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STUDIES ON PRODUCTION OF ALKALINE PROTEASES FROM *BACILLUS* SPECIES AND ITS APPLICATION IN DETERGENT INDUSTRY

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ABSTRACT
Fifty bacterial cultures were isolated from soil samples collected from village Malkondaji Dist. Latur. These were tested to produce alkaline proteases by using sterile milk agar plate. Out of 50 isolates 15 Isolates showed production of protease enzyme, which was identified as *Bacillus* species on the basis of biochemical characteristics. Crude enzyme obtains from starch casein broth showed optimum enzyme activity at pH10, 2 % of substrate concentration and temperature 60^0 C. This protease also showed promising results for the removal of blood and food stain from cloth.

KEY WORDS: Protease, *Bacillus* species.

INTRODUCTION

Owing to the industrial utilization of enzyme (Rao *et al.*, 1998) we decided to work on alkaline protease which an enzyme forming main bulk in industrial application (Rao *et al*., 1998). It constitute about 25% worldwide enzyme market in detergent industry ((Anwar *et al.*, 1998, Gupta *et al.*, 2002, Beg *et al.*, 2003, Horikoshi 1990). Generally alkaline are produce from microorganism using starch casein broth and can easily separate by downstream processing than plant and animal (Rao *et al*.,1998, Gupta *et al*., 2002). *Bacillus* is the dominant protease producer (Gupta *et al.*, 2002,Kumar and Takaji ,1999). The objective of present work to produce alkaline protease from soil isolates and apply it to remove blood and food stain as detergent application.

MATERIALS AND METHODS

Isolation of alkaline protease producer

The soil sample was collected from the farm area of the village Malkondji, Taluka Ausa, Dist. Latur, Maharashtra, India, were carried to laboratory in a polythene bag and kept in refrigerator for further use. (Ashokan *et al*; 2010 and Hamid Mukther Haq, 2008). 1% Soil sample is further diluted serially using sterile distilled water. Among which 10^6 dilution were used for further processing this dilutions were aseptically transferred on sterile nutrient agar plate and the plates were incubated at 37^0 C for 24 h.

Screening

After incubation the isolated bacterial colonies were spot inoculated aseptically on to the sterile skimmed milk agar plate (Deshmukh, 1997) and plates were incubated at 37^0C for 24h .After incubation the bacterial colonies with highest zone of casein hydrolysis are selected. Overall 15 colonies were selected and numbered as like 1, 2, 3…15, arranged according to the decreasing order of zone of hydrolysis by visual observations.

Inoculum preparation

50ml nutrient broth medium sterilized in a 250ml flask and were inoculated with 48h old culture of organism and the flask are kept on rotary shaker at 200 rpm at 37^0 C for 24h as inoculums.

Fermentation The 24h inoculums were subjected for the fermentation process and is carried out on laboratory scale by using starch casein broth media (Jeffery et al, 008) with pH 7.4-7.6 in 250ml flask on rotary shaker at 200 rpm at 37^0C for 24 h.

Enzyme assay The crude broth after centrifugation at 8000 rpm for 10 min. was used for protease activity study. The enzymatic assay was carried by using the method of MC Donald and Chan (1966) with certain modifications in time. Using this method the absorbance of 15 strains was measured among which strain 6 was selected for further study.

Optimization of various parameters and its estimation by Anson and Hegira method

The broth culture of 48h old was centrifuged and used as crude enzyme.

Effect of pH on enzyme activity

The activity of crude enzyme was studied at different pH buffer as pH from 7-9(phosphate buffer), pH 10 and10.34 (carbonate bicarbonate buffer) 1ml of enzyme and 1ml of buffer was kept for 20 min at 37^0C.

Effect of temperature on enzyme activity
The effect of various temperatures as 30^0C, 40^0C, 50^0C, 60^0C and 70^0C on enzyme activity were studied by above assay method.

Effect of incubation period on enzyme activity
The assay was carried out by verifying the incubation period for Enzyme and substrate activity. 0.1 ml of enzyme incubated along with 0.5 ml of substrate and 0.4 ml of buffer for different time span like 5 min, 10 min, 15 min , 20 min, 25 min, and 30 min, enzyme assay was carried out.

Effect of substrate concentration on enzyme activity
Same assay is carried out for different substrate concentration along with 0.1 ml of enzyme as incubated for 20 min with 0.5 ml of substrate with varying concentration (1%, 2%, 3%) along with 0.4 ml of buffer assay is carried out.

