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ARTICLE

Characterization of a metallo-protease produced in solid state fermentation by a newly isolated *Bacillus* strain

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ABSTRACT A metalloprotease was characterised from a newly isolated *Bacillus* strain. The enzyme activity was stimulated by mono- and divalent metal ions at 5 and 10 mM concentration and inhibited by EDTA, DTT and β ME (95, 90 and 80%, respectively) at 10 mM concentration. The enzyme inhibition caused by 10 mM EDTA was restored by 45-47% with 5 and 10 mM NaCl. These characteristics established the enzyme as a metalloprotease. Different agroindustrial substrates were used for economic production of the metalloprotease, among which rice bran was selected as best providing ≈1000 U protease /g of substrate in 72 h with 20% inoculum and 1:3 moisture content (w/v). The enzyme was thermostable (30-50°C) with maximum activity at 60°C. It also exhibited a broad pH activity range (6-9) with a maximum at pH 8.
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KEY WORDS

metalloprotease
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Solid-state fermentation

With the global enzyme demand rising 6.3 percent annually through 2013 (http://www.marketresearch.com/map/prod/2432362.html) and proteases representing the largest commercially exploited industrial enzymes. Among various proteolytic enzymes, there is a group of peptidases, metalloproteases, requiring metal for their activity. These metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by requiring a divalent metal ion like zinc, cobalt, manganese or nickel for their activity (Alvarez et al. 2006). Various microbial strains belonging to species, e.g. *Bacillus subtilis, Bacillus thermoproteolyticus, Bacillus megaterium, Bacillus cereus, Bacillus thuringiensis, Listeria monocytogenes*, *Streptomyces griseus* are reported to produce neutral metalloproteases which are sensitive to metal chelating agents such as ethylene diamine tetraacetic acid (EDTA) and o-phenanthroline (Miyoshi and Shinoda 2000, Bitar, et al 2008). Neutral metalloproteases find industrial importance as they cleave the amylase precursor into ρ and α amylases (Takekawa et al. 1991). Therapeutically, they selectively cleave fibronectin and type IV collagen and hence are important in dermatology (Stenn et al. 1989). Metalloproteases from *Bacillus thuringiensis* degrades antibacterial proteins produced by the insect host (Dalhammar and Steiner 1984). In addition, many extracellular metalloproteases play an important role in pathogenesis (Miyoshi and Shinoda 2000).

In wake of the wide ranged application of the microbial enzymes continuous efforts for their economic production to cope with industrial process is under focus. In this scenario, solid state fermentation has gained tremendous attention for the low cost production of industrially important enzymes by utilization of various types of waste as agro-industrial waste. Globally, 140 billion metric tons of biomass wastes are generated every year from agriculture. If managed carefully, however, solid state fermentation offers the best possible use of these agro-industrial wastes as substrate for metalloproteases production through different manipulations. The high protein and moisture content of these underutilized wastes facilitate their use as substrate in SSF with the supplementation of nitrogen sources (Saxena and Singh 2010). Advantages of solid-state fermentation include lower manufacturing costs with large volumes of production, less preprocessing energy and effluent generation, along with easy process management and better product recovery (Prakasham et al. 2006; Oliveira et al. 2006).

Present work reports metalloprotease production by a newly isolated *Bacillus* strain, having capability of utilizing agro industrial waste (wheat bran, gram husk, rice bran and mustard oil seed cake) as substrate. The optimization of the fermentation parameters and enzyme characteristics for its applicability and commercial evaluation has been established.

Materials and Methods

Microorganism

Proteolytic bacterial strains were isolated from soil collected from various sites at Delhi, NCR (National Capital Region), India and maintained in 50% glycerol at -20°C. The strains were screened for their protease producing property by the milk agar plate method. For this, the strains were streaked on

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milk agar plates containing 1% skimmed milk powder and incubated at 37°C overnight. The clear zone of hydrolysis around the streak indicates proteolytic property of the organism. The bacterial strain used in this study was selected on the basis of zone of hydrolysis exhibited on milk agar plates.

