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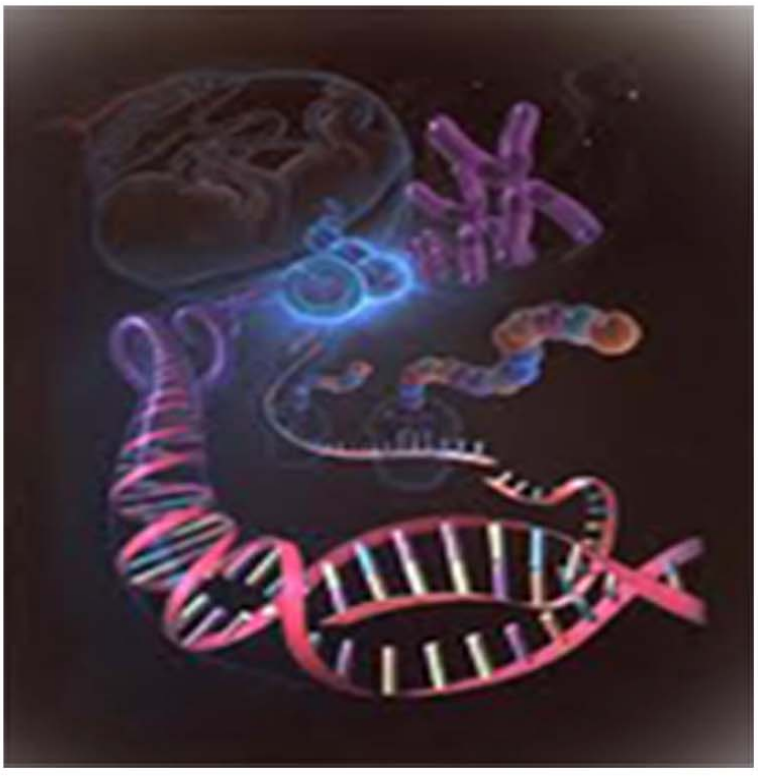

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Research Paper

CHARACTERIZATION STUDIES ON CASEINOLYTIC EXTRACELLULAR ALKALINE PROTEASE FROM A MUTANT *BACILLUS LICHENIFORMIS*

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After our previous purificational and optimisational studies on alkaline protease from a mutant of *B. licheniformis* Bl8, Characterisation of the purified enzyme was done from the culture supernatant by employing various parameters. The optimum pH and temperature for the activity of alkaline protease was previously found to be 10 and 50^{0}C and stable in the pH range 5.0 - 12.0. The thermo stability exhibited by protease ranges from 30-70^{0}C. Among various protease inhibitors PMSF strongly inhibited the enzyme activity revealing that the enzyme in the present study is serine alkaline protease. Ca^{2+} and Mn^{+2} had a slight enhancing effect on the activity of the enzyme. High level of hydrolytic activity was shown by casein and also found that purified alkaline protease digested the human blood clot, coagulated egg white to soluble form and also digested the chicken skin after prolonged incubation. The protease showed good compatibility and stability in the presence of $CaCl_2$ and glycine with detergents. The enzyme retained 20-40% activity with most of the detergents tested even after 3 h. The supplementation of the enzyme preparation in detergents completely removed the blood stain of the cloth. The enzyme followed a typical Michaelis-Menten kinetics and the apparent km value was found to be 3.2 mg ml^{-1}.

Keywords: Alkaline protease, Mutant *Bacillus licheniformis* Bl8 and Characterization

INTRODUCTION

Proteases are the enzymes that occupy a pivotal position both physiological and commercial fields. The detergent industry is the largest user of industrial enzymes, which accounts for more than one 1/3rd of the global market and is considered to be total of 1.6 billion US dollars. The genus *Bacillus* is most important organism for commercial protease production due to their high thermostability and pH stability (Juhasz and Skarka, 1990). In our earlier studies, we obtained a mutant strain of *B. licheniformis* Bl8 which produced 63.64% higher titers of alkaline protease over the wild strain, which is a significant increase

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of yield and found to be stable for the high yielding nature (Kaur *et al*., 1998). The present investigation deals with purification and characterization of alkaline protease of this mutant and its suitability for various commercial applications.

