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Production of a detergent-compatible alkaline protease from *Bacillus cereus* K-3

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Abstract
Proteases due to their huge application spectrum in various biotechnological processes have been the focus of intense research for many decades. However, most of the available proteases lack desired properties from industrial application view point; therefore, search for better and efficient thermostable alkaline proteases, is a continuous practise. In the current study, a total of 30 bacterial strains were screened for protease production. Isolate K-3, identified as *Bacillus cereus* (*B. cereus* K-3) produced substantial titre of protease that was thermostable and broad range pH stable. The optimum fermentation time for maximum protease production was 48 h under submerged fermentation. Among various carbon/nitrogen sources examined including agricultural residues, lactose and ammonium sulphate were found to be the best carbon and nitrogen source, respectively, for protease production. The optimum medium pH for maximum protease production was 9.0. The optimum temperature and pH for activity was 40°C and 9.0, respectively. However, enzyme showed considerable activity/stability over a wide range of temperature (50-70°C) and pH (8.0-12). Furthermore, *B. cereus* K-3 protease exhibited excellent compatibility with detergents and heavy metal ions. Considering these attributes *B. cereus* K-3 protease may have potential industrial applications.

Key words: *Bacillus cereus* K-3, protease, production, thermostable, alkalistable

1. Introduction
Considering polluting chemical based manufacturing/production technologies, there is immense emphasis on development of enzyme based environmentally-friendly/green technologies. However, enzymes being biological molecules are quite fragile for hostile industrial processes; therefore, there is quest for robust enzymes capable of functioning well under industrial conditions [1]. Microbial proteases represent the largest group of enzyme of commercial importance. Proteases are hydrolytic enzymes which find applications in wide range of industrial sectors viz. food, feed, pharmaceutical, leather, detergent, textile and several others [2]. Proteases constitute one of the major groups of industrial enzymes and accounts for 60% of the total enzyme sales with two thirds of them produced commercially from microbial origin [3]. Despite huge application potential, utilization spectrum of proteases is limited due to lack of industrially desirable characteristics among available proteases. Considering that most of the industrial processes are accomplished under hostile conditions including extremes of temperatures and pH, presence of inhibitors etc., proteases intended for industrial application must have robustness against solvents, surfactants, oxidants and stability at high temperature and pH [4]. Furthermore, such broad range thermostable, pH-stable, organic solvent resistant proteases may find novel applications in pharma, diagnostic, detergent, tannery, effluent treatment, and other industries. Hence attention is focused currently on the finding new protease producing microorganisms so as to meet the requirements of industry [1].

Protein engineering has been used to obtain proteases which show unique specificity and enhanced stability and/or to understand the structure-function relationships of the enzymes. But exploration of immense

microbial diversity for targeting the producers of novel proteases with industrially desirable characteristics still constitutes a significant research area [5]. Proteases have been isolated and characterized from several bacterial [6] and fungal spp. [7]. Enzymes from *Bacillus* spp. are known for poly-extremotolerance, capable of functioning in adverse ecological conditions [5]. Protease production from several *Bacillus* spp. has been reported. In biotechnological processes for protease production, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. cereus* have become most popular due to their excellent fermentation properties, high product yields and the complete lack of toxic by-products [6]. In addition, the vast diversity of proteases in contrast to the specificity of their action has attracted worldwide attention in attempt to exploit their physiological and biotechnological applications in various industries [8].

High cost of enzyme production is another major bottleneck for wide range industrial applications of proteases. About 30-40% of production cost of industrial enzymes accounts for the cost of substrate/growth medium [9]. Exploitation of agricultural residues as carbon/nitrogen sources for bulk production of industrial enzymes may play a pivotal role in reducing production costs [5]. Medium components (nitrogen and carbon sources), physiological factors (pH, incubation temperature and time), and biological factors influence the metabolic/biochemical behaviour of the microbial strain and subsequent production pattern. Hence, in commercial practice, the optimization of medium composition is important to have cost-effective product yield [5,10].

Considering the importance of robust proteases with process suitability for industrial applications, the current study reports a thermoalkaline protease producing bacterium *Bacillus cereus* K-3, and optimization of cultural conditions for enhanced protease production.

