INTERNATIONAL JOURNAL OF PLANT PROTECTION
VOLUME 9 | ISSUE 1 | APRIL, 2016 | 211-218

● e ISSN-0976-6855 | Visit us : *www.researchjournal.co.in*

IJPP

RESEARCH PAPER

DOI : 10.15740/HAS/IJPP/9.1/211-218

Production and optimization of extracellular alkaline protease from halotolerant chromate resistant *Bacillus circulans* isolated from Tannery solid waste

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ARITCLE INFO

Received : 25.01.2016
Revised : 28.02.2016
Accepted : 09.03.2016

KEY WORDS :
Bacillus circulans, Halotolerant, Alkaline protease, Chromate resistant, Tannery

ABSTRACT

Halotolerant alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline and high saline environment. These enzymes have extensive applications in industries like laundry detergents, pharmaceutical, food, leather and feather processing and proteinaceous waste bioremediation. Keeping it in view, the study was aimed to isolate chromate resistant, haloalkliphilic protease producing *Bacillus circulans* bacteria from the discharged tannery solid waste. A total of seven bacterial strains were isolated on selective milk agar plates (pH 8.0-9.0) from tannery solid waste on the basis of different colony morphology and higher tolerance capacity for chromate and NaCl. These strains exhibited variable alkaline protease activity and were tolerant to different concentration of both chromate (200-1300 µg/ml) and NaCl (1-9%). Out of seven, one strain TVD-5 was interestingly tolerant to high concentration of Cr(VI) (1300 µg/ml) and NaCl (8.0%) and exhibited vibrant clear zone diameter between (13-30 mm) on 1.0 per cent skim milk agar medium at pH 9.0 after 28 h incubation. This strain produced maximum protease of 390 Units/ ml during early stationary phase after 36 h of growth. The enzyme exhibited its optimal activity at pH 9.0, temperature 35°C and 8.0 per cent salinity, whereas, significantly active and stable in broad pH (7.5-11.0) and temperature (25-45°C) range and at NaCl concentrations ranging from 7.0 to 13.0 per cent. This bacterium may potentially be useful for simultaneous bioremediation of Cr(VI) containing wastes in the environment. Also, the proteases of this study may have many applications in different industries and environmental bioremediation.

How to view point the article : Verma, Tuhina and Agarwal, Swati (2016). Production and optimization of extracellular alkaline protease from halotolerant chromate resistant *Bacillus circulans* isolated from Tannery solid waste. *Internat. J. Plant Protec.*, **9**(1) : 211-218.

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INTRODUCTION

Proteases are the class of enzymes which occupy key position with respect to their applications in both physiological and commercial fields (Godfrey and West,

1996 and Gaur *et al*., 2015). Protease derived from microorganisms such as bacteria, fungi and yeast has found wide spread applications in many fields. Among various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases as they exhibit stability to chemical and physical changes in the medium and are potentially employed in industries (Fujiwara *et al*., 1993 and Miyaji *et al*., 2006). Recently, considerable focus has been given to the enzymes produced by alkali tolerant halophilic bacteria and their biotechnological potentials (Ventosa, 2004) due to an advantage that the enzymatic activity is retained even under harsh industrial processes (Mohapatra *et al*., 2003). These enzymes have extensive applications in industries like laundry detergents, pharmaceutical, food, leather and feather processing and proteinaceous waste bioremediation (Rao *et al*., 1998 and Gaur *et al*., 2014).

Leather industry is one of the major industries in India. Despite making significant contributions to the country economy, it also causes severe environmental pollution (Verma *et al*., 2008). Tanneries are mainly responsible for the release of huge amount of hexavalent chromium [Cr(VI), chromate] and proteinaceous substances through their solid waste in to the environment and hence are of great environmental concern (Verma *et al*., 2002). Protein being a macromolecule takes longer residence time for its degradation hence, finally adds significant stress on the environment and emits obnoxious smell due to generation of toxic gases. Further, Cr(VI) is well known carcinogen, mutagen and teratogen and is toxic to all form of life, thus listed as priority pollutant by environmental potential agency's (EPA, 2000). Also, the tanneries use sodium chloride to preserve the fresh skins from the microbial decomposition, thus the tannery wastes are saline in nature having basic pH. Removal of such hazardous waste is highly imperative which otherwise will lead to ecotoxicological risk.

