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Isolation and Characterization of Extracellular Thermoalkaline Protease Producing *Bacillus cereus* isolated from Tannery Effluent

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----------**ABSTRACT**----------
Twenty three bacterial strains were isolated from treated tannery effluent of Jajmau, Kanpur, India and exhibited variable protease activity as observed by caseinolytic zones on milk agar plates. However, one isolate TVP-9 showed a maximum clearance zone diameter of 39 mm at 37°C and 45 mm at 50°C after 24 h of incubation, indicating its thermostable nature and was selected as the best thermostable alkaline protease producing bacteria. The TVP-9 strain was identified as Bacillus cereus. The bacteria obtained maximum cell density at 28 h, whereas, produced maximum protease of 410 Units/ ml during early stationary phase after 36 h of growth. The enzyme exhibited its optimal activity at pH 9.0, temperature 35-50°C and 0.0 to 2.0% salinity, whereas, significantly active and stable in broad pH (7.0-11.0) and temperature (30-60°C) range and at NaCl concentrations ranging from 0.0 to 3.0%.

KEYWORDS: *Thermoalkaline protease, Bacillus cereus, Tannery effluent, Broad pH and temperature stable, Salinity tolerant.*

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I. INTRODUCTION

Proteases are the enzymes of utmost biotechnological interest and are present in all living organisms but bacterial proteases are the most preferred group of industrial enzymes as compared to animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement, faster growth rate, higher productivity and low production cost. Among various type of proteases, alkaline proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation (Bayoudh *et al*., 2000).Particularly, extracellular thermoalkaline bacterial proteases are important for the hydrolysis of waste proteins and enable the bacteria to absorb and utilize hydrolytic products (Srinivasan *et al*., 2009; Habib *et al*., 2012) by growing easily under extreme pH and temperature conditions. Recently, bacterial alkaline proteases have received attention as a viable alternative for bioremediation of protein rich tannery waste and their use in treatment of raw hide by replacing the hazardous chemicals especially involved in soaking, dehairing and bating of hides prior to tanning to produce quality leather without causing environmental pollution (Sudha *et al*., 2010; Ahmad and Ansari, 2013). Although proteases have been identified from different sources and many of these have found their ways into industrial and biotechnological applications, but still the proteases exploited is not sufficient to meet all the demands. A major cause for this is that many of the available enzymes could not resist the changes in environmental conditions in which they are supposed to work in industries. Thus, new promising bacterial strains that could survive under harsh environmental conditions could be isolated from native environments to increase the yield of such enzymes. Effluent from leather industry might give us a good source of potential bacteria that could produce proteases active at dual extremities of alkaline pH and high temperature because the tannery effluent discharged is alkaline in nature.

Gupta *et al.* (2002) have reported bacterial alkaline protease as an important proteases used in leather processing and laundry detergents. Mukhtar and Haq (2008) have also reported the production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent. In a study, (Sivaprakasam *et al.*, 2011), have reported the production of a salt tolearnt protease from *Pseudomonas aeruginosa* BC1 and its application in tannery saline wastewater treatment. Several bacterial species, belonging to a variety of genera such as *Bacillus*, *Pseudomonas*, *Aeromonas*, *Staphylococcus*, etc. are reported to produce alkaline protease having diverse industrial applications (Gupta *et al.*, 2002; Mukhtar and Haq, 2008; Saha *et al.*, 2011; Habib *et al.*, 2012; Ahmad and Ansari, 2013). These enzymes are mostly not significantly active at broad temperature and pH

range. There are still many unknown potential bacteria in the industrial waste dumping sites that may be a good source for thermostable alkaline protease. Thus, the present study was aimed to isolate and identify bacteria from the treated tannery effluent that could produce extracellular thermostable alkaline protease at promising rates under wide environmental conditions.