2. Materials and methods

2.1. Proteolytic bacteria and submerged fermentation for protease production

A total of 30 bacterial strains (isolates from extreme environments) obtained from the cultural repository of the Fermentation Biotechnology Laboratory of the School of Biotechnology, University of Jammu, Jammu, were used in this study. The bacterial isolates were examined for proteolytic activity on skimmed milk agar (SKMA) plates [11]. Bacterial cultures were streaked/spotted on SKMA plates. The plates were incubated at 37°C for 48 h, and examined for proteolytic activity. The cultures showing haloes or zones of clearance around spot/streak were considered presumptive proteolytic bacteria, and were selected for further study.

Bacterial strains which exhibited substantial proteolytic activity were examined further for protease producing potential under submerged fermentation. Freshly grown cultures of selected bacteria were inoculated in 100 ml of protease production medium containing (%, w/v): glucose 0.5, peptone 0.5, yeast extract 0.5, K_2HPO_4 0.4, Na_2HPO_4 0.1, $MgCl_2$ 0.01 and Na_2CO_3 0.6 (Hi-Media laboratories Ltd., India), at pH 9.0. Submerged fermentation was executed at 37°C for 48 h under shaking at180 rpm (New Brunswick, USA). Samples withdrawn from fermentation broth were centrifuged at 10,000×g for 10 min at 4°C (Eppendorf Centrifuge 5804-R, Germany), and the supernatant obtained was considered as crude enzyme and used for determining protease activity. For studying time profile for protease production, bacterial isolate K-3 was cultivated in protease production medium at pH 9.0 and 37°C under shaking (180 rpm). The culture broth was centrifuged and supernatant was assayed for protease activity after regular interval of time from 24 h to 120 h.

Bacterial isolate K-3 was observed for cultural morphology, gram-staining and spore staining. For further identification genomic DNA was extracted (HiPurA, Himedia kit) and PCR-amplified using universal 16S rDNA primers (forward primer 5′-AGRGTTTGATCCTGGCTCAG-3′, reverse primer 5′-GGCTACCTTGTTACGACTTT-3′). The amplified product was eluted (Axygen DNA gel extraction kit) and sequenced (Centre for instrumentation facility, University of Delhi, south campus, New Delhi). The DNA sequence data was analyzed for finding the closest homology for the microbe using BLAST.

2.2. Protease assay

Protease activity was determined by using casein (Sigma chemicals Ltd., USA) as the substrate. Assay mixture containing 0.5 ml of appropriately diluted enzyme and 0.5 ml of casein (0.65% in phosphate buffer 50 mM, pH 7.0) was incubated at 37°C for 20 min. The reaction was stopped with 2.5 ml of trichloroacetic acid (TCA, 5%, w/v), contents were centrifuged (10,000×g for 20 min at 4°C), and supernatant was examined

spectrophotometrically (UV-1800 Spectrophotometer, Shimadzu, Japan) for tyrosine content at 280 nm by comparing with tyrosine standard curve. One unit (IU) of protease activity was expressed as one µmol of tyrosine released in one min.

2.3. Effect of initial medium pH, and carbon/nitrogen sources on protease production

To determine the effect of initial medium pH on protease production, the production media pH was adjusted from 7.0-12. The fermentation was carried out by inoculating activated culture in production media at different pH and incubating at 37°C under shaking conditions. The enzyme activity was determined at regular intervals.

For elucidating the effect of carbon source on protease production, glucose of the protease production medium was replaced with different crude as well as refined carbon sources (0.5%, w/v) such as wheat bran, rice bran, wood waste, fructose, lactose or sucrose, (medium pH 9.0). The media were inoculated with 24 h grown culture and fermentation was carried out at 37°C under shaking conditions (180 rpm) and enzyme produced was assayed.

Similarly, to examine the effect of nitrogen source on protease production, usual nitrogen source of the production medium i.e. peptone and yeast extract were replaced with different refined and crude nitrogen sources such as ammonium sulphate, ammonium nitrate, potassium nitrate, gelatin, mustard cake or soybean meal (1.0%, w/v). The media were inoculated with 24 h old culture and fermentation was executed at 37°C under shaking (180 rpm) and protease titre was assayed.

