ISOLATION AND SCREENING OF HALOALKALINE PROTEASE PRODUCING BACTERIA FROM TANNERY SOLID WASTE

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Abstract

Twenty bacterial strains were isolated on selective milk agar plates (pH 9.0) from tannery solid waste on the basis of different colony morphology. These strains exhibited variable haloalkaline protease activity and were tolerant to different concentration of both chromate (350-1450 µg/ml) and NaCl (2-9%). Those having clearance zone greater than 20.0 mm were considered as significant isolate. Out of twenty, nine strains were interestingly tolerant to high concentration of Cr(VI) (850-1450 mg/l) and NaCl (6.5-9%) and exhibited vibrant clear zone diameter between 21-35 mm. All these isolates in this study were capable of reducing Cr(VI) aerobically and the reduction values ranged between 50.0-76.0% after 16-20 h of growth. Further, significant haloalkaline protease production was observed in these bacteria within 24 h under wide temperature (25-45°C) and pH (8.0-10.5) range. The potential of these strains to produce protease at higher rate in the presence of wheat bran as a cheap carbon source and yeast extract or beef extract as a nitrogen source makes them a potential candidate for industrial applications and removal of Cr(VI) and proteinaceous waste simultaneously from industrial waste of alkaline nature.

Key words: *Tannery waste, chromate resistant, bacteria, haloalkaline, protease*

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1. INTRODUCTION

Protease, a hydrolytic enzyme has attracted much attention because of its wide application in detergent, leather, food, pharmaceutical, agricultural industries and proteinaceous waste bioremediation. Of all industrially important enzymes, proteases constitute >65% of the total enzymes employed in various industrial and commercial purposes [1]. Among the various type of proteases, bacterial alkaline proteases are the most significant and widely studied group of enzymes compared with animal, plant and fungal proteases as they exhibit high productivity rate, cheaper production cost, stability to chemical and physical changes in the medium and are more demanding for industries [2, 3]. Interestingly, extracellular haloalkaline bacterial proteases are important for the hydrolysis of waste proteins with dual extremities of high salt and alkaline pH and enable the bacteria to absorb and utilize hydrolytic products under harsh conditions [4]. Recently, such 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 [5]. Further, many industrial processes are carried out in saline environments, where most of the normal bacterial alkaline proteases becomes unstable [6, 7]. Moreover, there is very limited information on the salt tolerant extracellular alkaline protease producing bacteria from the natural environment.

Leather industries are one of the major industrial sectors of India but are recognized as one of the highly polluting industries leading to soil and water pollution [8, 9]. Tanneries are mainly responsible for the release of huge amount of proteinaceous substances and toxic hexavalent chromium [Cr (VI), chromate] through their solid waste into the environment and hence are of great environmental concern. Protein degradation emits obnoxious smell due to generation of toxic gases such as NH_3, H_2S and CO_2 that creates serious human health problem. Hexavalent chromium [Cr (VI)] is well known carcinogen, mutagen and teratogen and cause cellular toxicity in humans and other living beings, thus listed as priority pollutant by Environmental Protection Agency [10]. Also, the tanneries use sodium chloride to preserve the fresh skins from decomposition, thus the tannery wastes are saline in nature having basic pH.

Bacteria inhabiting in such environments are a remarkable source for producing halotolerant alkaline proteases which will have diverse potential applications in the industries including environmental restoration [3]. In a study, Sivaprakasam *et al.* [4] have reported the production of a salt tolerant protease from *Pseudomonas aeruginosa* BC1 and its application in tannery saline wastewater treatment. Therefore, conducting research for the isolation of haloalkalophilic bacteria capable to produce extracellular proteases are of great importance. Also, these bacteria could be utilized for removal of both proteinaceous waste and Cr (VI) from industrial wastes. Keeping it in view, this study

was aimed to screen alkaline protease producing bacteria from the discharged tannery solid waste as it may result in isolation of novel haloalkaline proteases with unique physico-chemical characteristics with a view to facilitate its potential application in industries.