II. MATERIALS AND METHODS

2.1 SAMPLING OF TANNERY EFFLUENT AND ISOLATION OF ALKALINE PROTEASE PRODUCING BACTERIA

The treated tannery effluent was aseptically collected from the release point of common effluent treatment plant (CETP) of tanneries located at Jajmau, Kanpur, India in sterile glass bottles, transported on ice to the laboratory and processed for bacterial isolation within 6 h of collection. The alkali tolerant proteolytic bacteria were isolated from the treated effluent by the serial dilution method (APHA, 1998). The samples were serially diluted with sterile distilled water and the bacteria were isolated on the saline skim milk agar plates containing 2.5% (w/v) skimmed milk, 0.5% (w/v) peptone, 1.0% (w/v) NaCl and 1.5% (w/v) agar by the standard pour plate technique (APHA, 1998). The pH of the medium was adjusted to 9.0 after autoclaving with previously sterilized Na_2CO_3 (20% w/v). Plates were then incubated at 37^{o}C for 24-36 h. Colonies forming transparent zones around the bacterial colony due to hydrolysis of milk casein, after 24 h of incubation were taken as evidence for qualitative determination of protease producing bacteria.

Nine morphologically distinct bacterial colonies showing the clear zone diameter greater than 20.0 mm were selected and re-streaked several times on the same medium to obtain pure isolates. All the nine cultures were maintained on nutrient agar slants at 4°C and subcultured after every four weeks. The culture broth of these isolates were loaded in the wells created aseptically on milk agar plates (pH 9) and incubated for 4-6 h at 37°C to reassess the protease activity.

2.2 SCREENING OF BACTERIA FOR THERMOALKALINE PROTEASE PRODUCTION

Individual bacterial colonies were screened for thermostable alkaline protease production on skim milk agar medium. One hundred µl of overnight grown broth culture of each isolate was loaded in the wells created aseptically on milk agar plates (pH 9) and incubated for 36 h at 50°C. The isolate having maximum clearance zone was selected for further studies.

2.3 BACTERIAL GROWTH AND PREPARATION OF CRUDE THERMOALKALINE PROTEASE EXTRACT

The selected bacteria were inoculated in a 250 ml Erlenmeyer flask containing 50 ml broth which consisted of 0.5% glucose, 0.75% peptone, 0.25% yeast extract, 0.1% K_2HPO_4, 0.1% $MgSO_4$ 1.0% Na_2CO_3 and 1.0% NaCl and pH of the medium was adjusted to 9.0. The flasks were incubated on the orbital shaker (120 rpm) at 50°C for 48 h. The samples were withdrawn aseptically after regular interval of every 4 h upto 48 h of growth. The bacterial growth of every 4 h sample was assessed by turbidity measurement at 600 nm. Each sample was centrifuged at 10,000 rpm and 4°C for 5 min and the cell-free supernatant of each hour were collected and used as a crude enzyme extract for protease assay.

2.4 QUANTITATIVE ASSAY OF EXTRACELLULAR THERMOALKALINE PROTEASE ACTIVITY

The proteolytic activity was quantitatively assayed by the slightly modified method of Anson (1938). The reaction mixture contained 1.0 ml of crude enzyme extract and 2.0 ml casein [15% (w/v) in 20mM borate buffer, pH 9.0]. This mixture was incubated at 50°C for 20 min and the reaction was stopped by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid. The mixture was vortexed to ensure complete mixing and incubated for further 15 min at room temperature and then centrifuged at 10,000 rpm for 15 min. The supernatant was used to estimate the amount of free tyrosine released according to Lowry *et al.* (1951) using tyrosine as a standard. One unit of protease activity is defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per min per ml under the standard assay conditions.

2.5 BACTERIAL STRAIN SELECTION AND IDENTIFICATION

Depending on the maximum relative proteolytic activity one bacterial strain was selected for further studies and was identified following the scheme of Cowan and Steel (1993). Various morphological, physiological and biochemical tests were performed and results were interpreted according to Bergey's Manual of Determinative Bacteriology (Holt, 1994). Its identity was further authenticated from Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.6 EFFECT OF TEMPERATURE AND pH ON PROTEASE ACTIVITY

The effect of temperature on protease activity was studied by incubating the reaction mixture (pH 9.0) for 30 min at different temperature ranging from 30-70^{o}C using casein as substrate. The treated enzyme mixture was immediately transferred to 0^{o}C and temperature was again raised up to the assay temperature and the enzyme activity was determined as per the method of Anson (1938). The effect of pH on the rate of protease catalyzed reaction was determined by incubating the reaction mixture at 50°C and different pH values ranging from 6.0 to 12.0 and the remaining protease activity was measured under standard assay conditions.