MATERIALS AND METHODS

Microorganism and Cultural Conditions

The bacterial strain of *B. licheniformis* Bl8 isolated from alkaline soil of the milk processing unit and was mutated by UV irradiation (Kaur *et al*., 1998). Alkaline protease production was carried out in a 2L fermentator (Lark Bioreactor) containing 1L modified production medium which has been previousily mentioned. After the completion of fermentation the whole fermentation broth was centrifuged at 10,000 rpm at 40^{0}C and the clear supernatant was separated. The supernatant (crude enzyme) was presently subjected to characterization process after purification which was done earlier by various methods.

Further Characterisation of Purified Alkaline Protease

Effect of Metal Ions on the Activity of Alkaline Protease

The enzyme was incubated with various metal ion sources (10mM) viz, Ca^{2+}, Mg^{2+}, Co^{+2}, Cd^{+2}, Fe^{+3}, Na^{+}, Zn^{2+} and Cu^{2+} for 30min at 40^{0}C and relative protease activities were measured.

Effect of Different Substrates on the Alkaline Protease Activity

Substrates used here were Viz, bovine serum albumin, casein, egg albumin and gelatin. 1ml of enzyme was incubated with different protein substrates and the activities were measured.

Digestion of Natural Proteins

2ml of the enzyme was incubated with human blood clot, coagulated egg white and chicken skin in glycine-NaOH buffer (pH 9.5) at 37^{0}C for 10 h.

Detergent Stability of the Enzyme

The compatibility of purified alkaline protease with local laundry detergents was studied in the presence of 10mM $CaCl_2$ and 1M glycine.The detergents used were Ariel, Surf excel, Rin, Nirma and Wheel. The cleaning composition comprises one acid fluoride salt and one organic soil removing agent, stain remover, anti deposition agent. No enzymes have been reported, but the exact composition of the detergents has not been given by the specified companies. The detergents were diluted in distilled water (0.5% w/v), incubated with enzyme for 3 h at 40^{0}C, and the residual activity determined. The enzyme activity of a control sample was taken as 100%.

Destaining Property of Purified Alkaline Protease

This property was studied by dipping two pieces of blood stained cloth in detergent solution or detergent solution supplemented with enzyme followed by incubation for 10 min at 40^{0}C.

Effect of Substrate Conc. on Activity of the Enzyme

This was studied by incubating the 1ml of purified protease with different concs. of casein (substrate) viz, 1-10 mg respectively and the activity of enzyme was measured. From this, Km value of the enzyme was calculated.

RESULTS AND DISCUSSION

Characterization of Purified Alkaline Protease

Effect of various metal ions on the activity of the enzyme was shown in Table 1. Activities were expressed relative to the control. Activity of the control was taken as 100%. None of the metal ions showed considerable enhancing effect on

the activity of the protease. Ca^{2+} & Mn^{+2} had a slight enhancing effect on the activity of the enzyme. Of the different metal ions tested Zn^{+2} showed the maximum inhibition. Mn^{+2} showed the enhancing activity of alkaline serine proteases from some bacterial sources such as *B. sterothermophilus* F1 (Juhasz and Skarka, 1990) and *Nocardiopsis derssonvillei* (Laemmli, 1970).

Effect of different substrates on enzyme activity was studied. High level of the hydrolytic activity was shown by casein and with poor to moderated hydrolysis by BSA and egg albumin. However, the hydrolysis was hardly observed with gelatin and the results were shown in Table 2.

The ability of purified protease to digest some natural proteins was tested (Plate 1). These results showed that enzyme can convert the insoluble forms of human clot and coagulated white egg to soluble form. The enzyme was also able to digest chicken skin after incubation for a long time. The results suggest the usefulness of this enzyme for different application such as, extraction of collagen from skin for collagen replacement therapy, waste treatment and others.