Identification of the selected strain

Bacterial isolates were identified on the basis of their morphological (cell shape, Gram-staining nature, spore formation and motility) and biochemical characteristics (IMViC, oxidase, starch hydrolysis, catalase, casein hydrolysis test, nitrate reduction and glucose fermentation tests) according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974).

Solid state fermentation

The agro-industrial wastes to be used as substrate were procured from local market of Delhi (India) and grinded to size about 2-4 mm before use. Erlenmeyer flasks (100 ml) were employed for the experiments containing 5 grams of the substrate and 10 ml of liquid medium (0.1% KH_2PO_4, 0.25% NaCl, 0.01% $MgSO_4.7H_2O$, and 0.01% $CaCl_2$). The sterilized flasks were inoculated and incubated at 37°C for the desired period. The protease activity was checked periodically, after every 24 h.

Optimization of process parameters for enzyme production

Factors affecting enzyme production, as substrate, incubation time, moisture content and inoculum size were optimized. Enzyme production was performed by varying the substrate (wheat bran, gram husk, rice bran and mustard oil cake), the incubation time (24 h to 96 h), moisture content (1:2 to 1:5; w/v) and inoculum size (1%, 5%, 10% and 20% of bacterial culture with colony count at 4.5 x 10^6 cfu/ml).

Enzyme assay

Protease activity was assayed by the modified method of Kembhavi et. al (1993) using casein as substrate. Aliquots of enzyme suitably diluted were mixed with 50mM glycine-NaOH buffer (pH 9) to make 1 ml volume. One ml of 1% casein was added and incubated for 10 min at 60°C. The reaction was stopped by addition of 4 ml of 5% TCA. The mixture was allowed to stand at room temperature for 30 min to 1 h and centrifuged at 8000 rpm for 10 min. One ml of the supernatant was mixed with 5 ml of 0.5M Na_2CO_3 solution and 0.5 ml of Folin &Ciocalteu's reagent (1:2) was added. The mixture was incubated in dark to develop the blue colour, and then estimated spectrophotometrically at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate1 µg/ml tyrosine in 1 min under the experimental conditions. The experiments were carried out in triplicates and standard error was calculated.

Partial purification of enzyme

Partially purified enzyme was obtained by addition of solid $(NH_4)_2SO_4$ at 70% saturation to the crude enzyme obtained after solid state fermentation, with constant stirring at room temperature. The precipitate was collected by centrifugation at 12000 g for 20 min at 4°C. This precipitate was dissolved in 0.1 M phosphate buffer (pH 6.0) and dialysed overnight against the same buffer. This dialysate was used as the enzyme solution.

Effect of various physico-chemical factors on enzyme activity

Effect of temperature and pH on activity and stability of the enzyme

The optimum temperature of the enzyme activity was examined by varying the incubation temperature of the assay from 30°C to 70°C (at pH 9 for 10 min). The thermal stability of the enzyme was assessed by incubating the enzyme without the substrate fractions at various temperatures between 30ºC to 70ºC for 2 h. Enzyme samples were taken and assayed for activity at 30 min intervals..

Optimum pH for the enzyme activity was assessed by performing the assay with buffers ranging from pH 6 to 10 with casein (1%, w/v) as substrate. Phosphate buffer (pH 6-7) and glycine-NaOH buffer (pH 8-10) were used. Stability of the enzyme at different pH was assessed by incubating the enzyme in glycine-NaOH buffers of different pH for 2 h. The enzyme activity was measured at every 30 min.

Effect of NaCl on enzyme activity

To study the effect of NaCl on enzyme activity at different pH, aliquots of the enzyme were supplemented with NaCl (5 mM and 10 mM) at different pH (6-10) and assayed by the method discussed above.

Effect of metal ions and inhibitors on enzyme activity

Enzyme activity was assayed in the presence of 5 mM and 10 mM concentrations of various monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Hg^{2+}) metal ions, in chloride and sulphate salts. The activity of the enzyme was compared with those obtained in pure 50 mM glycine–NaOH buffer.