Some bacteria have exceptional ability to adopt and colonize such noxious polluted environment. Among all protease producing bacteria the strains of *Bacillus* genus gained importance because of extracellular enzyme production under submerged fermentation conditions (Subba Rao *et al*., 2008 and Verma and Baiswar, 2013). Such indigenous halotolerant chromate resistant alkaline protease producing bacteria can be utilized for biological treatment of Cr(VI) and protein rich industrial waste prior to its release in to the environment. Further, the enzymatically degraded proteinacious waste can be used as fertilizer for the plant growth. The present investigation therefore, has been undertaken to isolate and identify indigenous potent halotolerant alkaliphilic bacteria that could produce haloalkaliphilic protease at promising rates. Such strains can also be exploited to efficiently detoxify Cr(VI) and simultaneously hydrolyze proteins from tannery solid waste. Further, the protease production efficiency was elucidated under wide environmental conditions.

MATERIAL AND METHODS

Isolation and screening of chromate resistant halotolerant alkaline protease producing bacteria:

Tannery solid waste was collected from the dumping site of the tanneries of Jajmau, Kanpur, India in sterile bags and processed within 6-8 h of collection. The samples were serially diluted with sterile distilled water and the bacteria were isolated on the saline skim milk agar plates containing 1.0 per cent (w/v) skimmed milk, 0.5 per cent (w/v) peptone, 1.0 per cent (w/v) NaCl and 1.5 per cent (w/v) agar by the standard pour plate technique (APHA, 1998). The agar plates were also supplemented with varying chromate (100-250 mg/l) and NaCl (1.0-1.5%) concentration. The pH of the media was adjusted to 9.0 after autoclaving with previously sterilized Na_2CO_3 (20% w/v). Plates were then incubated at 35$\pm$1°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. Seven morphologically distinct bacterial colonies showing the clear zone diameter greater than 15.0 mm were selected and re-streaked several times on the same medium to obtain pure isolates. All the seven cultures were maintained on nutrient agar slants at 4°C and sub-cultured after every five weeks.

Tolerance of bacteria to various Cr(VI) and NaCl concentration :

The MIC of Cr(VI) for these seven strains was determined by the agar dilution method. The milk agar plates supplemented with different concentrations of Cr(VI) (200–1400 mg/lit.) and NaCl (1.5–9.0%) in combination were inoculated aseptically with about 2.9 x 10^5 colony forming units (CFU)/ ml bacterial cells of

exponential phase. Plates were incubated for 7 days at 35±1°C and observed for growth. The minimum concentration of Cr(VI), at which no growth observed, was considered the MIC of that isolate. Also, these strains were evaluated for tolerance to maximum NaCl concentration.

Assay of chromate reductase activity :

Chromate concentration in the culture supernatant was determined spectrophotometrically by the diphenylcarbazide (DPC) method using UV-Vis spectrophotometer (Shimadzu 1601, Japan) at 540 nm and the Cr(VI) concentration was determined by the standard curve of $K_2Cr_2O_7$ (100–1400 mg/lit.) (APHA, 1998). The initial (0 h) and final (after incubation) Cr(VI) concentration was determined by the DPC method and the Cr(VI) reduction efficiency of bacteria is determined in terms of " per cent Cr (VI) reduction". Total Cr [Cr(VI) + Cr(III)] in the culture supernatant was determined by atomic absorption spectrophotometer (AAS) at 357.9 nm, after digesting the supernatant with the mixture of nitric acid and perchloric acid (6:1, v/v).