Application of crude enzyme as detergent
The separate cloth pieces were taken for pickle stain and blood stain and were labeled as A (control), B (enzyme treated), C (enzyme +buffer treated), D (detergent treated), and E (enzyme + detergent treated). These cloth pieces were incubated for 2h at room temp After the incubation period the cloth were washed with tap water and dried (Singh et al., 1999).

Identification of strain were done by microscopic and biochemical characterization.

RESULTS AND DISCUSSION
In present work 50 isolates were isolated from the soil sample of Malkondji village dist. Latur. (Asokan S. *et. al.*, 2010). All 50 isolates were screened for protease production, Among them 15 were selected and numbered as 1-15, serially on the basis of zone of hydrolysis of casein (Plate 1)by visual observation. (Deshmukh, 1997) All 15 isolates subjected to starch casein broth for fermentation and casein hydrolysis was measured by Mc Donald and Chen method (Jeffery, 2008 and Safey *et al.*) and absorbance were noted at 700nm (table1) by spectrophotometer (Chemito make spectra scan UV 2600 double beam UV vis).
According to highest absorbance the strain number 6 (S6) were selected for further use.

Plate 1. Zone of hydrolysis by strain no. 6 on milk agar
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Table1. Absorbance of isolated strain

Strain number	Absorbance at 700nm
1	0.2318
2	0.1961
3	0.1500
4	0.1480
5	0.1721
6	0.3740
7	0.1209
8	0.1608
9	0.2697
10	0.2430
11	0.0539
12	0.1265
13	0.1423
14	0.0325
15	0.1534

