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Production and Purification of Alkaline Serine Protease from Marine *Bacillus* Species and Its Application in Detergent Industry

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Abstract: Green mussel (*Perna viridis*) collected from Kanyakumari coast, south west of India was screened for protease production by culturing on skim milk agar containing 5.0 and 10.0 mM of PMSF (phenyl methyl sulphonyl fluoride). The isolated *Bacillus* species was identified by Bergey's manual of determinative bacteriology. Moreover, the growth conditions for highest protease production were optimized with different fermentation period, pH, temperature, carbon and nitrogen sources. Purification of crude enzyme was carried out by ammonium sulphate precipitation, dialysis and DEAE cellulose column chromatography. The protease was active at pH 7 and 70°C and was found to be stable at pH 8 and 70°C. The apparent molecular weight of purified enzyme was 37kDa. Finally the applications of alkaline serine protease in detergent and solvent industry were tested and it was revealed that the purified enzyme can be used as an additive in detergent industry.

Key words: *Bacillus* Species · Protease · Optimization · Characterization · Purification · Detergent Industry

INTRODUCTION

The demand for industrial enzymes, particularly microbial origin, is ever increasing owing to their applications in a wide variety of processes [1]. Microbial proteases account for approximately 40% of the total worldwide enzyme sales [2]. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all characteristics desired for their biotechnological applications [3] Among the various proteases, bacterial protease was the most significant compared with animal, fungi and plant protease. Bacillus species were specific producers of extracellular protease [4].

There is a renewed interest in the study of proteolytic enzymes mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community [5]. They represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share accounting for about 60 % of total worldwide sale of enzymes [6, 7].

Proteases are broadly classified as endo or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine protease, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism [6]. Alkaline serine proteases of microbial origin posses considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures [8].

The enzyme optimization studies are done by carrying out the assay. The assay can be done using various methods [9-12]. The presents study used Chopra *et al*. [10] method for optimization studies. Studies on the purification and characterization of alkaline protease and its possible involvement in mobilization of storage proteins were carried out. The alkaline protease from the cotyledons of 4 day old germinating Indian bean (Dolichos lab L. varlignosus) seedlings was purified by ammonium sulphate fractionation, DEAE cellulose and finally casein alginate affinity chromatography. It has a monomeric enzyme with molecular mass of 40 KDa and exhibited sharp pH optima at 8.8 with casein [13].

The study finally dealt with the applications of alkaline serine protease in detergent and solvent industry. A protease isolated from *Pseudomonas aeruginosa* PD100 was active in the presence of SDS and Tween

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80 [14]. There was a report on the purification and partial characterization of thermostable alkaline protease from a newly isolated *Bacillus subtilis* PE-11. This enzyme improved the cleansing power of various detergents and removed bloodstains [15]. The enzyme activity of *Bacillus licheniformis* N-2 at different temperature, pH, metal ions and inhibitors renders its potential use in leather and detergent industries [16]. In this present study the purified enzyme was used for determination of detergents.

MATERIALS AND METHODS

Collection of Mussel Samples: The experimental green mussels (*Perna viridis*) were collected from Kanyakumari coast, south west of India.

Screening of Bacteria for Protease Production: The external surface of the mussel shell was scrapped and used as the sample. The sample was serially diluted and streaked on skim milk agar plates. The plates were incubated for 24 h at 37°C and enzyme activity was observed. Positive colonies those degraded skim milk was studied for protease production [17].

Screening and Identification of Protease Producing Organism: To screen serine protease producing organism the isolate was grown on skim milk agar plates containing 5.0 and 10.0 mM of PMSF (phenyl methyl sulphonyl fluoride). It was incubated at 37°C for 48 hr. The inhibitory effect of protease production was compared with the control (without PMSF) [18]. Based on the morphological and biochemical tests the bacterial isolate was identified [19].

Determination of Total Protein Content: The total protein contents of the samples were determined according to the method described by Lowry's method [20], the protein standard used was Bovine Serum Albumin (BSA) (1 mg/ml).