2. MATERIALS AND METHODS

2.1. Media and Chemicals

The media components and all reagents used in the study were of analytical grade and purchased from Hi-Media, Merck, Qualigens, India Ltd. and Sigma Aldrich chemicals, USA. Diphenylcarbazide (DPC) solution (0.25%, w/v) was prepared by dissolving 125 mg 1,5- diphenylcarbazide in 25 ml of high performance liquid chromatography (HPLC) grade acetone and stored in a brown glass bottle, for Cr(VI) determination.

2.2. Sampling and isolation of chromate resistant haloalkaliphilic protease producing bacteria

The tannery solid waste was collected from the dumping sites of the tanneries of Jajmau, Kanpur, India and landfilling sites of vicinity area in sterile containers, transported on ice to the laboratory and was processed for isolation of chromate resistant haloalkaliphilic protease producing bacteria within 6-8 h of collection. One gram of solid waste was serially diluted with sterile distilled water and the bacteria were isolated on the saline skim milk agar plates containing 1.0% (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 [11]. 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. Bacterial colonies showing transparent zones around their colony due to hydrolysis of milk casein, after 24 h of incubation were taken as evidence for qualitative determination of protease producing bacteria. Twenty morphologically distinct bacterial colonies showing the clear zone diameter greater than 12.0 mm were selected and re-streaked several times on the same medium to obtain pure isolates.

2.3. Screening of bacteria for tolerance to high Cr(VI) and NaCl concentration

All the twenty bacterial isolates were screened for resistance to high Cr(VI) and NaCl concentration by determining the MIC for Cr(VI) by agar dilution method having varying concentrations of NaCl. The milk agar plates supplemented with different concentrations of Cr(VI) (200–1550 mg/l) and NaCl (1.5–9.5%) 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 24 h at 35$\pm$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, the maximum NaCl tolerance concentration of these strains was recorded. Nine strains exhibiting high proteolytic activity and showing higher MIC values for Cr(VI) and tolerance to high NaCl concentration were selected for further study.

2.4. Assay of Cr(VI) reduction activity

Potential of selected nine strains were evaluated for the reduction of Cr(VI) to less toxic Cr(III) at the respective maximum chromate tolerance concentrations. Chromate concentration was determined spectrophotometrically in supernatant fraction of overnight grown cultures by diphenylcarbazide (DPC) method at 540 nm using UV-Vis spectrophotometer (Shimadzu 1601, Japan) and the Cr(VI) concentration was determined by the standard curve of $K_2Cr_2O_7$ (100–1500 mg/l) [11]. 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 "% 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).

2.5. Preperation of crude extracellular alkaline protease extract and enzyme assay

The crude extracellular alkaline protease of selected bacteria was prepared by inoculating each strain individually into 50 ml of sterilized skim milk broth (pH 9.0) in 150 ml Erlenmeyer conical flask and incubated at 35$\pm$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. Each sample was centrifuged at 10,000 rpm and 4°C for 5 min and the cell-free supernatant were collected and used as a crude enzyme extract for extracellular protease assay.

Enzyme activity was assayed using casein as the substrate with slight modification to the method of [12]. The reaction mixture consisted of 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2.0% (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% (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 [13]. 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.

2.6. Bacterial growth response

Growth response of selected strains was monitored at definite time intervals by measuring the absorbance at 600 nm. Each strain was individually inoculated into 100 ml

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 and absorbance was recorded. Also, the protease and Cr(VI) reduction activity of the bacteria was determined at definite time intervals throughout the bacterial growth.

2.7. Optimization of pH and temperature for protease production

The influence of pH on protease production was assessed by growing bacterial culture in the skim milk broth of varying pH ranging from 7.0 to 12.0 and the enzyme activity was assayed as described above. Similarly, the effect of incubation temperatures on protease production was determined by incubating the culture broth at different temperature *viz.* 15, 20, 25, 30, 35, 40, 45, 50, and 55°C following the standard method.

2.8. Effect of carbon and nitrogen source on protease production

The influence of various carbon and nitrogen source on enzyme production was investigated. The bacterial growth media was supplemented with various carbon sources such as maltose, glucose, fructose, sucrose, lactose, starch, glycerol and wheat bran at the level of 2.0% w/v. The effect of various nitrogen sources such as yeast extract, beef extract, urea, ammonium nitrate, sodium nitrate and ammonium sulphate on protease production were also studied. The peptone of the growth medium was replaced with these nitrogen sources. After incubation with 1.0% inoculum for 24 h at 35±1°C, the cell free supernatants obtained by centrifugation at 120 rpm were assayed for the protease production as described above.