2.4. Partial purification of B. cereus K-3 protease

Protease was developed by conducting submerged fermentation in 400 ml of optimized production medium. The biomass was separated by centrifugation at 10,000×g for 20 min at 4°C. Pellet was discarded and supernatant was fractionated by ammonium sulphate different saturation levels (20-100%). Pellet obtained after each saturation level was dissolved in Tris-Cl buffer (50 mM, pH 9.0). The protein content and enzyme activity of each fraction was determined, after dialysis. Fraction with maximum protease activity was further purified by ion exchange chromatography using diethylaminoethyl (DEAE) cellulose as matrix; elution was carried out with different concentration of NaCl (0.25 M-1.0 M). The fraction with highest protease activity was characterized for the effect of pH and temperature, metal ions and compatibility against various detergents.

2.5. Effect of pH and temperature on activity and stability of protease

For determining the effect of pH on protease activity, assay was conducted at different pH using appropriate buffers: (acetate buffer pH 4.0, citrate buffer pH 6.0, phosphate buffer pH 7.0 and 8.0, Tris-Cl buffer pH 9.0, glycine-NaOH buffer pH 10 and 11 and KCl-NaOH buffer pH 12; 50 mM). For examining pH stability, protease was preincubated for 1-2 h at different pH (4-12), and then assayed for residual activity.

Effect of temperature on activity of protease was examined by conducting assay at different temperatures (40-100°C). The thermostability of protease was determined by preincubating the enzyme at different temperatures ranging from 50-100°C for 40 and 80 min, and then assaying the residual activity.

2.6. Effect of metal ions and detergents on protease activity

The effect of various metals ions viz. Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Pb^{2+}, Cu^{2+}, Cr^{2+}, and Ni^{2+} on enzyme activity was studied by including their respective salts in assay mixture at final concentration of 5.0 mM. The compatibility of the protease was examined with various detergents viz. Surf excel, Nirma, Admiral, Active, Tide, Triton X-100 and SDS. Commercial detergent solutions were heated at 100°C to denature the endogenous enzymes. The enzyme assay mixture was added with 0.1 ml of 4% w/v detergent solution, and protease activity was assayed.

3. Results and discussion

3.1. Selection of proteolytic bacteria

Of the 30 bacterial strains examined for proteolytic activity, 3 isolates (K-3, KV-15 and M-1) which exhibited considerable activity on SKMA were selected. Protease(s) produced by these three isolates under

submerged fermentation were preliminarily examined for pH and thermostability. On the basis of results, isolate K-3 was then selected for further studies. The bacterial colonies were discrete, creamy white, flat and irregular. Microscopic examination revealed that bacterium was Gram-positive, rod shaped and sporulating. 16S rDNA sequencing of isolate K-3 showed 95% homology with that of other *Bacillus cereus* strains, thus validating its identity as *Bacillus cereus* (*B. cereus* K-3).

Among the various bacterial spp., interest in *Bacillus* spp. has gained much importance as their enzyme complement is robust enough to suit the relatively hostile industrial process conditions. *Bacillus* spp. accounts for 35% of total microbial enzyme sale [5,12]. Thus, *Bacillus* spp. are considered super secreting bio-factories for production of various industrial products including enzymes [13-14].

3.2. Initial medium pH and time profile for protease production

Optimum medium pH for protease production was found to be 9.0. The time course of protease production was investigated and maximum production was observed after 48 h (100 U/ml). After 48 h enzyme production got reduced, probably due to secreted unwanted toxic wastes and/or depletion of nutrients in the medium, which might decrease growth and enzyme production [5].

Maximum enzyme production time varies among different *Bacillus* spp. [5,15-16] and depends on microbial strain, cultural and environmental conditions and the genetic potential of the organism. Growth and metabolite (enzyme) production may be partially or completely associated [17-18].

3.3. Protease production using different carbon and nitrogen sources

Among different carbon sources used, protease production was found to be maximum with lactose (140 U/ml), followed by wood waste (102 U/ml). Other carbon sources like wheat bran, rice bran, sucrose and fructose showed considerable protease production as compared to dextrose, taken as control (Figure 1). The bacterium showed highest protease production in presence of ammonium sulphate as nitrogen source (122 U/ml) as compared to peptone and yeast extract of production medium. Other nitrogen sources did not give substantial protease yield as shown in Figure 2.