The effect of various protease inhibitors such as ethylenediaminetetraacetic acid [EDTA], dithiothreitol [DTT] and β-mercaptoethanol [β-ME] were determined by the addition of the corresponding inhibitors at a final concentration of at 5 mM and 10 mM to the reaction mixture, incubated at 37°C for 1 h without the substrate fraction and assayed under standard conditions.

Table 1. Morphological and biochemical tests performed for identification of selected bacterial isolate.

Morphological tests	
Grams staining	+
Cell shape	rods
Spore formation	+
Biochemical tests	
Indole production	–
Methyl red	–
Voges-Proskauer	+
Citrate utilization	–
Oxidase-test	–
Catalase-test	+
Starch hydrolysis	–
Nitrate reduction	–
Casein hydrolysis	+
Gelatin hydrolysis	+
Glucose fermentation	+
Lactose fermentation	+
Sucrose fermentation	+

Figure 1. Zone of hydrolysis on milk agar plate exhibited by the selected *Bacillus* isolate.

Reversal of enzyme inhibition by NaCl

Reversal of inhibition effect of EDTA by NaCl at varying concentrations was also studied. For this, EDTA at 1 mM, 2 mM, 5 mM, 10 mM and 20 mM concentrations were mixed with enzyme in presence of NaCl (5 mM and 10 mM) and glycine- NaOH buffer (pH 8). Casein (1%) was added and the enzyme assay was performed as discussed above.

Results and Discussion

The selected isolate (B80) was identified as a *Bacillus* strain on the basis of various microscopic and biochemical investigations (Table 1). The zone of hydrolysis exhibited by the selected strain is shown in Figure 1.

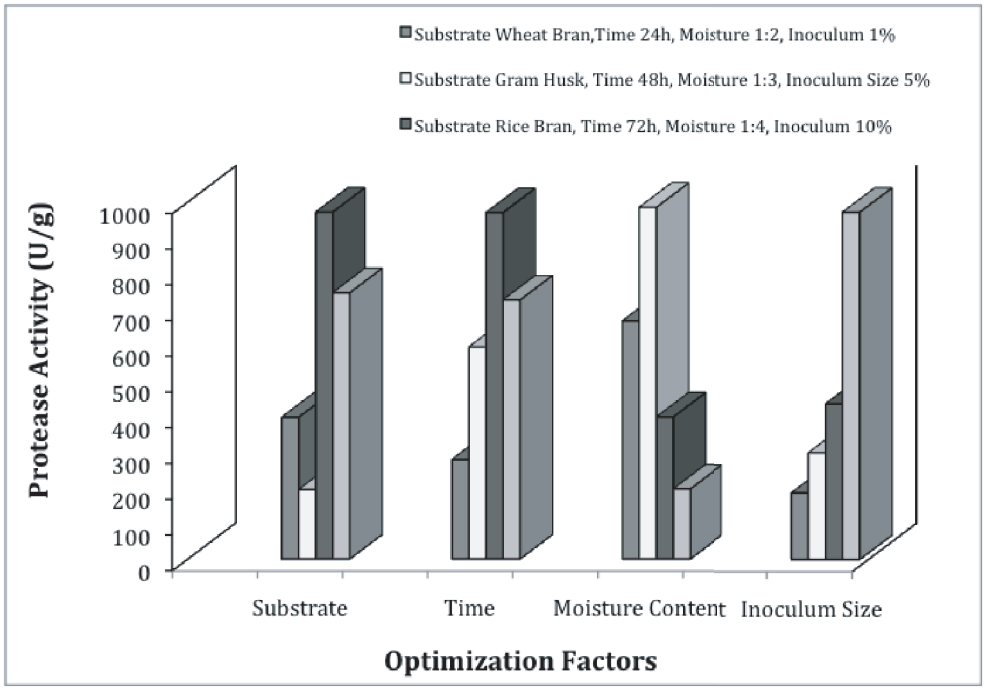

Figure 2. Effect of substrates, time, moisture content and inoculum size on enzyme production.

