pH range for cold as well as hot washes. Protease produced by *Bacillus* are mainly found to be active in the temperature range of 40 to 60°C though *Bacillus* Sp. RGR-14 produce protease that is stable in temperature range of 20 to 80°C and that of *B. cereus* MCMB -326 is stable in the temperature range of 25 to 65°C (Nilegaonkar et al., 2007; Oberoi et al., 2011). Thermophillic alkali-stable proteases from *B. tequilensis* in present study have advantage of showing 40% activity at 16°C and 45% at 80°C (Figure 5). In the present study protease production time which is after 6 h of incubation is best in comparison to the proteases production profile of Bacilli known so far. The proteases production starts with the beginning of log phase, just after 6 h of incubation and extends into stationary phase with 10% increase at 21 h of growth (Figure 1). There could be couple of explanations' for this. Enzyme production starts at the beginning of the log phase but gets degraded or inactivated simultaneously by other biomolecules being produced by the *Bacillus* or else the enzyme production is inhibited by feed back but is stable for the rest of the growth period. So far we have not come across any report in literature where in 90% of any enzyme is produced at beginning of the log phase by any *Bacillus* species. Most of the species of Bacillus produce protease after 48 h of growth (comparison is drawn in Table 2). Though there are reports wherein thermophilic Bacillus strain HSO8 and *Bacillus* sp. show growth and enzyme production after 16 h of growth (Huang et al., 2006, Darani et al., 2008). Cellular localization of the protease suggested it to be an extracellular enzyme and this result is in conformation of other *Bacillus proteases* (Kunamneni et al., 2003, Huang et al., 2006, Darani et al., 2008).

Effect of pH and temperature on growth and enzyme production

B. tequilensis MTCC 9585 grows in the pH range of 5 to 12 but is a mesophile. It shows maximum growth at pH 7 where as optimum pH for enzyme

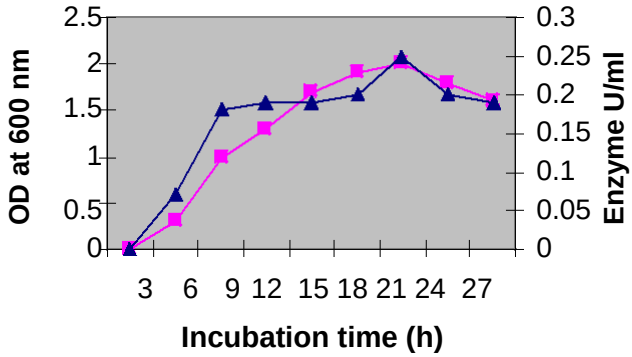

Figure 1. Growth Kinetics along with crude protease production of *Bacillus tequilensis* Samples were withdrawn at 3 h intervals for the determination of cell growth (A_{600}) (■) and protease production (▲).

production is 10. The reason for getting enhanced production could be attributed to the fact that the enzyme is alkaliphillic. The increase in the activity could be due to optimal activity of the enzyme at 10 pH or else optimal enzyme is production at this pH. This Bacillus strain retains 50% production and 40% growth at pH 11 and 40% production and 33% growth at pH 12 (Figure 2). There are reports ofprotease production at pH 10.7 by *Bacillus* sp (Darani et al., 2008) at pH 10.5 by *B. clausii* (Kazan et al., 2005) and at pH 10 by *B. pumilus* (Huang et al., 2003).

Four temperatures 4, 25, 37 and 50°C were selected to study their effect on growth and enzyme production by this *Bacillus*. B. *tenquilensis* is a mesophile vis a vis temperature also as it shows maximum growth at 37°C with about 50% of growth occurs at 50°C. Enzyme production is maximum of 37°C though about 30, 50 and 40% enzyme production is at 16, 50 and 60°C, respectively. This result is in conformity to most known bacillus proteases (Figure 3).