Protease Assay: Alkaline protease activity was determined by the standard assay [10]. The reaction mixture contained 5 ml of casein (prepared in 50 mM of Tris buffer, pH 8.0) and an aliquot of 1.0 ml of the enzyme solution and incubated for 30 min. The reaction was stopped by adding 5 ml of trichloroacetic acid solution (TCA) (0.11M). After 30 minutes the mixture was filtered. Two ml of the filtrate was added to 5.0 ml of 0.5 M sodium carbonate and 1.0 ml of Folin - Ciocalteu's phenol reagent and kept for 30 minutes at 37°C. The optical densities of the solutions were read against the sample blank at 660 nm using UV - Visible Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under assay conditions.

Growth Curve Determination of the Marine Bacterium *Bacillus* sp.: A loopful culture of the bacterial isolate was inoculated in the optimal medium. It was incubated at 37°C in an orbital shaker at 150 rpm. Five ml of the culture medium was withdrawn at a regular interval of 24 hr and its absorbance was measured at 600 nm by using UV - Visible [21].

Optimization of Protease Production

Effect of Fermentation Period on Enzyme Production: The test organism was grown in nutrient broth containing 1% casein and 3% NaCl. It was incubated at 37°C for 24, 48, 72, 96 and 120 hr in an orbital shaker at 150 rpm. The contents were then centrifuged at 10,000 rpm at 4°C for 10 min and protease activity was checked in the cell free extract.

Effect of pH on Enzyme Production: The test organism was grown in nutrient broth containing 1% casein and 3% NaCl. Effect of pH on enzyme production was studied by adjusting the culture media pH (6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) prior to sterilization. It was incubated at 37°C for 48 hr in an orbital shaker at 150 rpm. The contents were centrifuged at 10,000 rpm at 4°C for 10 min and protease activity was checked in the cell free extract.

Effect of Carbon Sources on Enzyme Production: To find the optimum carbon source for enzyme production, five carbon sources (1%) (starch, glucose, sucrose, lactose and xylose) were selected and added to nutrient broth. The organism was inoculated and incubated for 48 hr at 70°C and the enzyme activity was assayed in the culture supernatant.

Effect of Nitrogen Sources on Enzyme Production: To optimize the nitrogen source for enzyme production, five different nitrogen sources (1%) (yeast extract, gelatin, casein, urea and ammonium chloride) were added to nutrient broth and the organism was inoculated and incubated for 48h. The enzyme activity was assayed in the culture supernatant.

Enzyme Property Studies

Effect of pH on Enzyme Activity: To optimize the pH of enzyme activity, 1 ml of enzyme solution was reacted with 5 ml of casein solution [1 % (w/v)] in various pH values (6.0, 7.0, 8.0, 9.0 and 10.0) at 37°C for 30 min and the enzyme activity was assayed.

Effect of Temperature on Enzyme Activity: The influence of different temperatures on proteolytic activity of the crude enzyme was determined by holding the reaction mixture at various temperatures (30, 40, 50, 60 and 70°C) for 30 min during standard enzyme assay.

Purification of Protease from the Marine *Bacillus* sp.

Ammonium Sulphate Fractionation: Solid ammonium sulphate was added slowly to the crude protease enzyme with constant stirring till the percentages 12, 14 and 15 % (g %) of enzyme samples were obtained. The samples were stored at 4°C for 4 hr. The pellet recovered by centrifugation at 10,000 rpm for 10 min was resuspended in cold double distilled water. It was dialyzed for 24 hr against double distilled water (three changes) and buffer (Tris buffer, pH 8.0, 25 mM). It was stored at 2 to 8°C for further studies.

Purification of Protease by DEAE Cellulose Column Chromatography: The enzyme solution obtained in the above step was applied to DEAE-cellulose column (2.4 x 45 cm) pre equilibrated with 25 mM Tris buffer (pH 8). The enzyme was eluted with the same buffer at a flow rate of 10 ml/h.

Molecular Weight Determination: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 30 % (w/v) acrylamide slab gel with 25mM Tris/192 mM glycine buffer (pH 8) that contained 0.1% (w/v) SDS as the running buffer, as described by Laemmli [22].

Application Studies of Protease Enzyme from Marine *Bacillus sp.* In Industry

Effect of Ionic and Non-Ionic Detergents on Protease Activity: The effect of detergents on enzyme activity was studied using ionic and non-ionic detergents (1%) such as Tween 20, Tween 80, Triton X 100, SDS and polyethylene glycol dodecyle ether (BRIJ 35). One percentage of the above mentioned detergents were added to the enzyme sample and incubated for 30 min. Enzyme activity was assayed by standard method [10].