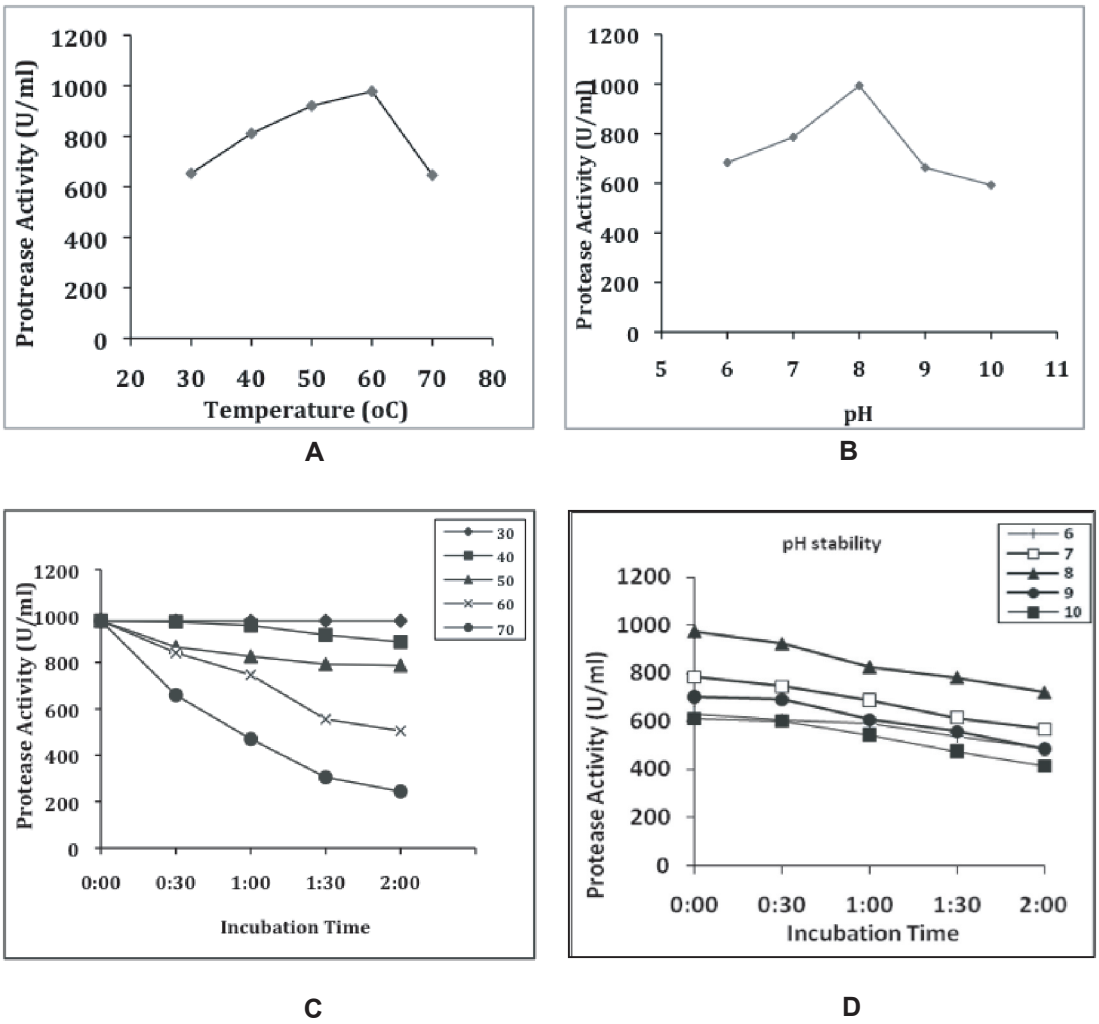

Figure 3. (a): Enzyme activity at different temperatures [30°C to 70°C] (b): Enzyme activity at different pH [6 to10]. (c): Stability of enzyme at temperatures 30°C to 70°C. (◆) 30°C, (■) 40°C, (▲) 50°C, (x) 60°C, (●) 70°C. (d): Stability of enzyme at pH 6-10 (+) 6, (□) 7, (▲) 8, (●) 9, (■) 10.

