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Research Article

Production, partial purification and characterization of alkaline protease from *Bacillus aryabhattai* K3

K.M. Sharma, R. Kumar*, S. Vats, A. Gupta.

Department of Biotechnology, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, India–136119.

Abstract

A number of bacterial strains were isolated from soil and were tested for alkaline protease production. The isolate K3, identified as *Bacillus aryabhattai*, exhibited maximum protease activity. Medium constituents and fermentation conditions were standardized for maximum alkaline protease production. The alkaline protease yield was enhanced to 6.66-fold (952 U/ml) under optimized culture conditions. Approximately 6.65-fold purification from the initial culture broth was achieved during ammonium sulphate precipitation (50%-70%) with a yield of 54.63% enzyme. The specific activity of the final partially purified enzyme was 3357.97 U/mg protein. The alkaline protease K3 exhibited its optimal activity at 50°C and at pH 9.0. The enzyme was stable in the pH range of 7.0-10.0. It remained fully active even after 2 h of incubation at 40°C, and retained 86% activity after 2 h incubation at 50°C. The protease was found to be inhibited by Na^{+}, K^{+}, Cu^{+2}, Fe^{+2}, Zn^{+2} and activated by Ca^{+2} and Mn^{+2} and Mg^{+2}. The protease was stable in presence of commercial detergents, retaining more than 90% activity in presence of Ariel, Surf Exel and Nip; more than 80% activity in presence of Tide and Vim; more than 70% activity in presence of Ghadi, after one hour of incubation.

Keywords: Alkaline Protease, Detergents, Fermentation, Precipitation.

1. INTRODUCTION:

Proteases are important industrial enzymes accounting for 60% of total global enzyme sales[1]. They represent one of the three largest groups of industrial enzymes[2]. Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acid[3]. They are indispensable for living organisms;
occur in a wide diversity of plants, animals and microorganisms[4]. Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre or are of metallo-type. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type[5]. Alkaline protease produced from thermophilic and alkalophilic *Bacillus* which can withstand high temperature, pH, chemical denaturing agents and non-aqueous environments have attracted a great deal of attention due to their multitude industrial application such as fertilizers, detergent, leather and pharmaceutical (preparation of ointments) industries and also in biotechnological application such as peptide synthesis, wastes management from various food-processing industries, silver recovery from used X-ray or photographic films and proteinaceous fodder from waste feathers or keratin-containing materials[4,5,6]. Proteases in association with lipase constitute part of lens cleaning solutions for removal of soil particles[7]. Despite the fact that many different proteases have been identified, only a few of them have been used in biotechnological and industrial applications as most are not resilient enough to meet industrial criteria. Thus, the search for new microbial sources is a continual exercise, where one must respect biodiversity[8]. In the present work a new proteolytic bacterial strain was isolated, the culture conditions for the alkaline protease production by isolated strain were optimized, the alkaline protease secreted was partially purified, characterized and studied for its compatibility with various commercially available detergents.

2. MATERIAL AND METHODS:

2.1 Isolation of alkaline protease producing microorganisms

Soil samples were collected from different site of Kurukshetra (Haryana). Sample preparation was done by the method of Seifzadeh et al. [9].These samples were suitably diluted and plated on skim milk agar plates (pH 9.0). Plates were incubated at 37°C for 48 h. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Isolate with highest activity was chosen for further studies and its Identification was done by Institute of Microbial Technology, Chandigarh.

2.2 Production and partial purification of alkaline protease

Protease production was carried out under submerged fermentation conditions using yeast extract casein medium as investigated by Naidu and Devi[10]. The production medium contained (g/l): Glucose, 10.0; Casein, 5.0; Yeast extract, 5.0; KH_2PO_4, 2.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.0. The pH of the medium was adjusted after sterilization to 9.0 using 10% (w/v) sodium carbonate (Na_2CO_3) solution sterilized separately[11]. After 48 h, cultures were centrifuged at 10,000 rpm for 20 min and supernatants were subjected to ammonium salt precipitation which was carried out in narrow range with 10% increments to attain different saturation levels (20-80%) using varying salt concentrations[14]. Each precipitated fraction was dissolved in minimal volume of 0.1M Glycine- NaOH buffer (pH 9.0) and protease activity and protein content were determined.