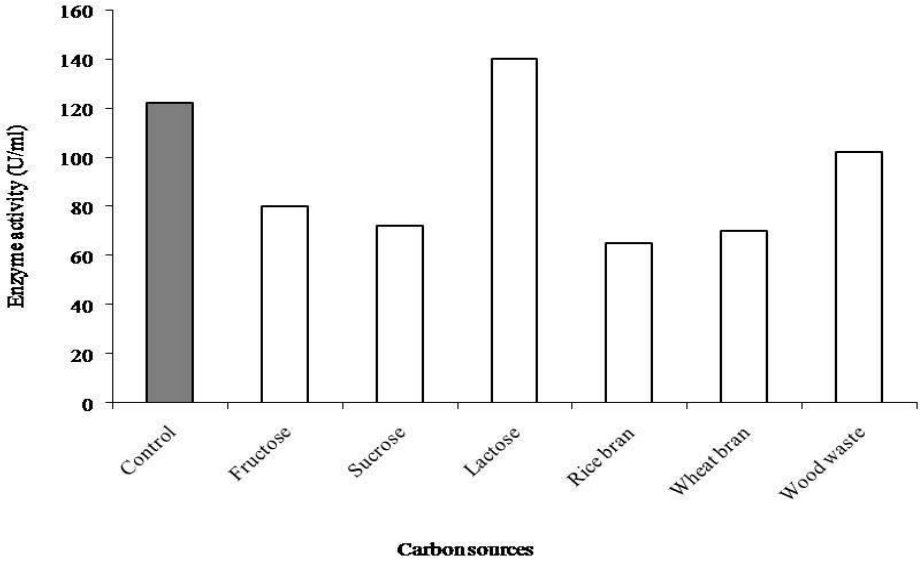

Figure 1: Protease production from *Bacillus cereus* K-3 using different carbon sources. The glucose of the protease production medium was replaced with various other carbon sources at 0.5% (w/v), and fermentation was executed.

Bacillus spp. have the capability to efficiently utilize various substrates for the economic production of commercial products thereby making them workhorses for industrial processes [14]. There are reports where *Bacillus* spp. are known to utilize crude substrates for protease production [19-20]. Corn starch as a carbon source was found to increase enzyme production from *Bacillus* sp. RKY3 [21]. The type and availability of

nitrogenous precursors in the medium influence the production of extracellular enzymes [11]. *B. subtilis* K-1 produced maximum protease when soybean meal was used as nitrogen source [5] while *Streptomyces* sp. DP 2 did so with mustard cake [11]. Besides, nitrogen source can significantly affect the medium pH during the course of fermentation, which in turn may influence enzyme activity and stability [2].

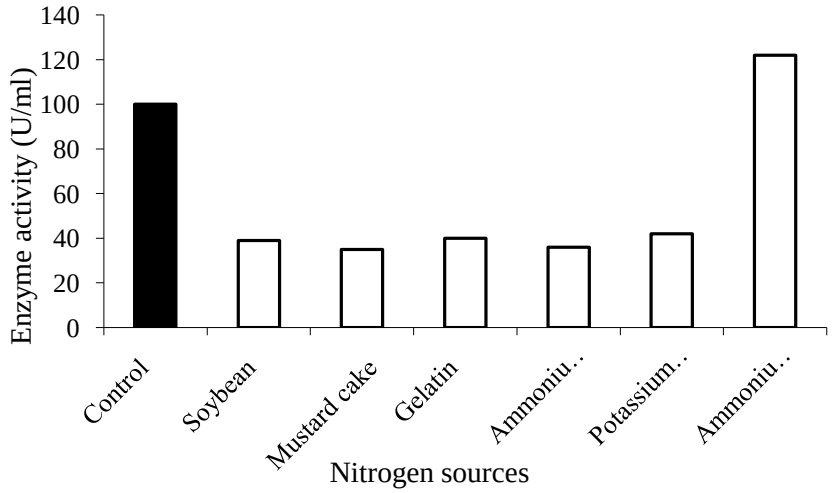

Figure 2: Protease production from *Bacillus cereus* K-3 using different nitrogen sources. Peptone and yeast extract of the protease production medium were replaced with various other nitrogen sources at 1.0% (w/v), and fermentation was executed.

3.4. Effect of pH and temperature on activity and stability of protease

B. cereus K-3 protease was partially purified using ammonium sulphate precipitation and DEAE-cellulose chromatography, and used for characterization. The enzyme was found to be active over a wide pH range of 7.0-10 with optimum at 9.0 (130 U/ml). The enzyme retained upto 65% stability at pH 11 and 12 (85 U/ml and 90 U/ml (Figure 3A)..