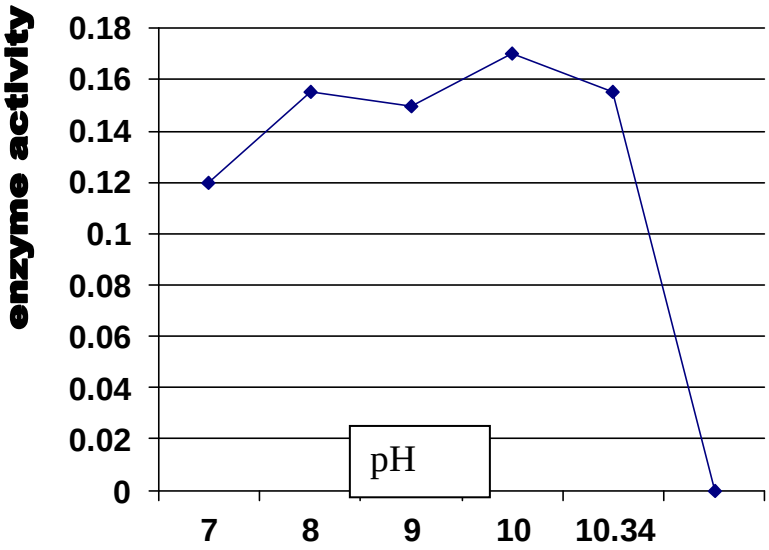

Figure 1. Effect of pH on enzyme activity

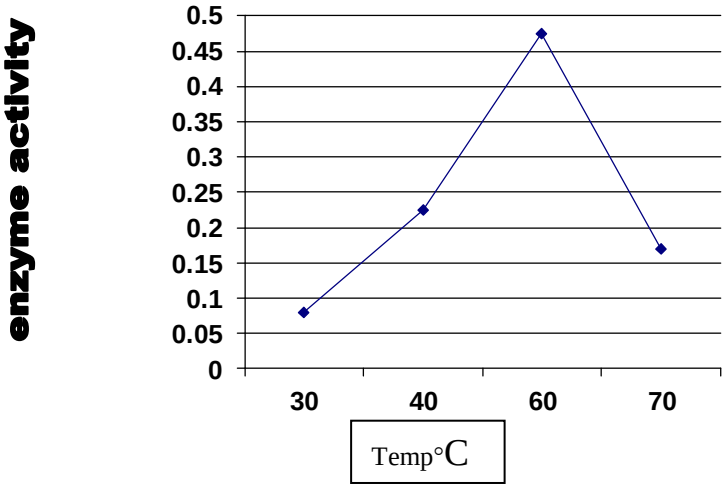

Figure 2. Effect of Temperature on enzyme activity

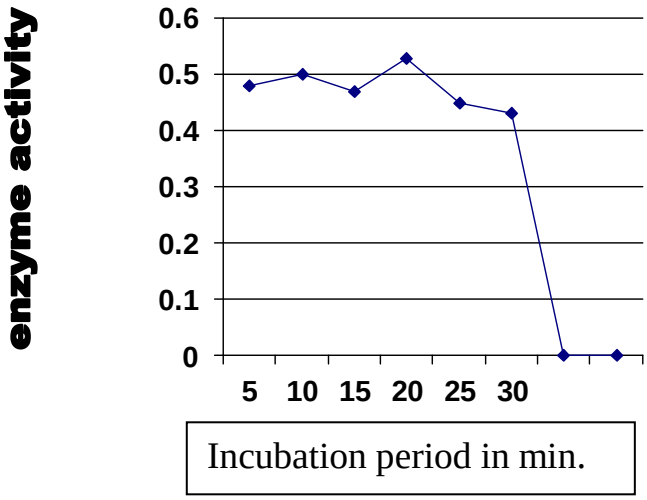

Figure 3. Effect of incubation period on enzyme activity

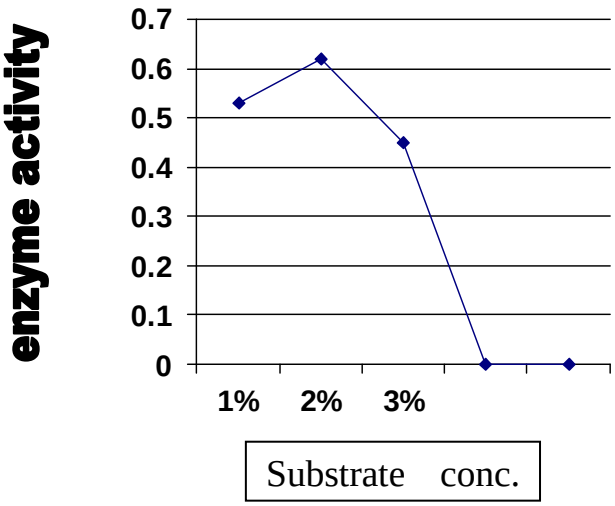

Figure 4. Effect of substrate conc. On enzyme activity

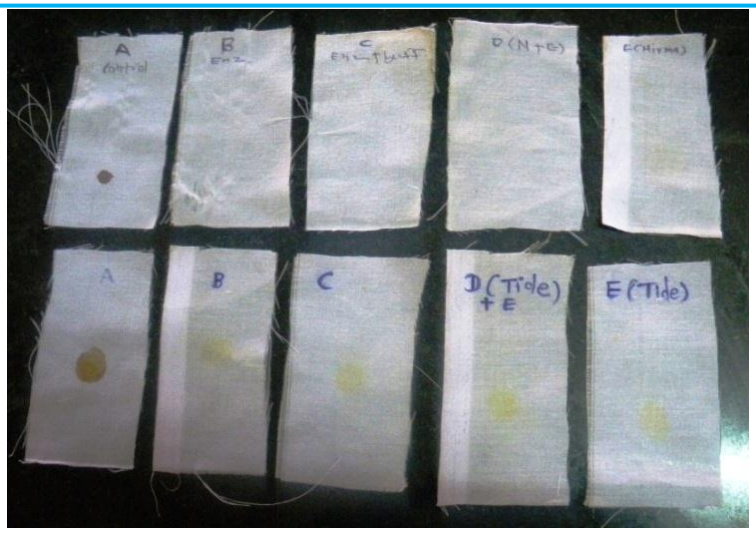

Plate 2. Blood and Food stain removing application
Note: 1st row blood spot ,2nd row food (pickle) spot
A (control), B (enzyme treated), C (enzyme +buffer treated),
D (detergent treated), and E (enzyme + detergent treated).

Table 2. Identification of the strain by microscopic,and biochemical test.

Characters		Biochemicals			
	Microscopic	Sugar fermentation	Some other biochemicals		
Test with results	Gram's nature-Gram positive rod	Lactose-positive	VP test-positive	Macconkey agar-positive	_
		Sucrose-positive	Citrate-positive	Urease-positive	Starch hydrolysis-positive
	Motility-motile	Glucose-positive	Methyl red-negative	H_2S-negative	Catalase-positive
	Spore arrangement-biterminus	Manitol-positive	_	Aerbic/unaerobic-aerobic	NaCl 6.5% Positive