Effect of substrate, time, moisture content and inoculum size on enzyme production

B80 showed maximum enzyme production on rice bran (972U/g) which was much higher than the activities 391, 106 and 746 U/g produced on wheat bran, gram husk and mustard oil cake, respectively (Fig. 2).

Though rice bran supplies almost the same amount of protein (10-15%) as wheat or gram husk, its protein is of considerably better quality (The Wealth of India 2001) and more easily utilized than the endosperm proteins in wheat bran (Landers and Hamaker 1994). Rice bran has been used as a fermentation substrate for the production of enzymes such as protease, amylase and lipase (Sumantha et al. 2006; Anto et al. 2006).

Bacillus usually produces extracellular protease during late exponential phase (Ward 1985). In our study, it was revealed that maximum level of protease was produced after 72 h (Fig. 2), after which the enzyme activity decreased considerably. Kumar and Parrack (2003) have also reported maximum protease production by *Bacillus* sp. in 72 h.

Inoculum size of 20% yielded 972 U/g of enzyme which was much higher than 435.6, 298.65 and 186.45 U/g produced with 10%, 5% and 1% inoculum, respectively (Fig. 2). Maximum enzyme production (985 U/g) was observed at moisture content of 1:3, which was much higher than 666, 398 and 196 U/g, produced at 1:2, 1:4 and 1:5 ratios, respectively (Fig. 2). High moisture reduce the porosity of the substrate thus limiting the oxygen transfer, while lower moisture content causes reduction in solubility of the nutrients of the substrate. Hence an optimal level of moisture is required for maximum enzyme production (Divaker et al. 2006).

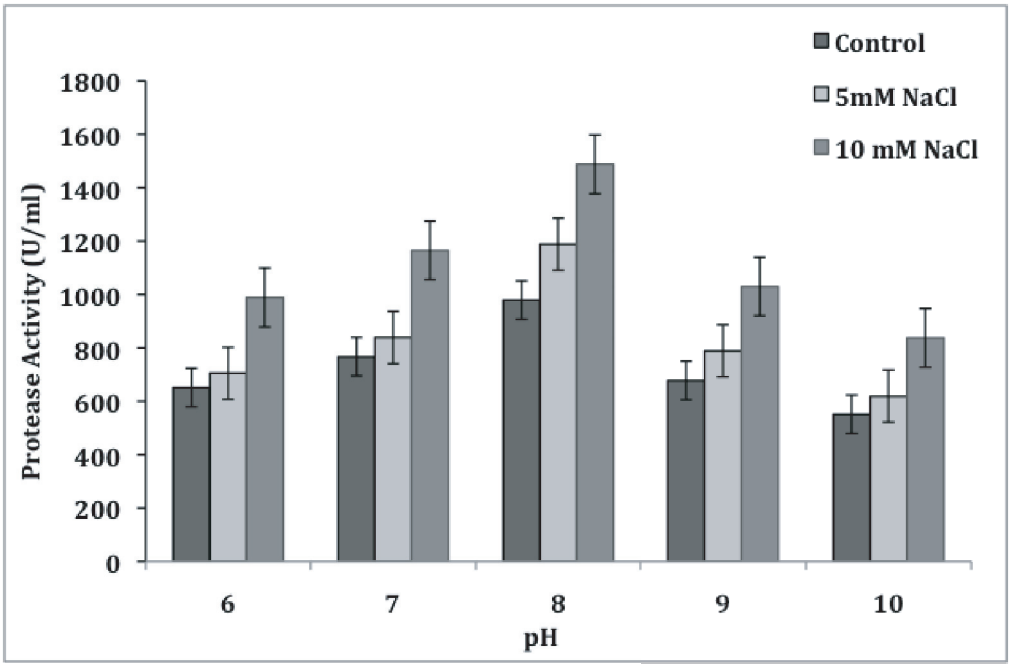

Figure 4. Relative protease activity in presence of NaCl (5mM) at different pH.