2.2 Measurement of protease activity and protein content

Protease activity was assayed by modification of method described by Kumar et al. [12]. 0.1ml of appropriately diluted enzyme solution was incubated with 1 ml of 1% casein in Glycine-NaOH buffer (0.1 M, pH 10.0) at 45°C for 10 min and the reaction was terminated by the addition of 5 ml trichloroacetic acid (5%). After 30 min, the mixture was filtered and 2 ml of filtrate was added to 5 ml 0.4 M sodium carbonate and 1 ml Folin- Ciocalteau reagent (1N). Absorbance was then measured at 660 nm. Concentration of L-tyrosine released in the filtrate was determined by comparing against standard curve of L-tyrosine constructed in a similar way. One unit of protease activity was defined as the amount of enzyme that released 1 µg of tyrosine per ml per minute under the above assay conditions. Total protein contents of the enzyme solution were measured according to the method described by Lowry et al. [13] using bovine serum albumin (BSA) as a standard.

2.3 Optimization of media:

2.3.1 Effect of pH and temperature of the medium on alkaline protease production

In order to investigate the influence of pH on protease production, production medium of different pH (6.0-12.0) were inoculated and incubated at 37°C with shaking at 150 rpm. The contents of the flask were harvested after 48 h and assayed for protease activity. Similarly, influence of temperature was investigated by varying the growth temperature (30-50 °C) at optimum pH, keeping the other parameters constant.

2.3.2 Effect of Carbon source on alkaline protease production

The effect of carbon source on protease production was studied by replacing glucose in the production medium with various simple and complex carbon sources including fructose, maltose, lactose, sucrose, glycerol, starch and galactose. A control represented by production medium with glucose as carbon source was performed at the same time. The flasks with medium were inoculated and incubated at 35°C for 48 h with shaking at 150 rpm. The enzyme activity was monitored thereafter. To study the effect of different concentrations of the best carbon source, the concentration of carbon source was varied from 5.0-20.0 g/l.

2.3.3 Effect of Nitrogen source on alkaline protease production

The effect of different nitrogen sources (10.0 g/l) viz. yeast extract, peptone, beef extract, tryptone, casein and their combinations, glutamic acid, glycine, ammonium acetate, sodium nitrate, ammonium chloride, potassium nitrate, sodium nitrate, ammonium sulphate, ammonium nitrate was determined by replacing yeast extract and casein in the production medium. A control experiment with yeast extract and casein as nitrogen source was also performed. Each flask containing the medium was inoculated and incubated at 35°C for 48 h under shaking conditions (150 rpm). The enzyme activity was monitored thereafter. Further, the selected nitrogen source was investigated at different concentrations (10.0-30.0 g/l).

2.4 Characterization of partially purified alkaline protease

2.4.1 Effect of pH on enzyme activity and stability

Optimum pH was determined by estimating the

protease activity over a pH range of 5.0 to 12.0, at 45 ºC using the following buffer systems: 0.1M sodium acetate (pH 5.0); 0.1M sodium phosphate (pH 6.0-7.0); 0.1 M Tris-HCl (pH 8.0); 0.1M glycine-NaOH (pH 9.0-12.0). The effect of pH on protease stability was studied by pre-incubating the enzyme in buffers of different pH values in the range of pH 5.0-12.0 for 2 h at 25ºC. The residual activities were determined at 45°C, pH 9.0 and expressed as percentage of the initial activity taken as 100%. The effect of temperature on protease activity was determined by estimating the protease activity at pH 9.0 and temperatures ranging from 20ºC-80ºC. Thermostability of the protease was examined by pre-incubation of the enzyme preparation (pH 9.0) at different temperatures (40°C-80°C) for varying time periods (30-120 minutes). The residual activities were determined at 50°C and expressed as percentage of the initial activity taken as 100%.

2.4.2 Effect of metal ions on enzyme activity

The effects of various metal ions (5 mM) on enzyme activity were investigated by pre-incubating the enzyme preparation for 30 minutes at 25°C in the presence of monovalent (Na^+, K^+) or divalent (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+}) metal ions. Thereafter, protease assay was performed at 50°C and pH 9.0. Relative activity was determined by considering the activity of the enzyme without any metallic ions as 100%.

2.4.3 Stability of enzyme in presence of commercial detergents

The suitability of the protease as a detergent additive was determined by testing its stability in the presence of commercial detergents including Ghari, Ariel, Tide, Surf Exel, Nip (Fena), Vim. Detergents were diluted in tap water to give a final concentration of 7 mg/ml to simulate washing conditions[15]. The partially purified enzyme was then added to different detergent solutions and incubated at 40°C for 2 h. The enzyme without adding any detergent and incubated under the same conditions was taken as the control. Suitable aliquots were withdrawn after 1h and 2h of incubation. Residual activity was measured at 50°C, pH 9.0 for 10 min and compared with the control.