It was observe that the present isolate was showing optimum enzyme activity at 60^0C temperature (Figure 2), pH10 (Figure 1) and after 48 h of incubation (Figure 3), at 2% of substrate concentration (Figure 4). Similar types of results were found by Banerjee *et al*., (1999) Oberai *et al*., (2011), Joo *et al*., (2003) Gupta *et al*., (2003). Also the similarity were found by Hung *et al.,* (2003), Singh *et al.,* (2011),Gupta *et al*., (1999) for optimum pH which is 10.The crude enzyme extract of present strain were applied for both pickle and blood stain removal. The stain was cleared more in enzyme and enzyme + buffer (plate 2) as compared to detergent proving its efficiency in stain removing as detergent (Banerjee *et al*., 1999**) .** According microscopic and biochemical characteristics (Table 2) Present strain show resemblance to *Bacillus* species (Bergey's D Manual, 1957; Asokan, 2010).

Conclusion
During present work it is concluded that there was successful isolation of a soil isolate identical to *Bacillus* species and able to produce alkaline protease having optimum activity at temperature 60^0 C, pH 10, after 20 min of incubation period at 2% of substrate concentration. And that can be successfully applied in blood and food (pickle) stain removing experiment.

REFERENCES
Asokan S. and Jayanthi C. (2010). Alkaline protease production from, *B. licheniformis, B. coagulance* from soil sample from Tanjaur Dist For the production of alakine protease. *J. Tissue Res.* **l10** (1) 21: 2123.

Banerjee U.C. Sani R. K. Azmi W. and Soni R. (1999). Application of thermostable proteases from *B. brevis* and its character as a Laundry detergent additive process. *Biochem.* **35**:213-219.

Bergey's D Manual of Determinative Bacteriology (1957). American Society for Microbiology, Willum and Wilkilan co. publisher Baltimer, USA,VII ED.

Deshmukh A.M. (1997). Handbook of media, stain, and reagent, In detergents: An Indian perspective. *Curr. Sci.* 75.

Safey E.M.E.L. and U.M. Abdule Raouf (2004). Production, purification and characterization of protease enzyme by *Bacillus sutilis''* Botany and Microbiology dept. faculty of science, Al-Azhar Univ.P0,71542 Assiut Egypt.

Strzelezyk E., Gorlach K. and Hrozyclci (1990). Labrotory of agricultural microbiology and technical University. Ul, Bernardyriska, 6, P. L, 85- 029 Byd SZCZ, *Poland. Bio-fertily soil.* 268-272.

Gupta R., Beg Q.K. and Lorenz P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiol. Biotech.* 59.

Hamid Mukhatar and Ikram –UL-Haq (2008). Described about the production of alkaline proteases from *B. subtilus* isolated from Soil collected from tannery area. *Pac. J. Bot.* **40**(4): 1073-1679.

Hande Genckal (2004). A light on alkaline protease production from bacillus spp. A report, Department of Biotechnology Bioengineering, Izmir Institute, Technology Izmir, Turky.

Horikoshi (1999). Alkaslophyle some applications of their products for Biotechnology. *Microbiol. Mol. Biol. Rev.* **63**(4): 735.

Huang Q., Peng Y., Li X., Wang H. and Zhang Y. (2003). Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus. Curr. Microbiol.* **4**6:169.

Jeffery L. S. H. (2008). Uses starch casein agar media to detect the protease activity from actinomycetes. *Af. J. Biotechnol.* **7**(20): 3697-3702.

Joo H., Kumar G., Park G., Kim K.T., Paik S.R. and Chang C. (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshi. Process Biochem.* **38**: 155.

Kumar C.G. (2002). Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus. Letters Applied Microbiol.* 34.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265.

Mala B. Rao., Aparna M. Tanksale., Mohini S. Ghate. And Vasanti V. Deshpande (1998). Molecular and biotechnological aspects of Microbeal proteases. *Microbiol. Molecular Biol. Review.* **62**:597-635.

MC Donald., C E and Chen L.I. (1965). Lowery modification of Folin reagent for the determination of protease activity. *Ann. Biochem.* **10**:175

Mohasin Ahamad Khan., Nadeem Ahamad., Ahamad Usman Jaffer., Idrees Ahamad Nasir and Muhammad Abdul Qadir. (2011). Isolation and screening of alkaline protease producingbacteria and physio-chemical characterization of the enzyme". Center of Excellence in Molecular Biology 87 West canal banc Thokar Niaz Baig, Lahore –5700, Pakistan.

Oberoi R., Beg Q.K., Puri S., Saxena R.K. and Gupta R. (2001). Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp.*World J. Microbiol. Biotechnol.* **17:** 493.