Bacterial growth and preparation of crude haloalkaliphilic protease extract :

Bacterial strains TVD-1, TVD-2, TVD-3, TVD-4, TVD-5, TVD-6 and TVD-7 was inoculated into 50 ml of sterilized skim milk broth (pH 9.0) in 250 ml Erlenmeyer conical flask and incubated at 35±1°C up to 30 h in an orbital shaker (120 rpm). The samples were withdrawn aseptically after regular interval of every 4 h up to 30 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 extracellular protease assay.

Quantitative assay of extracellular protease activity:

Enzyme activity was assayed using casein as the substrate with slight modification to the method of Sarath *et al.* (1989). The reaction mixture consisted of 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2.0 per cent (w/v) of casein and 0.15 ml of enzyme solution. The reaction mixture was incubated at 25ºC for 15 min thereafter stopped by adding 1.2 ml of 10.0 per cent (w/v) TCA then incubated at 37ºC for an additional 15 min, and the precipitate was removed by centrifugation at 8,000 rpm for 5 min. Further 1.4 ml of 1.0 M NaOH was added to 1.2 ml of the supernatant, and its absorbance was measured at 600 nm. The activity was determined by detecting the release of amino acids (tyrosine) from casein and the amount of tyrosine released was calculated from the standard curve constructed with tyrosine (Lowry *et al.,* 1951). 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.

Bacterial strain selection and identification :

Depending on the maximum relative proteolytic activity, Cr(VI) reduction efficiency and tolerant to high NaCl concentration the strain TVD-5 was selected as promising strain for further studies. Various morphological, physiological and biochemical tests were performed and results were interpreted according to Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Its identity was further authenticated from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Effect of pH, temperature and NaCl 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 15-50ºC using casein as substrate. The treated enzyme mixture was immediately transferred to 0ºC 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 35°C and different pH values ranging from 7.0 to 12.0 and the remaining protease activity was measured under standard assay conditions (Begum *et al*., 2007). The effect of various NaCl concentrations on the haloalkaliphilic protease activity was studied by incubating the reaction mixture (pH 9.0) with equal volume of different NaCl concentration ranging between 7.0-13.0 per cent. w/v and incubated for 20 min at 35°C. The residual activity of protease was then measured as per the standard method.

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under the following heads:

Isolation and screening of chromate resistant halotolerant alkaline protease producing bacteria:

Twenty bacterial isolates producing variable proteolytic zones on milk agar plates were isolated from the tannery solid waste samples by serial dilution methods. The zones of clearance by the isolates reflect their extent of proteolytic activity. Those having clearance zone greater than 15.0 mm were considered as significant isolate. Among 20 bacterial isolates, seven exhibited good proteolytic activity which was reassessed by loading their culture broth in the wells on milk agar plate (pH 9.0). The culture broth of good protease producers exhibited casein hydrolysis zone between 16-34 mm within 3-4 h of incubation at 35±1°C, thereby indicating an extracellular nature of the protease (Table 1). Further, Table 1 signifies that these strains were tolerant to 400-1300 mg/lit. Cr(VI) and 7.0-13.0% NaCl concentration. The results revealed that these strains were chromate resistant and haloalkaliphilic as they grew in the presence of high Cr(VI) and NaCl concentration and at pH 9.0. These bacteria are a relatively novel group of extremophiles due to various adaptive strategies (Gaur *et al.*, 2015). 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 waste (Verma and Baiswar, 2013). 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 and Sivaprakasam *et al.*, 2011) but the enzyme becomes unstable when the industrial processes were carried out at dual extremities of high pH and NaCl concentration.

Bacterial growth and assay of chromate reductase and haloalkaliphilic protease activity :