2.9. Statistical analysis

The experiments were performed thrice, each in triplicate. Standard deviation for each experimental result was calculated using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of chromate resistant halotolerant alkaline protease producing bacteria

A total of twenty protease producing chromate resistant haloalkaliphilic 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 (Table 1). Bacterial colonies showing zones of clearance around their colony due to hydrolysis of milk casein was taken as an evidence for qualitative protease production. These strains exhibited variable alkaline protease activity and were tolerant to different concentration of both chromate (400-1450 mg/l) and NaCl (2-9%). Those having clearance zone greater than 20.0 mm were considered as significant isolate. Out of twenty, nine strains TVD-4, TVD-5, TVD-6, TVD-8, TVD-9, TVD-11, TVD-12, TVD-17, and TVD-20 were interestingly tolerant to high concentration of Cr(VI) (850-1450 mg/l) and NaCl (6.5-9%) and exhibited vibrant clear zone diameter between 21-35 mm on selective skim milk agar medium at pH 9.0 after 24 h incubation and were selected for further study. The proteolytic activity of these nine strains were reassessed by loading their culture broth in the wells on milk agar plate (pH 9.0) and the casein hydrolysis zone also indicated the extracellular nature of protease. This reveals that these bacteria were chromate resistant and haloalkaliphilic as they grew in the presence of high Cr(VI) and NaCl concentration and at pH 9.0. Further, the chronic exposure to high levels of chromate, NaCl and alkaline environment have resulted in bacterial communities which belongs to a novel group of extremophiles that have an exceptional ability to adapt in chromate and NaCl polluted basic environments and have developed some mechanisms as an effective tool for survival in such stress environments [14].

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 [2]. 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 [15, 4] but the enzyme becomes unstable when the industrial processes were carried out at dual extremities of high pH and NaCl concentration.

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

S.No.	Bacterial strains	MIC of Cr (VI) (mg/l)	NaCl conc. (%)	Zone diameter (mm)
1	TVD-1	500	2	17
2	TVD-2	600	2.5	13
3	TVD-3	450	2	20
4	TVD-4	1150	6.5	25
5	TVD-5	1300	8	34
6	TVD-6	1200	7	27
7	TVD-7	750	4	32
8	TVD-8	1250	8	26
9	TVD-9	1500	9	45
10	TVD-10	700	5	30
11	TVD-11	900	5.5	23
12	TVD-12	1100	6.5	27
13	TVD-13	550	4	18
14	TVD-14	850	2	24
15	TVD-15	700	5	17
16	TVD-16	800	4	20
17	TVD-17	1000	5.5	23
18	TVD-18	400	2	14
19	TVD-19	650	2	19
20	TVD-20	950	6.5	21

3.2. Growth, chromate reduction and haloalkaliphilic protease activity of selected bacteria

Bacterial growth of selected strains was measured at 600 nm in selective skim milk broth and a typical sigmoidal growth curve was obtained (Figure not shown). Onset of stationary phase of these strains was between 16-20 h of growth and during this phase, the protease production was found maximum in all the nine strains (Figure 1). Production of protease during the stationary phase of growth is in accordance with the studies of other workers [4, 14]. In addition to the alkaline protease production, the chromate reduction potential of these bacteria was also determined and the results are depicted in figure 1. All the nine bacterial isolates in this study were capable of reducing Cr(VI) aerobically and the reduction values ranged between 50.0-76.0% after 16-20 h of growth. Results indicated that the Cr(VI) reduction and NaCl tolerance ability of these strains were growth dependent and gradually increased as the incubation time progressed up to 16-20 h and their value decreased in the stationary phase, whereas, the protease activity was maximum during stationary phase 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 [16]. Further, incubation resulted into a lesser growth as well as lesser alkaline protease production. These findings are in agreement with the study of other researchers who reported maximum protease activity after 24 h [17]. 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 [15, 2].