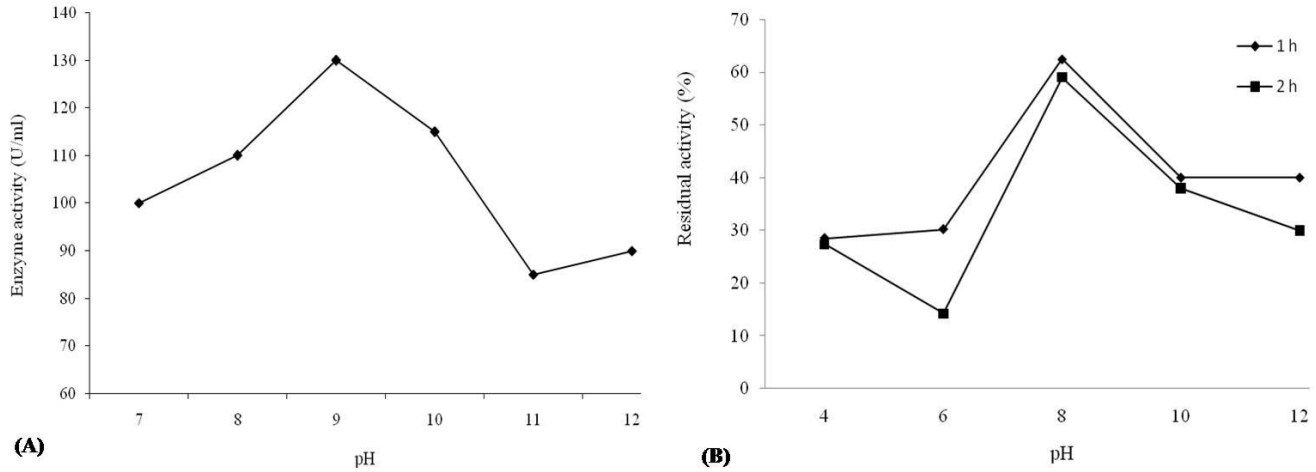

Figure 3: Effect of pH on activity of *B. cereus* K-3 protease (A), and pH-stability (B). Effect of pH was studied using different buffers of appropriate pH. pH-stability was examined by preincubating the protease at different pH for 1 and 2 h, and then assaying the residual activity.

pH-stability analysis of *B. cereus* K-3 protease indicate that protease has maximum stability at pH 8 for 1-2 h (residual activity 62 and 59%). However, at acidic or high alkaline pH activity decreased. Furthermore, activity reduction was observed to be more drastic towards acidic range as compared to alkaline (Figure 3B). The

function of protein is absolutely dependent on its three-dimensional structure. Changes in pH may alter electrostatic interactions between charged amino acids; thereby inducing conformational changes in the three-dimensional structure of the enzyme leading to reduced substrate binding and/or catalytic activity. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrates [5,11,16]

B. cereus K-3 protease expressed maximum activity at 40°C (105 U/ml); a gradual decrease in enzyme activity was observed at higher temperatures (Figure 4A). Considerable activity was seen at 100°C (46 U/ml). Thermostability of *B. cereus* K-3 protease showed that enzyme was thoroughly stable at 50-70°C for 40 min (residual activity 100%), however, there is abrupt decrease in activity at higher temperature (>80°C). However, 80 min pre-incubation of enzyme lead to gradual activity reduction at 60°C and above as presented in Figure 4B.