3. RESULTS AND DISCUSSION:

3.1 Isolation and Screening

Total of fifty morphologically different bacterial strains were isolated from different soil samples. Isolated cultures were separately screened for their proteolytic activity. Twenty isolates showed proteolytic activity, as indicated by zone of clearance on skim milk agar. Figure 3.1 shows zone of clearance around the colony of the bacterial isolate K3 which exhibited widest zone of clearance. The twenty proteolytic strains were then subjected to quantitative screening to select the most promising enzyme producer under submerged fermentation conditions.The alkaline protease activity of the isolates was found to be ranging from 40.94 U/ml to 143.61 U/ml. Isolate K3 was selected for further study as it exhibited highest alkaline protease activity.

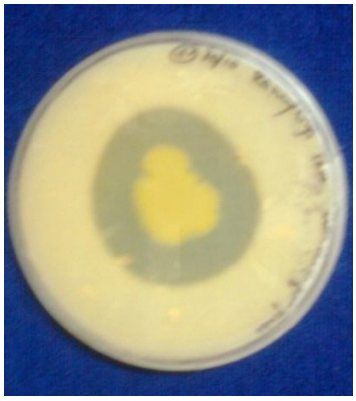

Figure 3.1 Zone of hydrolysis around the colony of K3 on skim milk agar plate.

3.2. Growth kinetics and protease production

Protease production by *Bacillus aryabhattai* K3 with reference to its growth is shown in Figure 3.2. The study indicated that the growth was exponential up to 36 h followed by stationary phase. The production of protease could be noticed from the early exponential phase of growth. The bacterium was producing only small quantity of enzyme in early stages. A steady increase in enzyme production could be seen as the growth progressed from early exponential to early stationary phase. Protease secretion reached a maximum in 48 hours at stationary phase. Thereafter a decline in activity was observed. This suggests that production of protease by this *Bacillus* sp. is dependent on cell growth and incubation time has been reported to play substantial role in enzyme production Folasade M. Olajuyigbe[16].

3.3 Optimization of conditions for alkaline protease production

3.3.1 Effect of medium pH on alkaline protease production

The pH of the medium had significant effect on alkaline protease production by *B. aryabhattai* K3 as shown in Figure 3.3. The bacterium produced very low levels of alkaline protease when grown in the medium of pH 6.0. The production of protease increased with increase in pH of the medium and peaked to 362.58 U/ml at pH 8.0. With further

increase in pH, there was a decline in enzyme titre. Similarly *B. cereus* SV1 protease showed optimum activity at pH 8.0 and Protease from halo-alkaliphilic *Bacillus* sp. 17N-1 showed activity at pH 6.5 to 8.5 with an optimum at pH 7 reported by Bajaj and Jamwal[17]. Likewise Arulmani et al. [21] published a report on a protease from *Bacillus laterosporus*-AK1which was active in the pH range 7.0-12.0 and had an optimum pH of 9.0.

3.3.2 Effect of incubation temperature on alkaline protease production

The culture was grown at various temperatures to find out the most suitable temperature for alkaline protease production by *B. aryabhattai* K3. The optimum temperature for protease production was found to be 35°C (417.37 U/ml) as shown in Figure 3.4. Protease titre reduced significantly beyond 40°C. This is in contrast to *B. subtilis* SHS-04 in which Optimum temperature for cell growth was 37°C but op- timum protease production was recorded at 45°C reported by F. M. Olajuyigbe[16].

3.3.3 Effect of carbon sources on alkaline protease production

Different carbon sources (10 g/l) used in the production medium for alkaline protease production by *B. aryabhattai* K3 resulted in wide variation of enzyme levels as presented in Figure 3.5. Among these, maximum titre (622.64 U/ml) was attained in the medium supplemented with lactose. Similarly Bajaj and Jamwal[17] reported that *Bacillus pumilus* D-6 exhibited higher productivity of protease in culture media using lactose as carbon source.

3.3.4 Effect of nitrogen sources on alkaline protease production

Figure 3.6 depicts the effect of different nitrogen sources (10 g/l) on alkaline protease production by *B. aryabhattai* K3. The alkaline protease production was better with organic nitrogen sources than with the inorganic nitrogen sources and amino acids. Combination of yeast extract and casein (control) stimulated the highest protease yield (624.48 U/ml) followed by a combination of yeast extract and beef extract (483.58 U/ml). Bajaj and Jamwal[17] published a report on a protease from *B. Pumilus* D-6 found that urea showed 80% maximum protease activity while ammonium sulphate enhanced enzyme production by 40%, and gelatin and soybean meal did so to the tune of 26 and 24%, respectively.