2.7 EFFECT OF NaCl ON PROTEASE ACTIVITY

The effect of various NaCl concentrations on thermostable alkaline protease activity was studied by incubating the reaction mixture (pH 9.0) with equal volume of different NaCl concentration ranging between 0.0 to 5.0% w/v and incubated for 20 min at 50^{o}C. The residual activity of protease was then measured as per the standard assay procedure so as to determine its use for tannery saline waste water treatment.

III. RESULTS AND DISCUSSION

3.1 ISOLATION AND SCREENING OF THERMOSTABLE ALKALINE PROTEASE PRODUCING BACTERIA

The treated tannery effluent was found to be alkaline (pH 8.3), which supports the growth of alkaliphilic bacteria. Twenty three bacteria producing variable caesinolytic zones on milk agar plates were isolated from treated tannery effluent. The clear zone of hydrolysis around each bacterial colony reflects their extent of extracellular proteolytic activity and was due to casein hydrolysis by protease enzyme (Habib *et al.*, 2012). Out of 23 isolates, nine exhibited vibrant clear zone diameter greater than 20.0 mm on 2.5% skim milk agar medium at pH 9.0 after 24 h incubation. These isolates were supposed to exhibit high potential for alkaline protease activity which was reassessed by loading their culture broth in the wells on skimmed milk agar plates (pH 9.0). Culture broth of all the nine strains cleared more than 20.0 mm zone within 5 h of incubation at 37°C, thereby indicating an extracellular nature of protease (Table 1). Further, one isolate TVP-9 showed a maximum clearance zone diameter of 39 mm at 37°C and also had better potential (45 mm) for alkaline protease activity at 50°C after 24 h incubation (Table 1), indicating its thermostable nature and was selected as a promising thermostable alkaline protease producing bacteria for further studies. In tanneries, such isolates may be useful for dehairing and bating processes during tanning operations and also for hydrolysis of proteinaceous waste in the discharged tannery effluent. Several researchers have also isolated the alkaline protease producing bacteria from tannery waste reflecting their potential in waste water treatment and leather manufacturing as an accepted green alternative to the chemical process (Mukhtar and Haq, 2008; Sivaprakasam *et al.*, 2011) but the enzyme becomes unstable when the industrial processes were carried out at dual extremities of high temperature and pH.

Table 1: Alkaline protease activity of tannery bacterial isolates as casein hydrolysis zone diameter observed on skim milk agar medium

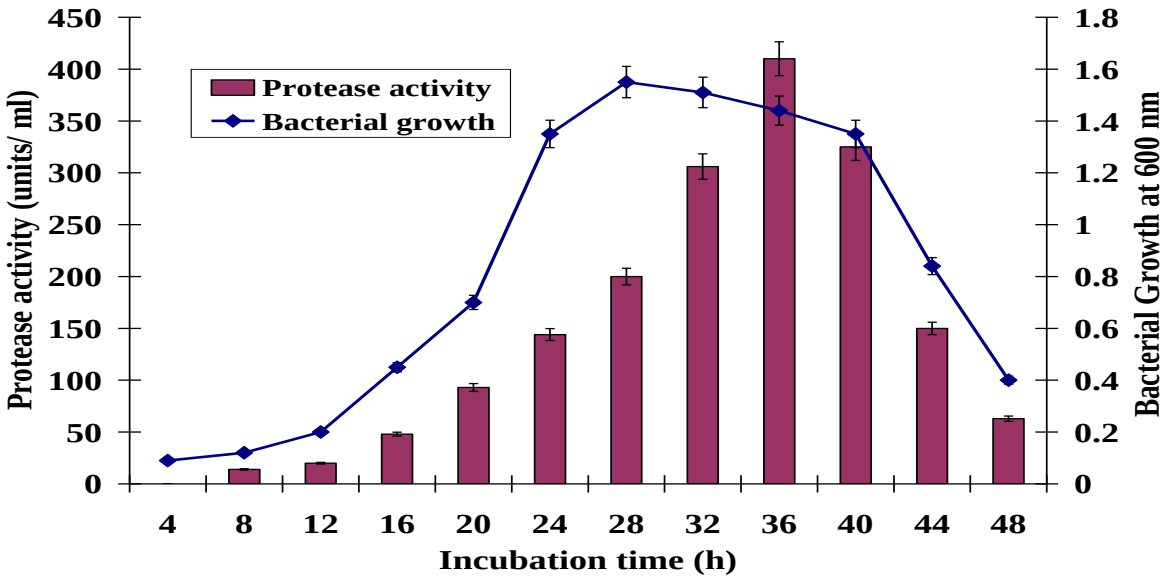
S. No.	Bacterial Strain	Zone diameter(mm) after 24 h incubation	
		at 37 ^{o}C	at 50 ^{o}C
1.	TVP-5	23	25
2.	TVP-6	33	34
3.	TVP-9	39	45
4.	TVP-11	27	16
5.	TVP-14	24	28
6.	TVP-15	32	15
7.	TVP-18	21	12
8.	TVP-20	25	23
9.	TVP-23	30	14