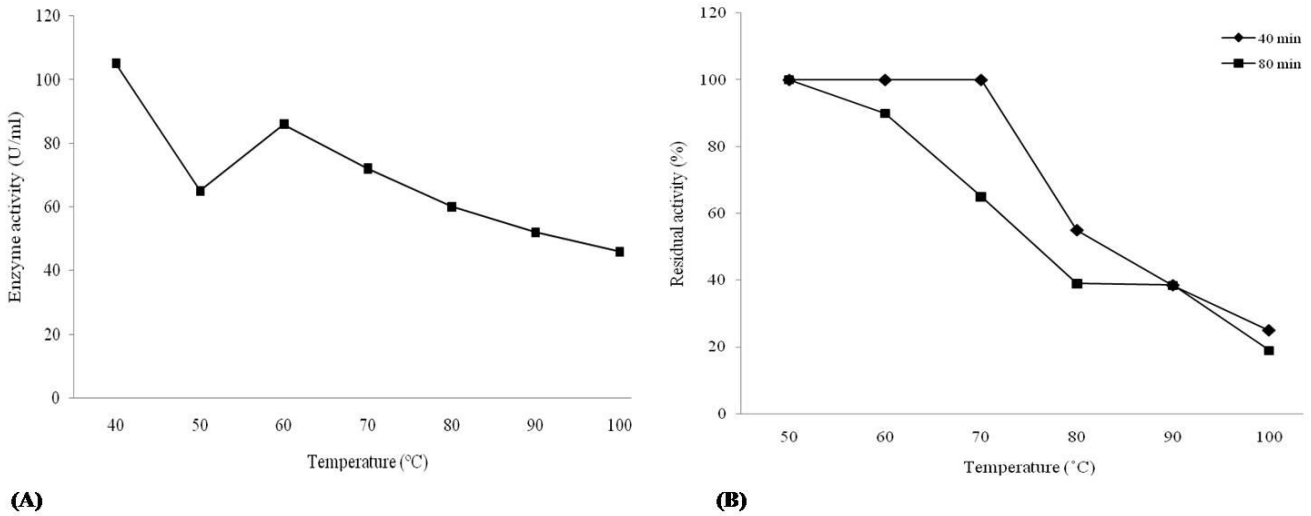

Figure 4: Effect of temperature on activity (A) and stability (B) of *B. cereus* K-3 protease. Activity assay was conducted at different temperatures (40-100^{o}C).Thermostability was analysed by preincubating the enzyme at different temperatures (50-100^{o}C) for 40 and 80 min, and assaying the residual activity.

Temperature optima and stability studies suggested that the enzyme could be used in industrial processes proceeding at extreme temperatures. There are several reports of thermostable protease production from *Bacillus* spp. like *B. pumilus* KS12 [22], *B. licheniformis* KBDL4 [23] and *B. cereus* SIU1 [24]. *B. subtilis* I-2 protease showed maximum activity at pH 8.0 and stability at pH 7.0-10.0, which suggested potential application of this enzyme in various biotechnological industries [25]. Thermostability refers to prolonged stability of enzyme at high temperatures. The thermostability mechanisms for thermozymes are varied and depend upon molecular interactions such as hydrogen bonds, electrostatic and hydrophobic interactions, disulfide bonds, and metal binding which can promote a superior conformational structure for the enzyme [11].

3.5. Effect of metal ions and detergents on enzyme activity

The enzyme activity increased in presence of Ca^{2+} (237 U/ml), Mg^{2+} (260 U/ml), Fe^{2+} (240 U/ml), Zn^{2+} (293 U/ml), Cu^{2+} (243 U/ml) and even Hg^{2+} (272 U/ml), however, certain metal ions decreased the activity like Cr^{2+} (47 U/ml) and Ni^{2+} (70 U/ml) as shown in Figure 5. The increased activity in the presence of metal ions could be attributed to their role in stabilizing the enzyme structure and thus increasing its stability. More than a quarter of all known enzymes require the presence of metal atoms for full catalytic activity. Metal ions can influence the activity of enzymes by multiple ways: they may accept or donate electrons to activate electrophiles or nucleophiles; they themselves act as electrophiles; they may mask effect of nucleophiles to prevent unwanted side reactions; they may bring together enzyme and substrate by means of co-ordinate bonds and may hold the reacting groups in the required three dimensional orientation; they may simply stabilize the catalytically active conformation of the enzyme [16]. *B. cereus* NS-2 fibrinolytic protease activity was increased in the presence of Fe^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+}. However, Pb^{2+} and Hg^{2+} strongly inhibited protease activity [26].