3.4 Partial purification of alkaline protease

The results of partial purification of protease from *Bacillus aryabhattai* K3 are summarized in Table 3.1. Approximately 6.65-fold purification from the initial culture broth was achieved during ammonium sulphate precipitation (50%-70%) with a yield of 54.62% enzyme. The specific activity of the final partially purified enzyme was 3357.97 U/mg protein. The partially purified enzyme was thereafter designated as alkaline protease K3. Gupta et al[18] reported approximately 4.62-fold purification of protease from *Virgibacillus pantothenticus* MTCC 6729 by ammonium sulphate precipitation (80%) with a recovery of 36.90% enzyme.

3.4 Effect of metal ions on enzyme activity

Effect of metal ions on the activity of alkaline protease K3 is presented in Figure 3.7. The addition of Ca^{+2} and Mn^{+2} and Mg^{+2} increased the enzyme activity by about 36%, 22% and 8% respectively. These cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures[8]. Other metal ions which had a negative impact included Na^{+}, K^{+}, Cu^{+2}, Fe^{+2} and Zn^{+2}.A significant inhibitory effect on the protease activity was observed with Hg^{2+} (0% relative activity). Sathyavrathan and Krithika[19] reported that Bacillus licheniformis NRRL-NRS-1264 shows lower enzyme activity when supplemented with Ca and Mg. The most striking feature was that enzyme activity remained un-fazed even in presence of inhibitors like Pb2+ and Hg2+ which are considered universal inhibitors of enzyme activity reported by Bajaj and Jamwal[17].

3.5 Stability of alkaline protease K3 in presence of commercial detergents

In order to evaluate the feasibility of using alkaline protease K3 as a detergent-additive, its stability towards some detergents available in the local market, was studied. Relative activity of the protease after incubation with different detergents is presented in Figure 3.8. The protease was stable in presence of commercial detergents, retaining more than 90% activity in presence of Ariel, Surf Exel and Nip; more than 80% activity in presence of Tide and Vim; more than 70% activity in presence of Ghadi, after one hour of incubation. The enzyme retained more than 50% activity with all the detergents tested even after 2 h of incubation. Thus, it can be used as a detergent addditive. Kumari and Rani[20] reported that Wheel, Surf Excel and Nirma shows 80, 82 and 80% relative activity respectively after 1 hr of incubation where as Rin and Ariel shows between 50-60% relative activity after 1 hr of incubation and after 2 hr of incubation Wheel and Surf Excel shows 60% relative activity.

4. CONCLUSION

Owing to its stability at alkaline pH, high temperature and also in presence of commercial detergents; it can be used as an additive in detergents. Although many alkaline proteases have been reported from microbial origins, to our knowledge this is the first report on production, partial purification and characterization of an alkaline protease from this bacterium. Further purification and characterization of the protease can be carried out to test its suitability for other industrial applications. Study can be conducted in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme.

Table 3.1 Summary of partial purification of alkaline protease:

Purification step	Total Protease Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude extract	95200.00	1.8850	188.50	505.04	1.00	100
$(NH_4)_2SO_4$ (50%-70%) (dialyzed)	51998.24	1.2388	15.485	3357.97	6.65	54.62

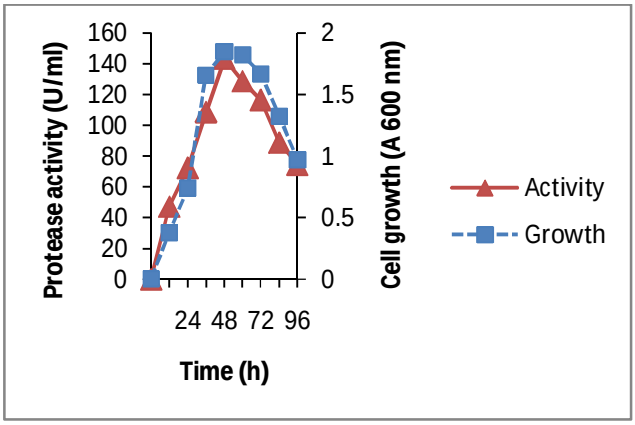

Figure 3.2 Time profile of cell growth and alkaline protease production (1% (v/v) inoculum, pH 9.0, 37°C, 150 rpm).