3.2 ASSAY OF EXTRACELLULAR ALKALINE PROTEASE AND BACTERIAL IDENTIFICATION

The rate of alkaline protease production and increase in bacterial cell density by TVP-9 was investigated in a shake flask study upto 48 h of growth (Figure 1). It was found that the bacterial growth and the enzyme production were gradually increased as the incubation time progressed upto 28 h. The bacteria obtained maximum cell density at 28 h and then entered the stationary phase which lasted till 44 h of incubation, whereas, the protease activity was maximum (410 Units/ ml) at 36 h of growth and thereafter the enzyme activity started to decline. This correlation was attributable to an increased need for turnover of cell proteins at the slower growth rate (Muthuprakash and Abraham, 2011). A very little extracellular protease production was observed

during the lag and early log phase of the bacterial growth. This suggests that proteases are largely produced during the post exponential phase or onset of stationary phase of their growth. Other researchers have also reported similar observation regarding protease production (Kornberg *et al*., 1968; Mukhtar and Haq 2008; Khan *et al*., 2011). The requirement of protease for bacterial sporulation has been demonstrated by the use of protease inhibitors (Dancer and Mandelstam, 1975).

Fig. 1.Growth behaviour of TVP-9 bacterial strain and rate of extracellular thermoalkaline protease production during 48 h of growth.



Based on the morphological, physiological and biochemical characteristics TVP-9 was identified as *Bacillus cereus* (Table 2). Its identity was confirmed from the Institute of Microbial Technology, Chandigarh, India. Although, proteolytic enzymes are produced by many microorganisms but *Bacillus* species are reported to secrete high quantity of proteases for various commercial and industrial purposes in leather, food, detergent, laundry, photography, pharmaceutical industry, bioremediation, etc (Mukhtar and Haq, 2008; Khan *et al*., 2011; Saha *et al*., 2011; Josephine *et al*., 2012; Ahmad and Ansari, 2013). Further, due to the fast growth rate and enhancement of protease production by genetic manipulation has been well established in *Bacillus* species by various researchers, has further underlined the significance of this enzyme (Joshi, 2010; Singh *et al*., 2010; Ahmad and Ansari, 2013).

Table 2: Morphological, physiological and biochemical characteristics of thermoalkaline protease producing TVP-9 bacteria isolated from tannery effluent

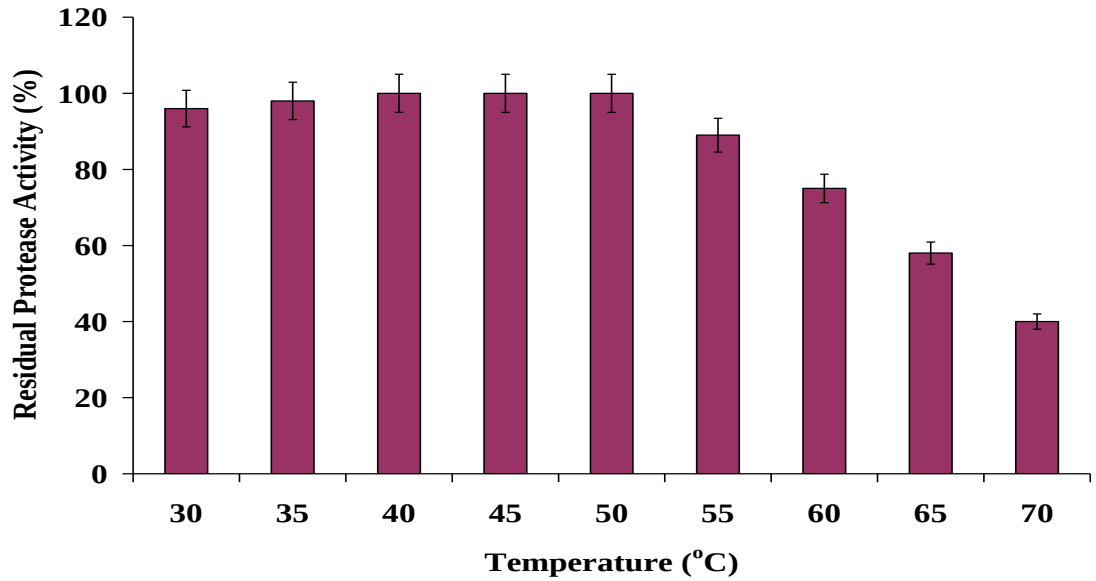
Characteristics	Results
Colony morphology	Cream, smooth, round
Cell morphology	2-4 µm, Rods
Gram staining	+
Spore (s)	+, sub-terminal
Motility	_
Growth at temperatures	20-70 °C
Growth at pH	5-12
Growth on NaCl (%)	2.0-9.0
Methyl red test, Gelatin hydrolysis, Starch hydrolysis, Urea hydrolysis, Nitrate reduction, Ornithine decarboxylase, Arginine dihydrolase, Cytochrome oxidase, Catalase test	+
Growth on MacConkey agar, Indole test, Voges Proskauer test, Citrate utilization, H_2S production, Lysine decarboxylase	_