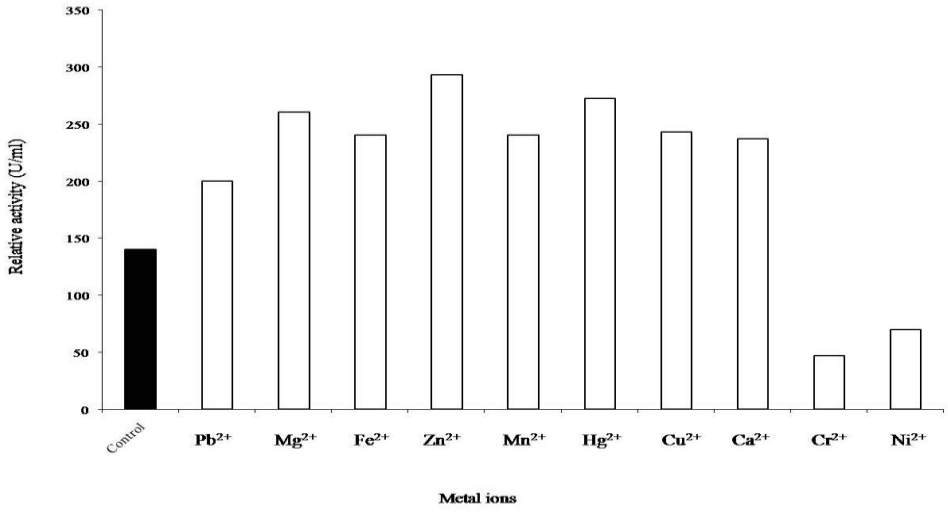

Figure 5: Effect of metal ions on *Bacillus cereus* K-3 protease activity. Different metal salts were included in the enzyme assay mixture at final concentration of 5.0 mM, and activity was assayed.

The enzyme showed reasonable activity in presence of detergents as compared to that of control (100 U/ml) except Triton X-100, which enhanced protease activity by 3-fold (288 U/ml, Figure 6). This may be due to formation of micelles resulting insolubility of enzyme in detergent solution. Enzymes can be denatured and/or show very low reaction rates in solvent or detergent media because of the unfolding, structural disfunctioning, and stripping of the essential water layer from the enzyme molecule.

The stability of alkaline proteases in the presence of denaturants like surfactants, oxidants, detergents and organic solvents is highly desirable characteristic for their applications in industries. The reasonable activity of protease in presence of commercial detergents suggested its potential use as detergent additive. The TKU006 protease from *B. cereus* retained substantially activity in the presence of Tween 20, Tween 40, Triton X-100 and SDS [27]. Alkaline protease from *Bacillus pumillus* was stable not only towards the nonionic surfactants like Triton X-100 and Tween 80 but also the strong anionic surfactant, SDS [2].

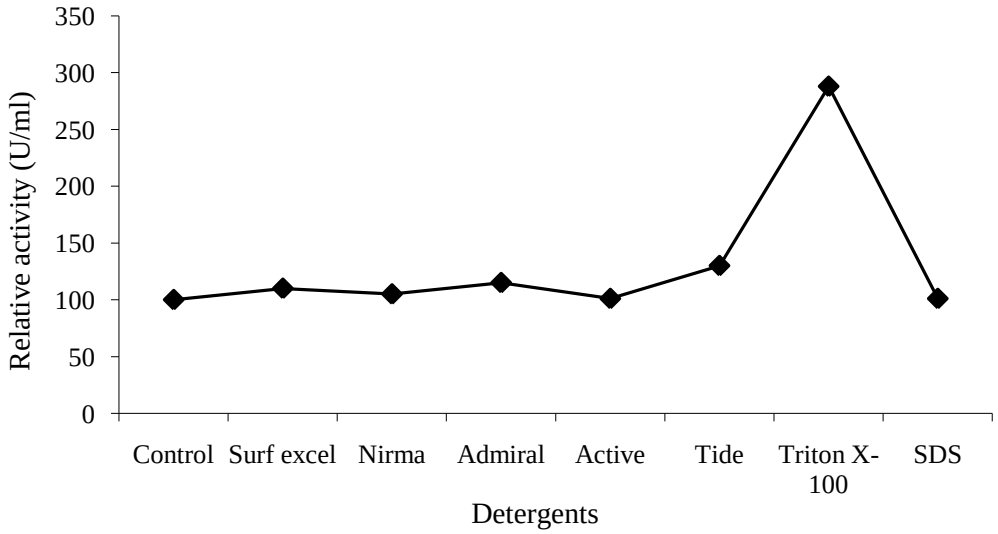

Figure 6: Detergent-compatibility of *Bacillus cereus* K-3 protease. Protease activity assay was executed in presence of different detergents.

Conclusion

It may be concluded that *B. cereus* K-3 successfully utilized agrowastes for protease production. Furthermore, thermostability, alkalistability, detergent-compatibility and heavy metal resistance of the protease make it a suitable candidate for potential applications in various biotechnological industries like detergent, leather, food/feed, photographic etc. Besides, the enzyme may serve as a model system and may provide new insights about the molecular basis of thermostability and alkalistability and may pave the way for engineering of novel enzymes.

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