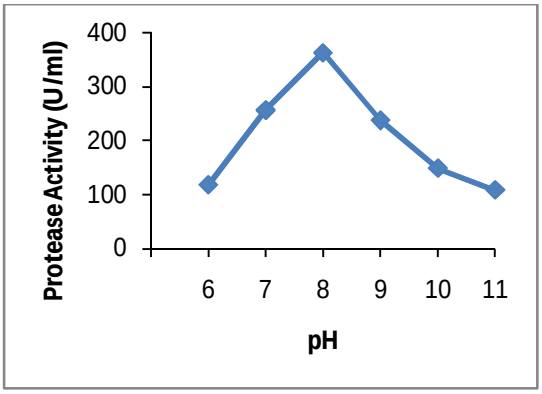

Figure 3.3 Effect of pH on alkaline protease production (5% (v/v) inoculum, 37°C, 48 h, 150 rpm).

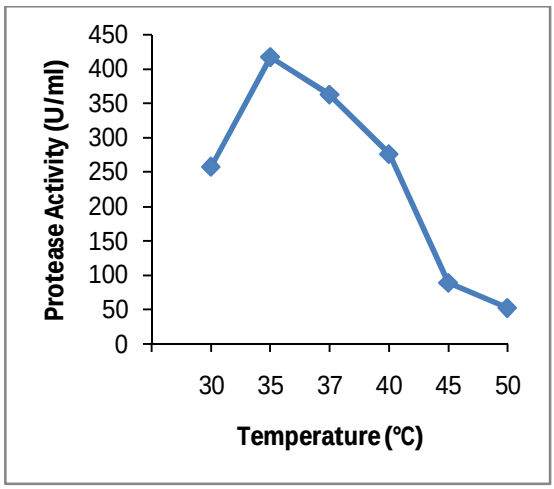

Figure 3.4 Effect of incubation temperature on alkaline protease production (5% (v/v) inoculum, pH 8.0, 48 h, 150 rpm).

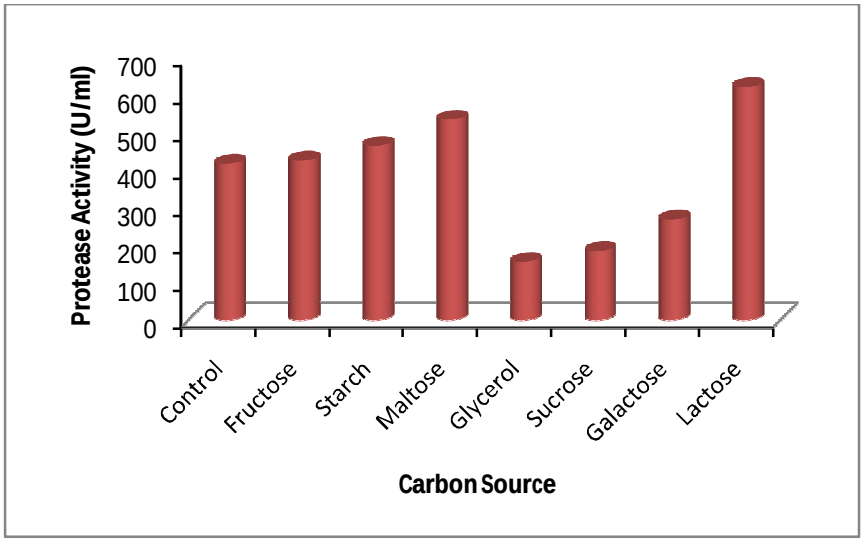

Figure 3.5 Effect of carbon sources on alkaline protease production (5% (v/v) inoculum, pH 8.0, 35°C, 48 h, 150 rpm).