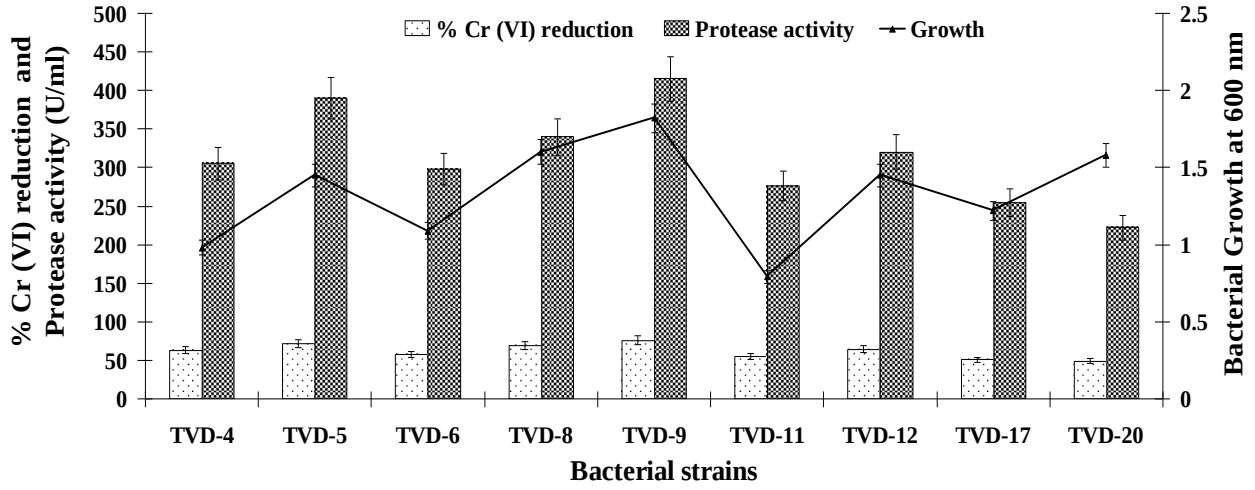

Fig-1: Growth response, Cr(VI) reduction (%) and protease activity (Unit/ml) of selected bacterial strains. Error bars represent mean ± standard deviation. Each point is mean of three independent experiments.

3.3. Effect of temperature on protease production

Enzyme production was recorded by varying the incubation temperature form 15-55°C. Figure 2 revealed that the bacterial isolates yielded maximum protease production between 25-45°C. Further, increase in temperature resulted in the decrease of enzyme and biomass production. Temperature was found to significantly regulate the synthesis and secretion of bacterial extracellular protease by changing the physical properties of the cell membrane [18, 19]. Therefore, temperature is a critical parameter that should be considered in order to obtain maximum protease production [20]. Further, the temperature requirement of the organism is based on the nature of organisms. Many reports showed bacterial and fungal alkaline protease production at lower and moderate temperatures (20-30°C) [21, 6].