Effect of Polar and Non-Polar Solvents on Protease Activity: The effect of solvents on enzyme activity was studied by incubating the culture supernatant with polar and non-polar solvents (1%) such as acetone, benzene, chloroform, hexane and toluene. Enzyme activity was assayed by standard method [10].

RESULTS AND DISCUSSION

In the present study, *Bacillus* species was isolated from the outer shell of the green mussel (*Perna viridis*) by serial dilution technique and plated on nutrient agar medium containing 3 % NaCl. In earlier studies fifty three bacterial strains were isolated on alkaline agar medium from various soil fields of Korachi [23].

Screening and Identification of Marine Protease Producing Organism: Different colonies were noted on skim milk agar plates and a clear zone around one colony after 24 hr of incubation at 37°C was taken and used for further studies. The organism was identified as belonging to the genus *Bacillus*.

Screening of Alkaline Serine Protease from Isolated Species: The activity of protease enzyme is totally inhibited by PMSF, which clearly show that the enzyme belongs to serine group of alkaline proteases [24]. The present study also showed that the protease activity was strongly inhibited by PSMF at 5mM and 10mM concentration by the absence of zone formation when compared with the control (without PMSF). So this enzyme belongs to serine group of proteases.

Determination of Total Protein Content: The total protein content was checked in the supernatant (Table 1). The total protein content was also checked in all purification steps.

Optimization of Cultural Parameters

Effect of Fermentation Period on Enzyme Production: The optimization of the fermentation medium for maximization of thermostable neutral protease production by *Bacillus sp*. HS08 was reported [25]. The maximum protease production by bacteria seen in 48 to 72 hr of incubation was reported [26, 27]. In the present study the highest protease activity of 0.57 U/ml was seen at 48 hr of fermentation period.

Table 1: Determination of total protein content of examined samples.

Fermentation Period (hr)	Protein content (mg/ml)
24	40.60
48	43.89
72	25.88
96	24.29
120	22.16

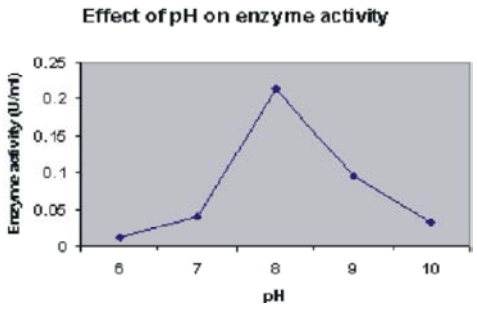

Fig. 1: Effect of pH on protease activity

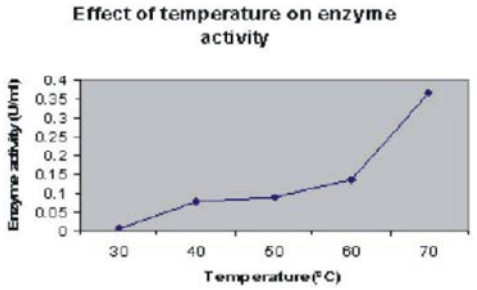

Fig. 2: Effect of temperature on protease activity.

Effect of pH and Temperature on Enzyme Production: The maximum protease production is seen in media with pH 9.0 and 10.0 [28, 29]. The highest protease production is also seen in the culture with pH 10 (alkaline medium) from *Bacillus pumilus* [30]. The present investigation showed that *Bacillus sp* produced maximum protease at 8.0 (Fig. 1). Similar result was reported by Folasade *et al*. [31].

The protease activity is relatively stable in the temperature range 60 - 65°C and retains 85.2% of its activity at 70°C [32, 33]. The enzyme is active in alkaline condition, indicating its potential use in detergent formulations [34, 5]. In the present study the protease enzyme activity was higher at higher incubation temperature. The enzyme was stable up to 70°C and the highest enzyme activity was reported at 70°C (Fig. 2).

Partial Purification and Enzyme Characterization: Enzyme activity was maximum in fraction 2 (0.4748 units/ml). About 14 % of ammonium sulphate was added to culture supernatant to obtain fraction 2. It was noticed that 14-15 % of ammonium sulphate was optimum for alkaline serine protease fractionation (Table 2).