Effect of pH and Temperature on the activity of proteases

The maximum protease activity of partially purified was recorded at pH 10 but 87% of the activity was retained at pH 11 and pH 12. Interestingly the protease is stable in the pH range of 7 to 10. Huang and his group (2003) have reported extracellular protease from Bacillus which also shows highest activity at pH 10. There are several other reports of alkaliophillic protease production from *Bacillus sp*, *Thermus aquaticus; Xanthomonas maltophila; Vibrio metscnikovii and bacillus* Sp. (Durham et al., 1987; Debette, 1991; Kwon et al., 1994; Matsuzawa et al., 1998; Darani et al., 2008). However this enzyme is interesting as it shows more than 80% activity at alkaline pH of 11, 12 and is most stable at pH 10 and is also active at this pH (Figure 4).

The enzyme in the crude extract shows activity in wide temperature range retaining 40% of the activity retained at two extreme temperatures of 16 and 80°C with maximum activity shown at 60°C. The stability profile indicates that the enzyme is most stable at 37°C (Figure 5). There are many comparative reports of thermophillic and thermostable bacilli such as *B. subtilis* PE-11 (Kunamneni et al., 2003) which has temperature optima at 60°C, *B. stearothermophilus* AP-4 and *Bacillus* sp. B21-2 protease with temperature optima of 55 and 60°C (Rahman et al., 1994) and bacillus strain HS08 where optimal activity was found at 65°C (Huang et al., 2006). Comprehensive comparison is drawn in Table 1, where it's adavantage over commonly used proteases produced by *B. licheniformis* and *Bacillus stearothermophilus* is quite evident as it is alkaliophilic and shows activity in temperature range of 16 to 80°C.

Effect of metal ions

Protease from *B. tequilensis* is a metallo- protease as metal ions affect its function. The activity of the enzyme is inhibited by Cu^{2+} and Zn^{+} ion but its activity is promoted by K^{+}, Ca^{2+} and Mg^{2+} (Figure 6). The activity of alkaline serine protease from *B. pumilus* is enhanced by Ca^{2+}, $Mg^{2+}$and Na^{+} where as Cu^{2+} and Zn^{2+} inhibit the activity slightly (Huang et al., 2003). In contrast to our results Zn^{2+} does not inhibit activity of the protease produced by *B. stearothermophilus* (Dhandapan et al., 1994). The cations have been reported to increase the thermal stability of the Bacillus proteases. Nevertheless there are reports where neither metal ions nor EDTA showed any significant effect on the enzyme activity (Kunamneni et al., 2003).

Wash test performance and shelf life of protease

The visual examination of pieces of cloth (cotton) with various detergent and detergent along with crude enzyme

Table 2. Morphological, physiological and biochemical characteristics *B.tequelensis* MTCC 9585.

Character	Results
Colony morphology	
Configuration	Round
Margin	Entire
Elevation	Raised
Surface	Smooth
Pigment	Cream
Opacity	Opaque
Microscopic analysis	
Gram's Reaction	**+**
Cell Shape	Rods
Size (µm)	1-4 µm
Spores	**+**
Position	**Central**
Shape	**Oval**
Sporangia bulging	**Non bulged**
Motility	**+**
Growth parameters	
Aerobic conditions	+
Anaerobic conditions	Less growth
Optimum temperature	37°C
Optimum pH	7
Biochemical analysis	
Growth on MacConkey	Nil
Indole test	-
Methyl red test	-
Voges Proskauer Test	+
Citrate utilization Test	+
H2S production	-
Gas production	-
Casein hydrolysis	-
Starch hydrolysis	-
Urea hydrolysis	+
Nitrate reduction	+
Arginine dihydrolase	-
Catalase test	+
Oxidase Test	+
Tween 40, 80 hydrolysis	+
Acid production from	
Dextrose	+
Maltose	+
Xylose	+
Mannitol	+

shows washing and stain removal efficiency of the detergent increases by adding the enzyme (Figure 7). Many workers have tested efficiency and compatibility of the specific protease production with detergents available in the market, in presence of stabilizers like $CaCl_2$ and glycerin (Kunamneni et al., 2003). The specialty of our