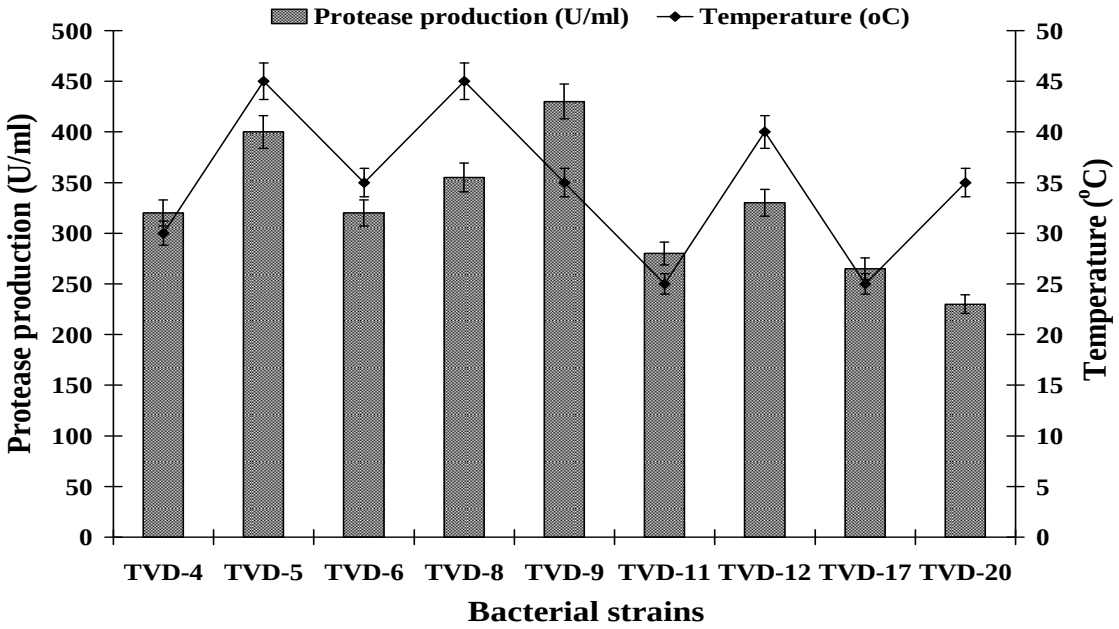

Fig-2: Effect of temperature on protease production of selected bacterial strains. Error bars represent mean ± standard deviation. Each point is mean of three independent experiments.

3.4. Effect of pH on protease production

The influence of medium pH on protease production was studied by varying the pH from 7.0-12.0 and a constant respective incubation temperature for each strain showing maximum growth. It is evident from Figure 3 that the maximum protease production in these nine strains was achieved at pH 8.0-10.5. Beyond this pH range, decrease in protease production and biomass production was observed. Further, the remarkable protease production in the pH range of 8.0-10.5 revealed alkaliphilic nature of these strains as well as protease enzyme. Effect of pH is one of the major factors for the growth of microbes in the medium that affects the productivity of microbes [22]. Also, pH of the growth medium plays a vital role by inducing physiological changes in microbes and their enzyme secretion [23]. It also strongly affects enzymatic processes and transport of compounds across the cell membrane [7]. Such proteases are very suitable for application in several industries including leather manufacturing. Moreover, the tannery wastewater is alkaline in nature which favors the potential usage of the isolated protease for bioremediation studies [24, 25]. Further, the molecular basis of pH affecting bacterial metabolism in culture broth is obscure. Since proton motive force in chemiosmosis is affected by the medium pH value, it is possible that under optimum pH range, the relative metabolic efficiency is high [26].

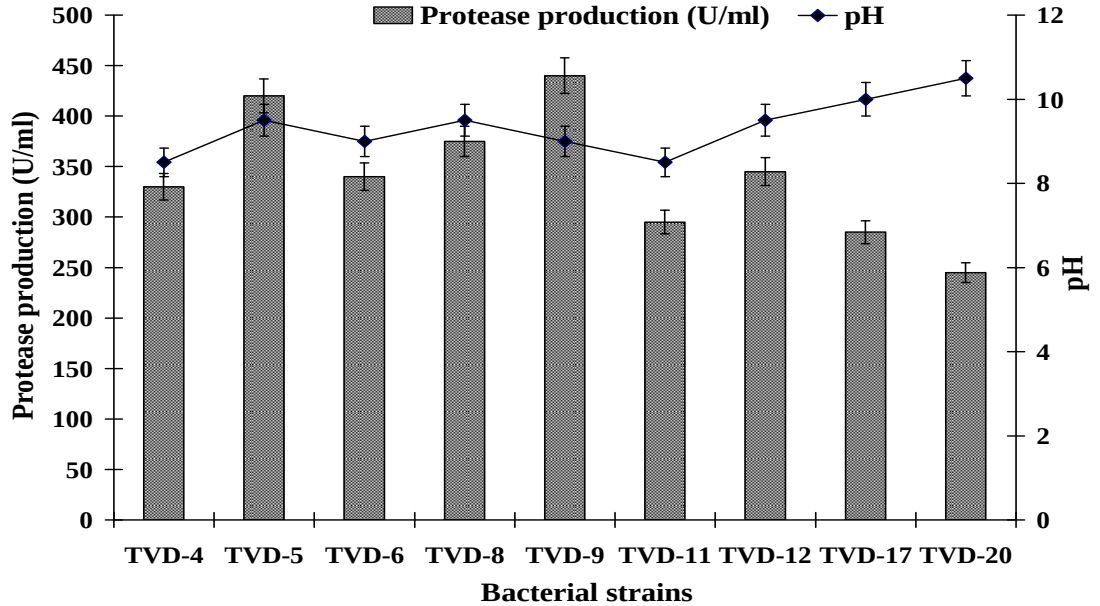

Fig-3: Effect of pH on protease production of selected bacterial strains. Error bars represent mean ± standard deviation. Each point is mean of three independent experiments.