(+) Positive; (–) Negative

3.3 EFFECT OF TEMPERATURE AND pH ON PROTEASE ACTIVITY

The alkaline protease of TVP-9 retained ~100% activity in the range of 30-50°C, hence the optimum temperature for maximal enzyme activity and stability was between 30-50°C indicating its thermostable nature (Figure 2). Further, the enzyme retained 89%, 75% and 58% activity at temperature 55, 60 and 65°C, respectively. Above 65°C, there was a sharp decrease in the enzyme activity and stability. The result seems to be very interesting as the broad optimal temperature range of this study, is a very suitable characteristic for its industrial acceptability including tanneries and a common feature for getting the bacterial alkaline protease commercialized. Further, in India wastewater treatment plants generally operate at 30-40°C temperature. Hence, the isolated protease could be used for an effective bioremediation of tannery waste water. Abusham *et al.* (2009) have reported an alkaline protease of *Bacillus subtilis* strain R having 100% stability between 35-55°C. In contrast to it, Khan *et al.* (2011) also reported highest protease activity of 380 U/ ml by an alkalophilic *Bacillus* isolate at 50°C. However, the enzyme activity was significantly decreased at 40-60°C temperature. Muthuprakash and Abraham (2011) have reported 55°C as an optimum temperature for bacterial alkaline protease production and there was a drop in enzyme activity at temperature below or above this value.

Fig. 2. Effect of incubation temperature on protease activity of TVP-9 bacterial strain



The effect of pH on alkaline protease stability was also determined. Figure 3 reveals that the extracellular alkaline protease of TVP-9 strain was found to be significantly active (66-81%) over a broad pH range of 7.0-11.0 at 50°C with a maximal of 100% enzyme activity and stability at pH 9.0. Further, the enzyme activity decreased rapidly at pH levels below 7.0 and above 11.0. This reveals the highly alkaline nature of the protease which makes it suitable for application in alkaline environments of industries including leather manufacturing. Generally, the pH of tannery wastewater is of slightly alkaline nature which favors the potential usage of the isolated protease for bioremediation studies. These findings are in accordance with the earlier report of Manachini *et al*. (1988) showing alkaline protease stability of *Bacillus thermoruber* in a broad pH range of 7.5-11.0 with maximum activity at pH 9.0. In a study, Almas *et al*. (2009) reported, the remarkable activity of alkaline protease of *Bacillus* strain SAL 1 in the pH range of 7.0-10.0 with an optimum at pH 9.0. Khan *et al.* (2011) reported an alkaline protease of *Bacillus* species stable in the pH range of 10-12 having maximium stability and activity of 380 U/ ml at pH 11.5.