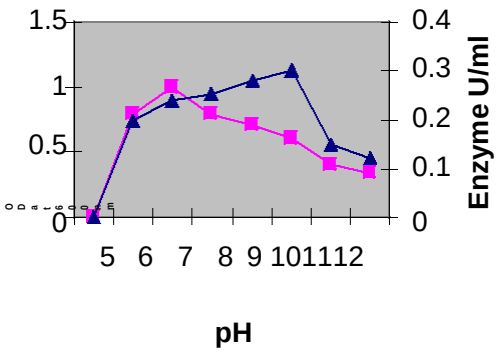

Figure 2. Effect of pH on growth and protease production of *Bacillus tequilensis* Samples were taken after 21 h at 37°C under shaking conditions (100 rpm), for the determination of cell growth (A_{600}) (■) and protease production (▲)

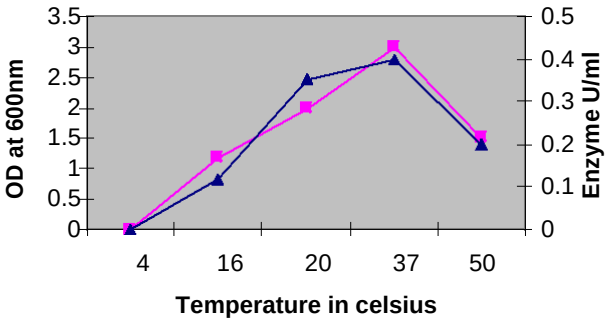

Figure 3. Effect of temperature on growth and protease production of Bacillus *tequilensis.* Samples were taken after 21 h under shaking conditions (100 rpm), for the determination of cell growth (A_{600}) (■) and protease production (▲)

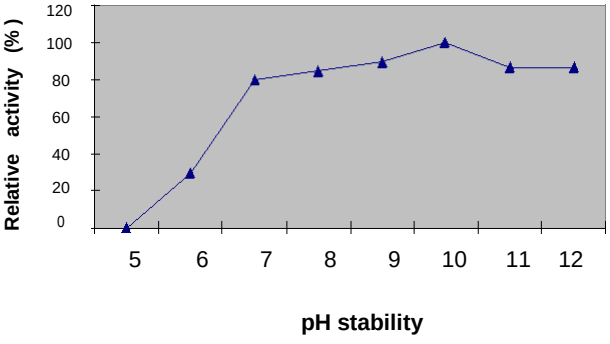

Figure 4. pH stability profile of protease/s in cell free extract of *B. tequilensis*

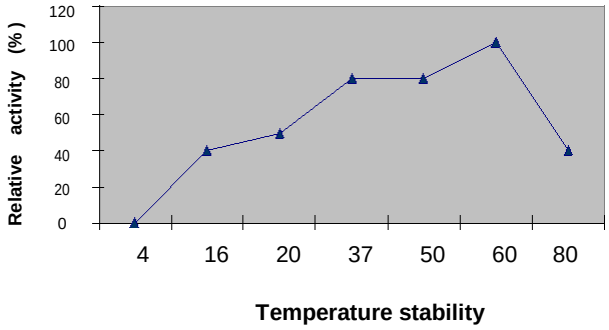

Figure 5. Temperature stability profile of protease/s in cell free extract of *B. tequilensis.*

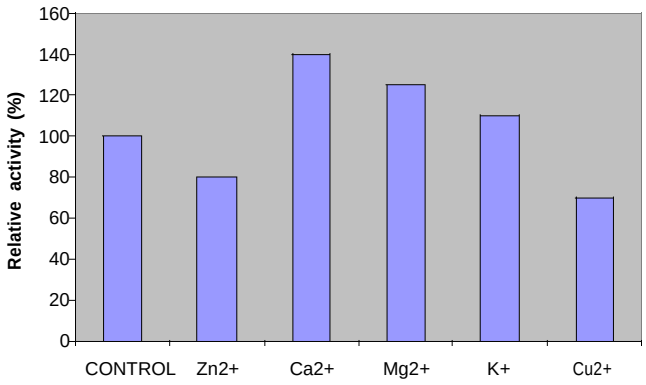

Figure 6. Effect of metal ions on the activity protease/s in cell free extract of *B. tequilensis.*

