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RESEARCH ARTICLE

Purification of extracellular protease by *Bacillus* sp. Isolated from Lonar meteoritic crater

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ABSTRACT

The extracellular proteases was purified by halophilic *Bacillus sp.* which having potential to grow at high salt conditions. The obtained protease has wide industrial applications mainly in food industry, detergent, medical and biotechnology. The extracted crude protease was purified by using ammonium sulphate, dialysis and ion exchange chromatography. The 80% ammonium sulphate gives the up to 2.4 fold purification of protease. The dialyzed sample was introduced on anionic exchanger as DEAE cellulose having a strong affinity towards the protease and gives 4.2 fold purification.

Key words: Bacillus sp., protease, chromatography, dialysis.

INTRODUCTION

Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, and *B. majovensis* (Beg & Gupta, 2003). Alkaline proteases produced by halophilic, thermophilic and alkaliphilic bacilli can withstand high salt, temperature, pH, chemical denaturing agents and in non-aqueous environments. Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments (Norazizah *et al.,* 2005).

METHODS AND MATERIAL

Enzyme production medium

The overnight incubated bacterial culture (2% v/v) was inoculated in 250 ml Erlenmeyer flask having protease production medium (100ml)

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containing (g/l): Peptone 3, Glucose 10, $CaCO_3$ 0.3, NH_4NO_3 4, KH_2PO_4, 1.5, $MgSO_4$ 0.5, $ZnSO_4$ 0.36 and pH was adjusted to 9.0. Incubate the synthetic medium on orbital shaking incubator for 24 h at 37°C with 120 rpm. After incubation cells were separated by centrifugation at 12000 g for 20 min at 4°C and cell free filtrate (CFF) containing protease was collected for analysis of proteolytic activity and protein contents (Adinarayana *et al.,* 2003).

Enzyme assay

Protease activity was measured by using casein as substrate (Huang *et al.,* 2006). A mixture of 400 µl casein solution (2% w/v in 50 mM Phosphate buffer pH 7.0) and 100 µl extracted enzyme was added in each tube and incubated for 10 min at 50°C. The reaction terminated by addition of 1ml trichloroacetic acid (TCA) (10% v/v). The mixture allowed to centrifuge at 14,000 g for 20 min and 1 ml supernatant was removed carefully. Tyrosine/tryptophan content was determined by using Lowery method. The blank was prepared by adding 1ml of TCA before addition of an enzyme. One unit of protease activity (U) is defined as the amount of enzyme that hydrolyzed casein to liberate one µmole tyrosine per min under the above assay condition (Norazizah *et al.,* 2005).

Determination of total protein content

The total protein contents of the samples were determined according to the method described by Lowry (Lowry *et al.,* 1951); the protein standard used was BSA (Merck). Protein standard solution, in the range of 0.5 to 5 mg/ml was prepared in triplicate to obtain a standard curve. Samples (cell-free supernatant) were diluted to 1 ml with distilled water so that the protein content would be within the range of the standards. Alkaline copper sulphate reagent (5 ml) was added to each tube and mixed well. The solutions were kept at room temperature for 10 minutes followed by the addition of 0.5 ml Folin & Ciocalteu's Phenol reagent (Merck) working solution. Each tube was rapidly mixed, and incubated in dark for 30 minutes. Absorbance of the samples was measured spectrophotometrically at 570 nm using UV/Vis spectrophotometer (Systronic- model 119) (Lowry *et al.,* 1951).

Precipitation of protease by ammonium sulfate Dialysis

The obtained ammonium sulfate precipitate was introduced into dialysis membrane-50 & 60 for dialysis against glycine-NaOH buffer (pH 9.0) for 12 h on magnetic stirrer (clock wise direction) having 250-300 rpm. The obtained protease was collected and measured its protein content as well as enzyme activity by standard assay conditions (Huang *et al.,* 2006).

Preparation of column

The column is fixed in the upright position and its bottom is sealed with glass wool. The column is now filled about one third of its height with the mobile phase. A thick suspension called slurry, of the degassed stationary phase (gel, adsorbent, or resin) is gently poured into the column by closing its outlet. The slurry is generally added till 3/4th of the column by particular resin. The outlet is now opened and the column is stabilized by washing with mobile phase. A circular filter paper disc is then placed on the surface of the column to prevent disturbance during sample addition (Huang *et al.,* 2006).

Ion exchange chromatography

The partially purified sample from gel permeation was introduced in to the DEAE cellulose column, equilibrated with 10 mM glycine-NaOH buffer (pH 9). The close affinity proteins were eluted by elution buffer having 0.5 M NaCl dissolved in 0.2 M glycine-NaOH buffer (pH-9). Adjust the flow at 1 ml/min. The 40 fractions were collected and determined its enzyme activity and protein content by standard assay conditions (Huang et al., 2006).

RESULT AND DISCUSSION

The results of protease purification from *Bacillus sp.* are summarized in following Table 1. After fixed fermentation time period, the fermented broth was centrifuged at 12000g for 20 min at 4°C to obtain a cell free extract containing crude enzyme. The initial activity of culture supernatant was 3100 U and 2300 mg of total protein. Among all the used concentration of ammonium sulphate the 80% w/v final concentration showed a good result for fractionation of enzyme. The optimum ammonium sulphate precipitation at 80% w/v final concentration showed 2.4 fold increases in specific activity compared to the unconcentrated supernatants. According to Sadia et al. the fractionation of protease by ammonium sulphate was carried out at 60% and 70% saturation recovered 3.6 and 3.9 fold purification (Sadia *et al.,* 2009). The 80% w/v ammonium sulphate was optimum for protease fractionation (Guangrong *et al.,* 2008).

Table 1 : : Purification of an extracellular protease from *Bacillus sp.*

Purification	Total protein (mg)	Total activity (U)	Recovered activity (%)	Specific activity (U/mg)	Purification (Fold)
Crude enzyme	2400	3100	100	0.9	1
Ammonium Sulphate	750	2800	87.9	3.1	3.4
Dialysis	630	2300	84.8	3.4	3.7
Ion exchange	120	1400	51.5	4.7	5.2

The obtained pellet dissolved in minimal volume of 0.2M glycine NaOH buffer (pH 9) and dialyzed in presence of same buffer. Dialysis was performed by using dialysis membrane 50 and 60 for the exclusion of exclude the metals and salts as well as it also helps to lose some proteins from their pore size. The 0.2 fold increases it**s** specific activity when compared with 80% ammonium sulphate fractionation. The dialyzed sample referred to a**s** partially purified alkaline protease and applied gel chromatography for purification. Chromatography is of fundamental importance for enzyme purification. Molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (biospecific affinity).

The dialyzed sample was introduced on anionic exchanger as DEAE cellulose having a strong affinity towards the protease. Ion-exchange chromatography is a separation technique, based on the charge of protein molecules. Enzyme molecules possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged) (Barrett *et al.,* 2004).

The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration. Because of the concentrating effect, samples can be applied in dilute form. All the impurities (proteins) were washed during washing treatment except affinity protease. The resultant bound protease was further eluted by increasing NaCl concentration. The protease was purified by DEAE-cellulose 52 showed 13.2 fold its activity (Yong *et al.,* 2011). Moreover, an extracellular protease produced from *Bacillus sp.* was purified by using a DEAE sephacryl (Sadia et al. 2009), DEAE-sepharose CL-6B (Kumar, 2002 & Huang *et al.,* 2006) found excellent purification rate. In the present investigation the ion exchange chromatography showed 5.2 fold increase of its purity.

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