The bacterial growth response of these seven strains was determined up to 30 h after regular interval of 4 h by measuring the absorbance at 600 nm. Also, these bacteria were screened for their chromate reduction potential by DPC method and for alkaline protease production by the casein digestion method and the results are depicted in Fig. 1. All the seven bacterial isolates in this study were capable of reducing Cr(VI) aerobically and the reduction values ranged between 45.6-71.6 per cent after 24 h of growth. It was found that the bacterial growth, protease production and chromate reduction were gradually increased as the incubation time progressed up to 20 h and then entered the stationary phase which lasted till 28 h of incubation, whereas, the protease activity was maximum (390 U/ ml) at 28 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). Fig. 1 shows that among the seven strains, one strain TVD-5 attained maximum cell density after 28 h of growth and exhibited maximum chromate reductase and haloalkaliphilic protease activity and hence was selected for further studies. Further, incubaton resulted into a lesser growth as well as lesser alkaline protease production. These findings are in good agreement with the study of Jadhav *et al.* (2013) who have reported maximum protease activity after 24 h. Other researchers have also reported little extracellular protease production during the lag and early log phase of the bacterial growth, whereas, it is largely produced during the post exponential phase or onset of stationary phase of their growth (Mukhtar and

Table 1 : MIC of Cr(VI), NaCl tolerance efficiency and alkaline protease activity of bacteria isolated from tannery solid waste

Sr. No.	Bacterial strains	MIC of Cr (VI) (mg/lit.)	NaCl concentration (%)	Zone diameter (mm)
1.	TVD-1	500	2	17
2.	TVD-2	600	2.5	16
3.	TVD-3	450	2	20
4.	TVD-4	1150	6.5	25
5.	TVD-5	1350	8	30
6.	TVD-6	1200	7	28
7.	TVD-7	750	4	23

Haq 2008 and Verma and Baiswar, 2013).

Bacterial strain selection and identification :

On account of morphological, physiological and biochemical characteristics the strain TVD-5 was found to be Gram-positive, rod shape and was .identified as the *Bacillus circulans* (Table 2). Among all bacterial species, *Bacillus* sp. plays an important role in the

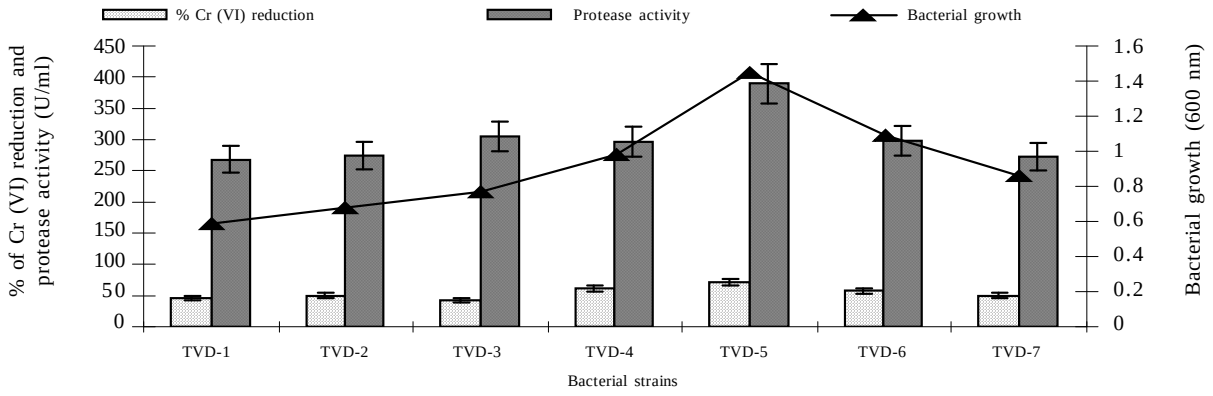

Fig. 1 : Growth response, per cent Cr(VI) reduction and protease activity (Unit/ml) of selected bacterial strains. Error bars represent mean ± standard deviation. Each point is mean of three independent experiments

Table 2 : Morphological, physiological and biochemical characteristics of aloalkaliphilic, chromate resistant protease producing TVD-5 bacteria

Charcteriastics	Results
Gram stain	+
Cell shape	Rod
Endospore formation	+ (central)
Motility	+
Fluorescence (UV)	-
Growth at temp (°C)	35
Growth at pH	7-12
Growth on NaCl (%)	7-11%
Growth on Mac Conkey agar	+
Indole Test	-
Methyl Red Test	+
Citrate Test	-
Casein hydrolysis	+
Starch hydrolysis	+
Tween 40 hydrolysis	+
Nitrite reduction	-
H_2S production	-
Cytochrome oxidase test	+
Catalase test	+
Oxidation/ Fermentation	Fermentation
Gelatin liqnefaction	+
Acid form Adonitol	-
Acid form Arabinose	+
Acid form Dextrose	+
Acid form Fructose	+