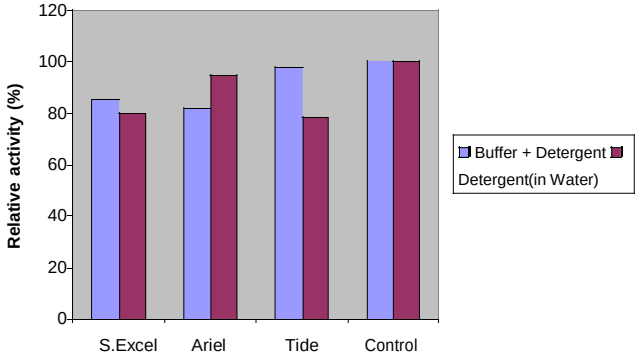

Figure 7. Compatibility of crude protease/s of *Bacillus tequilensis* with commercial detergents. Enzyme is incubated with detergent (either in buffer or water).

enzyme is that it retains its activity in hot as well as cold water depending upon the requirement and in addition its shelf life is longer in comparison to known proteases. The three detergents were already containing the enzyme as quoted by their manufacturers. Therefore, their suitable controls were also run and their activities were found to be very low compared to those obtained by supplementation with crude proteases of *B. tequilensis*. The crude enzyme preparation when stored at 10˚C was found to stable over the period of 9 months (Figure 8).

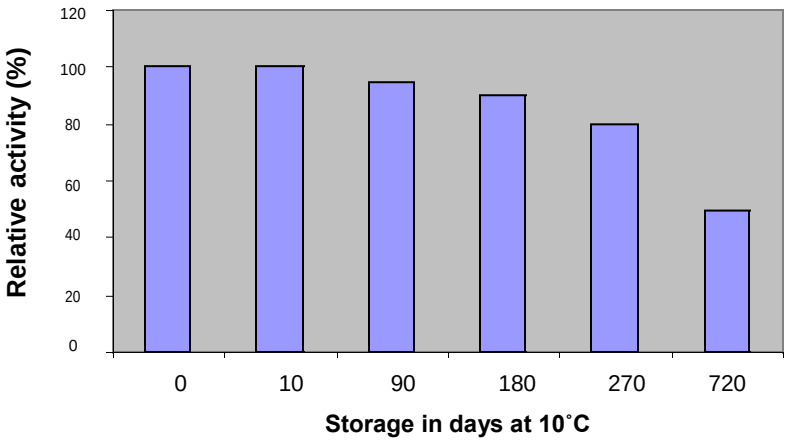

Figure 8. Effect of storage time (in days) at 10˚C on the protease/s activity of *Bacillus tequilensis.*

This revealed that the enzyme may be suitable supplement to detergents.

Conclusion

The production of 90% enzyme just after 6 h of growth, the fast growth rate of Bacillus, production of protease that is alkaliphilic and thermophilic but protease showing activity in vast temperature range with compatible destaining properties gives this enzyme an edge over commonly used proteases in the detergent formulations.

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REFERENCES

Beison F, Tiss A, Riviere C, Verger R (2000). Methods of lipase detection and assay: a critical review. European J. Lipid Sci. Technol., 102: 133-153.

Cappuccino JG, Sherman N (2007). Microbiology: A Laboratory Manual, Ed. 8.

Darani KK, Falahatpishe HR, Jalali M *(*2008). Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. Afr. J. Biotechnol., 7(10): 1536-1542.

Debette J (1991). Isolation and characterization of an extracellular proteinase produced by a soil strain of *Xanthomonas maltophila*. Curr. Microbiol., 22: 85-90.

Dhandapani R, Vijayaragavan R (1994). Production of thermophilic extracellular alkaline protease by *Bacillus stearothermophilus*. Ap-4. World J. Microbiol. Biotechnol., 1: 33-35.

Durham DR, Stewart DB, Stelwag EG (1987). Utility of subtilisin GX as a detergent additive. J. Bacteriol., 169: 2762-2768.