Enzyme activity and stability in the presence of some available commercial detergents was also studied with a view to exploit the enzyme in detergent industry. A good protease is expected to be stable in the presence of commercial detergents. In the present study, the protease showed excellent stability and compatibility in the presence of locally available detergents (wheel, Ariel, Surf excel, Rin, Nirma) at 40^0C in the presence of $CaCl_2$ and glycine as stabilisers. The proteases showed good stability and compatibility in the presence of Nirma, Surfexcel and wheel (Table 3 and Figure 1). Enzyme activity and stability in the presence of detergents was reported earlier (Manachini *et al*., 1982). They observed that the enzyme retains 84.5% of activity in the presence of Vim and more than 40% in the presence of Nirma super, Wheel and Nirma. Interestingly, in the present study, the enzyme retained around 20-40% activity with most of the detergents tested even after 3 h of incubation (Rahman *et al*., 1994).

The results of destaining experiment showed the complete removal of stain in detergent solution supplemented with enzyme where as blood stain was not completely removed from cloth dipped in detergent solution alone (Plate 2). This suggested usefulness of this enzyme in detergent industry. From this study, the protease could be recommended as an additive in detergents to improve the washing performance.

Table 1: Effect of Metal Ions on the Activity of Alkaline Protease

Metal ion Source	C	$CaCl_2$ (Ca^{2+})	$MgCl_2$ (Mg^{2+})	$CoCl_2$ (Co^{2+})	$CdCl_2$ (Cd^{2+})	$FeCl_3$ (Fe^{3+})	NaCl (Na^{+})	$ZnCl_2$ (Zn^{+2})	$CuCl_2$ (Cu^{+2})
Relativeactivity(%) (10mM)	100	102.1	101	90	90	80	98	82	90

Table 2: Effect of Different Substrates on the Alkaline Protease Activity

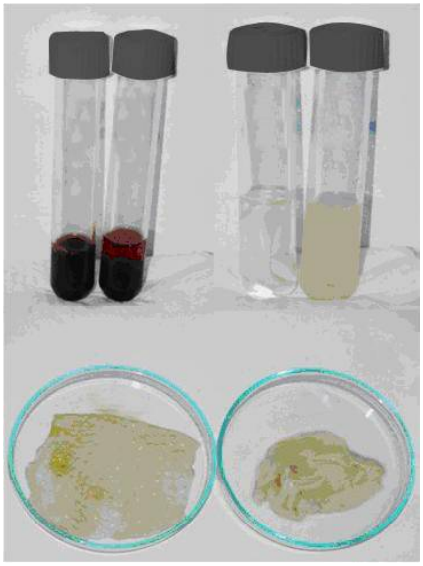
Substrate (1 ml)	Casein	Bovine serum albumin	Egg albumin	Gelatin
Relative enzyme activity (%)	100	54	32	12

Table 3: Detergent Stability of the Enzyme

Detergent	(%) Retention of enzyme activity						
Time	0 hr	0.5 hr	1	1.5	2	2.5	3.0
Control	100	100	100	100	100	100	100
Wheel	100	90	80	70	60	50	46
Ariel	100	74	62	51	43	36	20
Surf excel	100	84	82	64	62	48	35
Rin	100	65	59	47	36	30	22
Nirma	100	90	86	74	50	48	40

Plate 1: Digestion of Natural Proteins

A Blood clot B Egg white



Chicken skin

Plate 2: Washing Performance of Alkaline Protease

(A) Blood stained Cloth (B)Stained cloth after detergent wash (C)Stained cloth after detergent and enzyme wash