(+) Positive; (–) Negative

production of alkaline protease owing to their chemoorganotrophic nature (Singh *et al*., 2010). Several species of *Bacillus* are industrially employed to produce thermostable alkaline protease as they grow easily under extreme conditions and are easy to manipulate (Gaur *et al*., 2014). Further, *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. (Almas *et al*., 2009 and Jadhav *et al*., 2013).

Effect of pH, temperature and NaCl on protease activity :

Table 3 depicts that the *Bacillus circulans* strain TVD-5 of this study could grow and produce haloalkaline protease over a wide range of pH (7.0–12.0). However, maximum protease production was observed at pH 9.0 (390 Unit/ ml). The extracellular alkaline protease of this strain was found to be significantly active (365-350 Unit/ ml) over a broad pH range of 7.5-11.0. Further, the enzyme activity decreased rapidly at pH levels below 7.5 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. Almas *et al*. (2009) also 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.

The protease of *Bacillus circulans* TVD-5 was completely stable in the broad temperature range of 25-45°C during 1 h incubation with the maximum protease activity of 400 Unit/ ml at 35°C (Table 3). However, with further increase in every 5°C temperature above 45°C, there was a significant decrease in the enzyme stability and activity. Temperature was found to influence extracellular enzyme secretion possibly by changing the physical properties of the cell membrane (Rahman *et al*., 2005). The protease of TVD-5 strain is more thermostable than protease studied by several other researchers. These properties are considered to be very important for industrial protease production. Hence, it is evident that the protease of *Bacillus circulans* TVD-5 is more thermostable and could be applied for several biotechnological and industrial purposes. Gaur *et al.* (2009) have reported an alkaline protease of *Bacillus* strain having 100 per cent stability at 40°C. In contrast to it, Mukhtar and Haq (2008) also reported highest protease activity of 380 U/ ml by a *Bacillus subtilis* isolate at 50°C.

The effect of NaCl on protease activity of TVD-5 is represented in Table 3. The alkaline protease of this strain was found significantly stable in the range of 7.0 to 12.0 per cent NaCl concentration, when incubated for 20 min at 35°C and the maximum protease activity of 405.54 Units/ ml was observed at 8.0 per cent NaCl concentration, indicating that the protease of TVD-5 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.

Conclusion :

A chromate tolerant haloalkaliphilic strain of *Bacillus circulans* TVD-5 was isolated for enhanced

Table 3: Effect of pH, temperature and NaCl on the protease activity of TVD-5

Sr. No.	pH	Protease activity (U/ml)	Temperature (°C)	Protease activity (U/ml)	NaCl conc.(%)	Protease activity (U/ml)
1.	7	340	15	335	7	386.43
2.	7.5	365	20	350.76	8	405.54
3.	8	376	25	367	9	395.32
4.	8.5	380	30	388.34	10	380.22
5.	9	390	35	410	11	362
6.	9.5	385	40	407.45	12	327
7.	10	379	45	395	13	310
8.	10.5	368	50	330.21		
9.	11	350				
10.	11.5	335				
11.	12	320				

production of alkaline protease from tannery solid waste. The protease was significantly active and stable in broad pH (7.5-11.0) and temperature (25-45°C) range and at NaCl concentrations ranging from 7.0 to 12.0 per cent. This organism appears to have greater potential for enhanced enzyme production through optimization of nutritional and physical parameters. *Bacillus circulans* TVD-5 seems to be an interesting candidate for application in biotechnological processes, such as treatment of chromium contaminated proteinaceous saline wastes.

Acknowledgement :

Authors are extremely thankful to the "Science and Engineering Research Board" (SERB), Department of Science and Technology (DST), Government of India (GOI), New Delhi for financial support as "Young Scientist Research Project" for this work.

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