3.4 EFFECT OF NaCl ON PROTEASE ACTIVITY

The effect of NaCl on protease activity of TVP-9 is represented in Table 3. The alkaline protease of TVP-9 was found nearly 100% stable in the range of 0.0 to 2.0% NaCl concentration, when incubated for 20 min at 50°C. Further, a significant amount of protease activity of 370 and 320 U/ ml was observed at 2.5, and 3.0% salt concentration, respectively indicating that the protease of TVP-9 is halotolerant. Sodium chloride at still higher concentrations further reduced the protease activity and stability. Salt tolerance of alkaline proteases makes their industrial application possible under saline conditions. Dodia *et al*. (2008) reported an alkaline protease secreting haloalkiliphilic bacterium (Gene bank accession number EU118361) having high enzyme

activity in the range of 0–4 M NaCl concentration. The protease stability at high salt concentrations is a desirable characteristic, as NaCl is used as a core component in granulation of protease prior to addition in detergents (Dodia *et al*., 2008). Sivaprakasam *et al*. (2011) has also reported the production of salt tolerant protease from *Pseudomonas aeruginosa* strain BC 1 and its use in treatment of tannery saline waste water. Our findings revealed the suitability of *Bacillus cereus* alkaline protease of this study in industrial applications especially in tanneries, laundary formulations, pharmaceuticals, etc.

Fig. 3. Effect of pH on protease activity of TVP-9 bacterial strain.

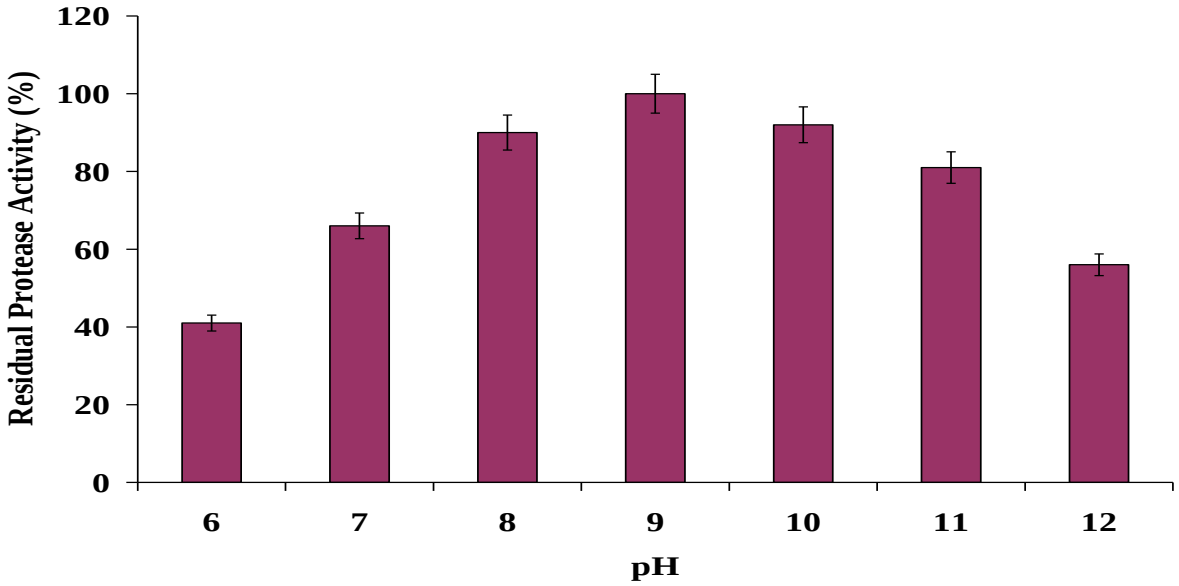


Table 3: Effect of various NaCl concentrations on thermoalkaline protease activity of TVP-9 strain

S. No.	NaCl concentration (%)	Protease activity (Units/ ml)
1.	0.0	405
2.	0.5	405
3.	1.0	410
4.	1.5	410
5.	2.0	410
6.	2.5	370
7.	3.0	320
8.	3.5	140
9.	4.0	60

IV. CONCLUSION

An extacellular thermotolerant alkaline protease producing *Bacillus cereus* strain was isolated from the tannery waste waster. The protease was significantly active and stable in broad pH (7.0-11.0) and temperature (30-60^{o}C) range and at NaCl concentrations ranging from 0.0 to 3.0%. Due to the remarkable performance of alkaline protease of this strain such as activity at high pH and temperature, high pH stability, thermostability and halotolerance makes it a potential candidate for application in industries such as tanneries, food, pharmaceutical, detergent, waste treatment plants, etc.

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