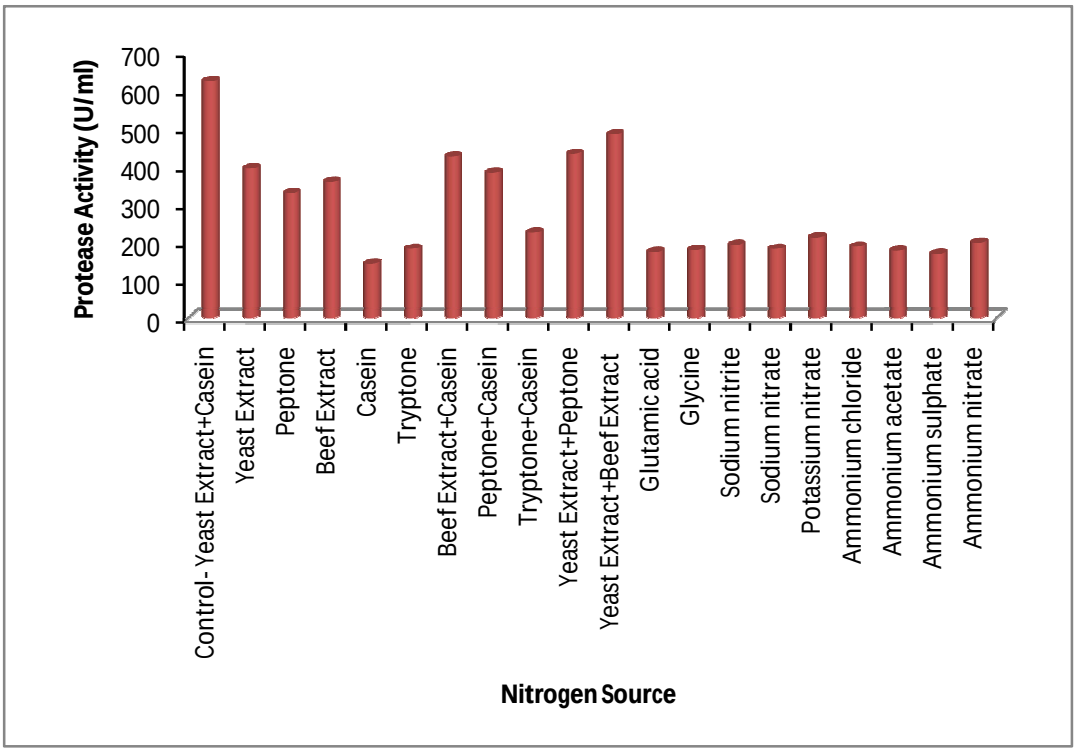

Figure 3.6 Effect of nitrogen sources on alkaline protease production (5% (v/v) inoculum, pH 8.0, 35°C, 48 h, 150 rpm).

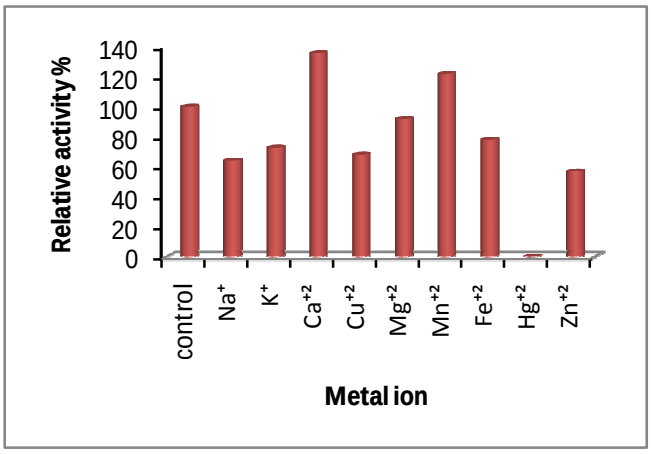

Figure 3.7 Effect of metal ions on activity of alkaline protease K3.

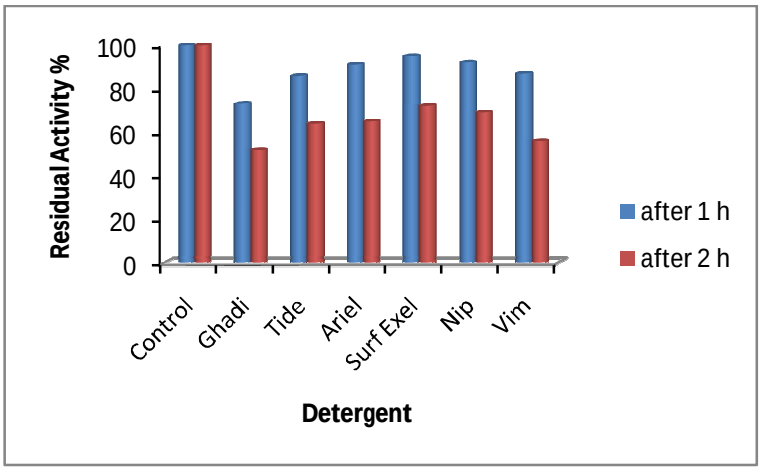

Figure 3.8 Stability of alkaline protease K3 in presence of commercial detergents.

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