Horikoshi K (1990). Enzymes of alkalophiles In: Microbial Enzymes and Biotechnology (second edition). St. Louis, MO: Elsevier Appl. Sci., 275-294.

Huang GR, Ying TJ, Huo P, Jiang JX (2006). Purification and characterization of a protease from *Thermophilic bacillus* strain HS08. Afr. J. Biotechnol., 5: 2433-2438.

Huang Q, Peng Y, Wang LH, Zhang Y (2003). Purification and characterization of an extracellular alkaline serine protease with dehairing function from *B. pumilus*. Curr. Microbiol., 46: 169-73.

Ibrahahim ASS, Nefisa MA, Shayeb EI, Mabrouk SS (2007). Isolation and Identification of Alkaline Protease Producing Alkaliphilic Bacteria from an Egyptian Soda Lake J. Appl. Sci. Res., 3: 1363-1368.

Jaswal RK, Kocher GS (2007). Partial characterization of a crude alkaline protease from *Bacillus circulans* and its detergent compatibility. Int. J. Microbiol., 4(1): 1-5.

Kazan D, Denizci AA, Oner MNK., Erarslan A (2005). Purification and characteriztion of serine alkaline protease from *Bacillus clausii* GMBAE 42. J. Ind. Microb. Biotech., 32(8): 335-344.

Kunamneni A, Elliaiah P, Prasad DS (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11AAPS Pharm. Sci. Tech., 4(4) article 56.

Kwon YT, Kim JO, Moon SY, Lee HH, Rho HM (1994). Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. Biotechnol. Lett., 16: 413-418.

Matsuzawa H, Tokugawa K, Hamaok M, Mizoguchi M, Taguchi H, Terada, Kwon S, Ohta T (1998). Purification and characterization of aqualysin I, a thermophilic alkaline serine protease produced by *Thermus aquaticus* YT-1Eur. J. Biochem., 171: 441-447.

Nascimento WC, Martins ML (2006). Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergent. Braz. J. Microbiol., 37: 307-311.

Nilegaonkar SS, Zambare VP, Kanekar PP, Dhakephalkar PK , Sarnaik SS (2007). Production and partial characterization of dehairing protease from Bacillus cereus MCM B-326. Bioresour. Technol., 98(6): 1238-1245.

Oberoi R, Beg QK, Puri S, Saxana RK, Gupta R (2001). Characterization and wash performance analysis of an SDS-resistant alkaline protease from a Bacillus sp. World J. Microbiol. Biotechnol., 97: 493-497.

Olajuyigbe FM, Ajele JO (2005). Production dynamics of extracellular protease from *Bacillus* species. Afr. J. Biotechnol., 4: 776-779

Patel RK, Dodia MS, Joshi RH, Singh SP (2006). Halo-alkaline Protease from a Newly Isolated Haloalkaliphilic *Bacillus sp*. Isolated from Seawater in Western India. World J. Microbiol. Biotechnol., 22(4): 375-382.

Rahman R, Razak C, Ampon K, Basri M, Yunus W, Salleh A (1994). Purification and characterization of a heat stable protease from *Bacillus stearothermophilus* F-1. Appl. Microbiol. Biotechnol., 40(6): 822-827.

Rao K, Lakshmi NM (2007). Alkaline Protease from *Bacillus firmus* 7728

Afr. J. Biotechnol., 6: 2493-2496.

Sarath G, Motte RS, Wagner FW (1989). Protease assay methods. In R.J Beynon and J.S Bond (eds), *Proteolytic enzymes*: (A practical approach), IRL PRESS, Oxford, New York: Tokyo.

Sneath PH (1986). Bergey's manual of systematic bacteriology (2). Baltimore: Williams and Wilkins.

Tang XM, Lakay FM, Shen W, Shao WL, Fang HY, Prior BA, Wang ZX, Zhuge (2004). Purification and characterisation of an alkaline protease used in tannery industry from *Bacillus licheniformis* J. Biotechnol. Lett., 26: 1421-1424.

Usharani B, Muthuraj M (2010). Production and characterization of protease enzyme from *Bacillus laterosporus* Afr. J. microbiol. Res., 4(11): 1057-1063.