Effect of various physico-chemical factors on enzyme activity

Effect of temperature and pH on activity and stability of the enzyme

The highest enzyme activity was detected at 60°C (Fig 3a). The enzyme was found to be stable for 2 h at 30°C. At 40 °C and 50°C the enzyme lost only 16-22% of its activity after 2 h. However, after 1 h at 60 °C and 70°C the enzyme retained only 70 and 50%, respectively (Fig. 3c).

The enzyme was active between pH 6.0 and pH 9.0 with maximum activity at pH 8.0 when incubated for 10 min at 60°C (Fig. 3b). In this pH range the enzyme retained about 75-80% of its initial activity (Fig. 3d). At pH 10 the enzyme retained about 60% its initial activity.

Effect of NaCl and its relation with pH

The enzyme activity enhanced in presence of NaCl at 5 mM and 10mM concentrations, at all pH (6-9) with an optimum at pH 8, where about 20% increase in the enzyme activity was observed (Fig. 4). Higher salinity may promote binding of a hydrophobic substrate to an enzyme or of hydrophobic residues to each other within the enzyme to ensure optimal folding for enzymatic activity. It was observed that NaCl had no effect on the optimum pH of the enzyme activity, similarly, pH did not effect the enhancement of the enzyme activity by NaCl, with the enzyme showing the maximum activity at pH 8 both in presence and absence of NaCl in different concentrations.

Table 2. (a). Effect of various metal ions and protease inhibitors on enzyme activity.

	Relative protease activity	
	5 mM	10 mM
Control	100	100
Na^{+}	136.9	152
K^{+}	109	126.7
Ca^{2+}	126	133
Mg^{2+}	145.5	131.6
Zn^{2+}	126.3	128.3
Mn^{2+}	145.7	182
Cu^{2+}	179.4	139
Hg^{2+}	132.3	124
Fe^{3+}	105	102
EDTA	25	5
DTT	49	10
B-ME	52	20

(b): Reduction in EDTA inhibition of enzyme activity with NaCl.

EDTA Concentration (mM)	Protease activity (%)	Protease activity with NaCl (%)	
		5 mM	10 mM
None	100	136.9	152
5	10	42	51.6
10	5	41.8	48.7

Effect of metal ions and inhibitors on enzyme activity

The increase in enzyme activity in presence of NaCl suggested that the enzyme requires a metal ion as a cofactor. Relative protease activity in presence of different metal ions is shown in Table 2a. The enzyme activity increased in presence of all the metal ions (Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Zn^{++}, Mn^{++}, Cu^{++} and Hg^{++}). Maximum 80-82% enhancement occurred in the presence of Cu^{2+} (5 mM) and Mn^{2+} (10 mM) ions. This result suggests that the investigated enzyme is a metalloprotease that requires a metal ion for its activity.

The enzyme activity was inhibited by EDTA and DTT and β-mercaptoethanol (Table 2b).

Inhibition with EDTA and DTT at 10 mM concentration was 95% and 90%, respectively, while at 5mM the inhibition was 75 and 51%, respectively. Inhibition of 80% and 48% was measured in the presence of 10 mM and 5 mM β-mercaptoethanol, respectively. The nearly complete inhibition of enzyme activity by EDTA and DTT indicates that it is a metalloprotease. Similar results have been reported by Tang et al (2010). The protease activity was 90% inhibited by DTT, thus the enzyme might contain S–S bond(s) as a part of its monomeric structure (Nilegaonkar et al. 2007).

Reversal of EDTA inhibition by NaCl

In the presence of 10 mM EDTA, addition of 5mM and 10 mM NaCl resulted in restoration of enzyme activity with 42% and 49%, respectively. Similarly, in the presence of 5 mM EDTA, addition of 5mM and 10 mM NaCl resulted in restoration of enzyme activity with 42% and 52%, respectively.

Conclusion

The results suggest that the characterised *Bacillus* strain has a high biotechnological potential for metalloprotease production in solid-state fermentation by using cheap substances (e.g. rice bran, mustard oil cake and wheat bran). The metalloprotease studied was highly active and stable in presence of metal ions and in broad pH and temperature range. The enzyme could be potentially used for recovering high quality, value-added ingredients from the proteins of rice bran.

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