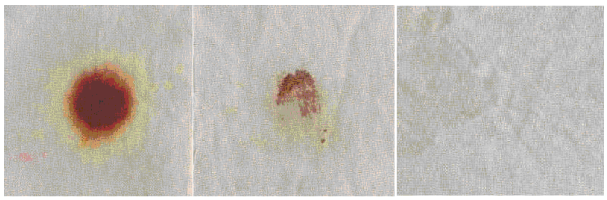


Figure 1: Effect of Detergents on the Stability of Purified Alkaline Protease

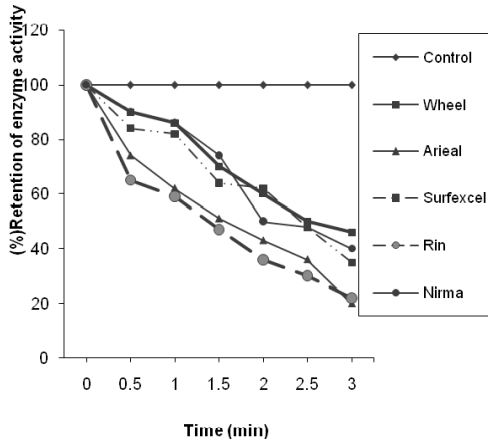
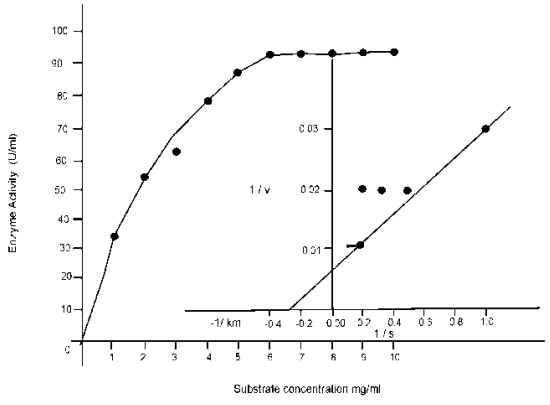


Figure 2: Effect of Substrate Conc. on Purified Alkaline Protease (Inset Line weaver-Burk Plot)



Effect of substrate conc. on enzyme activity was also studied (Figure 2). The results showed that the enzyme followed a typical Michalis-Menten kinetics from a double reciprocal plot. The apparent Km of the enzyme for casein was found to be 3.2 mg/ml. The Km values of 0.4 and 1.3 towards casein (mg ml^{-1}) have been reported for alkaline proteases of *B. alcalophilus var. halodurans* (Rao *et al*., 1998) and *Brevibacterium linens* (Strongin *et al*., 1979), higher values 3.7, 7.4 and 9.4 mg ml^{-1} have been reported for the proteases of *B. polymyxa* (Kaur *et al.,* 1998) *Halomonas* sp., Es10 (Tobe *et al*., 1975) and *B. licheniformis* N3 (Udandi Boominadhan and Rajendran Rajakumar, 2009). The lower km value of 3.2 mg ml^{-1} in the present study indicates lower affinity of the enzyme from mutant *B. licheniformis* (Bl8). Apparent km of the enzyme for casein was found to be 2.9 mg ml^{-1} for mutant *B.polymyxa*. It has lower Km value than the wild *B. polymyxa* protease (Manachini *et al*., 1982).

CONCLUSION

The purified alkaline serine protease exhibited desirable properties such as high pH and temperature optima, detergent stability, good washing performance. So this enzyme can be suggested for application in detergent industry, after proper evaluation of the performance in field trails. The results also suggested that the usefulness of this enzyme for different application such as, extraction of collagen from skin for collagen replacement therapy, waste treatment and others. The suitability of this protease for other commercial applications was not investigated. However, it is reasonable to assume the promising nature of this enzyme for other commercial applications.

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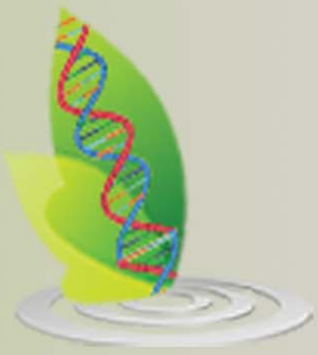

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