Table 2: Enzyme activity in ammonium sulphate fractions

Fraction number	Ammonium sulphate added (g/100 ml)	Enzyme activity (U/ml)
1	12	0.3831
2	14	0.4748
3	15	0.4572

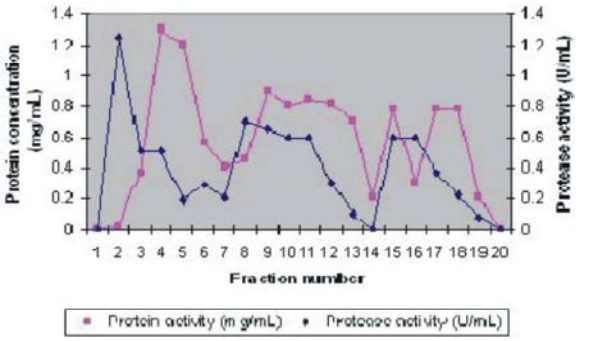

Fig. 3: Graph showing the purification profile of alkaline serine protease by DEAE Cellulose chromatography

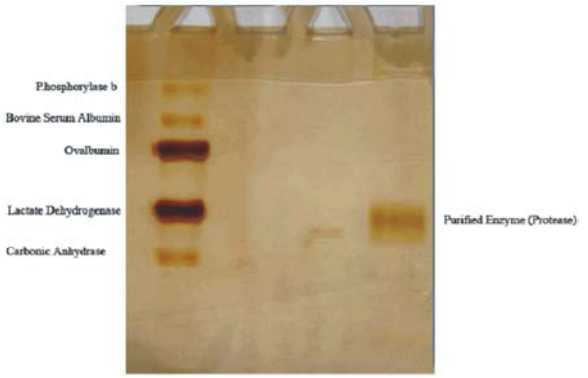

Fig. 4: Molecular mass determination of *Bacillus* protease by SDS PAGE
Lane 1: Molecular marker, Lane 2: Purified enzyme

The active fraction obtained from the previous step (ammonium sulphate precipitated sample) was dialyzed and loaded onto DEAE cellulose chromatography. The column was packed and equilibrated with Tris buffer (25 mM, pH 8.0). The bounded protein was eluted with buffer containing 50 -500 mM of NaCl (step elution). DEAE cellulose chromatography results revealed one major peak obtained in fraction 2 which showed the highest enzyme activity. This sample was loaded to check the purity of enzyme (Fig. 3).

The major extracellular protease is a neutral protease, which is 33.5 kDa and pI 7.4 [35]. SDS PAGE analysis revealed that the molecular weight of *Bacillus* species alkaline serine protease was 37 kDa (Fig. 4).

Table 3: Effect of ionic and non-ionic detergents on protease activity

Detergents (1 %)	Relative activity (%)
Tween 20	204
Tween 80	299
Triton X100	109
SDS	225
BRIJ 35	115

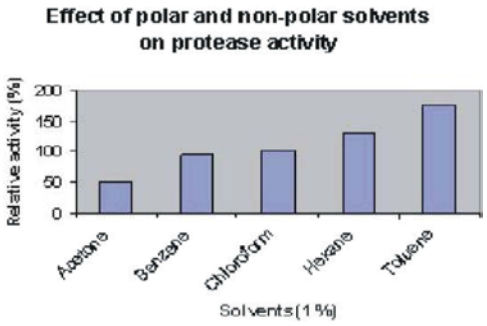

Fig. 5: Effect of polar and non-polar solvents on protease activity

Application Study

Effect of Ionic and Non-Ionic Detergents on Protease Activity: The enzyme activity was the highest (299 %) in Tween 80 and SDS (225 %) and lowest (109 %) in Triton X 100 (Table 3). The extracellular production of the enzyme, its thermostable nature and compatibility with most commercial detergents are features which suggest its application in detergent industry [36]. In the present study it was also shown that the alkaline serine protease isolate from marine *Bacillus* species was able to degrade both ionic and non-ionic detergents.

Effect of Polar and Non-Polar Solvents on Protease Activity: The enzyme activity was seen highest (177 %) in toluene and hexane (131%) and lowest (51%) in acetone (Fig. 5).

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