3.5. Effect of carbon and nitrogen sources on protease production

The effect of various carbon sources on protease production was evaluated. Table 2 revealed that out of nine bacterial strains in seven strains (TVD-4, TVD-5, TVD-9, TVD-11, TVD-12, TVD-17, and TVD-20) wheat bran (2.0% w/v) was found to be the best carbon source, allowing maximum protease production (300-450 U/ml) followed by maltose which supported maximum protease production in TVD-6 (360 U/ml) and TVD-8 (390 U/ml) strain. The addition of carbon sources in the form of either monosaccharides or polysaccharides could influence the production of enzyme [27]**.** However, Gaur *et al*. [1] reported that addition of starch to the culture medium induced protease synthesis. In contrast, other sugars such as glucose, fructose, maltose and lactose reduced protease production although growth was observed optimal [15].

Table-2: Effect of various carbon sources on protease production of selected bacteria (Unit/ml)

S.No.	Bacterial strains	Maximum Protease production (Unit/ml) after 24 h incubation						
		Maltose	Fructose	Sucrose	Lactose	Starch	Glycerol	Wheat bran
1	TVD-4	340	325	305	288	270	255	360
2	TVD-5	420	435	415	390	378	355	430
3	TVD-6	300	315	268	360	265	250	340
4	TVD-8	355	390	375	382	360	330	330
5	TVD-9	410	385	425	450	430	380	450
6	TVD-11	295	310	290	245	275	230	320
7	TVD-12	275	320	325	340	355	335	360
8	TVD-17	282	300	315	280	245	260	340
9	TVD-20	190	220	195	225	240	265	300

The effect of various organic and inorganic nitrogen sources on protease production was also studied and the results are presented in Table 3. Among the various nitrogen sources, high yield of protease production was observed in yeast extract ranging between 360-475 U/ml in TVD-4, TVD-5, TVD-6, TVD-9, TVD-12, and TVD-17 strains. However, TVD-8 and TVD-20 showed maximum protease production (425 and 280 U/ml) in presence of beef extract followed by TVD-11 which used urea as a nitrogen source to achieve maximum protease production of 320 U/ml. Many researchers have reported that organic nitrogen sources were better suited to *Bacillus* sp. for growth and enzyme production than inorganic sources [1, 3]. The best nitrogen source for protease production was beef extract for *Bacillus* sp., while yeast extract and tryptone were comparable [1]. Several researchers have also reported that organic nitrogen sources were found better for enzyme production than inorganic sources [3, 14].

Table-3: Effect of various nitrogen sources on protease production of selected bacteria (Unit/ml)

S.No.	Bacterial strains	Maximum Protease production (Unit/ml) after 24 h incubation					
		Beef extract	Yeast extract	Urea	Ammonium nitrate	Sodium nitrate	Ammonium sulphate
1	TVD-4	330	350	310	300	295	320
2	TVD-5	390	425	405	370	355	400
3	TVD-6	365	375	335	320	315	340
4	TVD-8	425	408	410	310	343	350
5	TVD-9	445	475	365	390	380	415
6	TVD-11	245	270	310	220	243	260
7	TVD-12	355	380	326	275	290	310
8	TVD-17	310	315	330	440	280	460
9	TVD-20	280	245	232	205	230	240

4. CONCLUSION

In this paper, we reported the isolation and screening of bacteria from tannery solid waste which is chromate resistant and capable to produce haloalkaliphilic protease. Nine strains were found to be tolerant to high concentration of Cr(VI) (850-1450 mg/l) and NaCl (6.5-9%) and was capable of producing proteolytic enzyme under highly alkaline conditions. Interestingly, these bacteria were able to produce haloalkaline protease under wide temperature (25-45°C) and pH (8.0-10.5) range. Further, significant protease production was obtained within 24 h in presence of wheat bran as a carbon source and yeast extract or beef extract as nitrogen source. The potential of these strains to grow on cheap carbon source and also producing high amount of haloalkaline protease in short time makes it a promising candidate for industrial application and environmental restoration such as treatment of chromium contaminated proteinaceous saline wastes.

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