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Kinetic Characterization and Immobilization of Partially Purified Extracellular Alkaline Protease from Rhizospheric Soil Bacterium *Bacillus subtilis* strain EN4

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Protease constitutes a large and complex group of enzyme which plays an important nutritional and regulatory role in nature. Among five strains of endosulfan degrading bacterial isolates from rhizospheric soil samples of Crop Research Centre, GBPUAT, Pantnagar, strain EN-4 was found as the best producer of extracellular alkaline protease and selected for present study. The bacterium was identified as *Bacillus subtilis* strain EN4 on the basis of 16S rDNA gene sequencing. This protease had optimum activity at pH 10 and temperature 50°C. The enzyme was stable at alkaline pH range and upto a temperature of 70°C. The K_m and V_{max} values of EN4 protease were 0.55M and 22.2 U/ml, respectively. Partially purified alkaline protease was immobilized on calcium alginate beads with 59% activity recovery. The properties of immobilized alkaline protease were determined. The optimum pH and temperature for immobilized alkaline protease were 9 and 60°C, respectively.

Key words: *Bacillus subtilis,* Alkaline protease, Immobilization, Characterization.

Extracellular enzymes, produced by diverse groups of organisms viz fungi, yeasts and bacteria, have wide range of industrial applications. Proteases are among the most viable commercial enzyme covering 65% of global market (Patel *et al.,* 2006). They have wide range of applications in industrial processes eg, in detergent, food, pharmaceutical, leather, and silk industries (Adinarayana *et al.,* 2004). Proteases (E.C. 3.2.21.24) are classified as acid, alkaline and neutral on the basis of pH optimum of its enzyme activity. Alkaline proteases are particularly important as they are stable over a wide range of pH (7.0-11.0) and temperature (30-60°C) (Giri *et al.,* 2011; Joo *et al.,* 2002; Gupta *et al.,* 2002; Oberoi *et al.,* 2001).

Despite of the use of several enzymes in industry, the present status is not sufficient to fulfill all industrial and biotechnological needs. As industrial processes are carried out under many harsh conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. Therefore, it would be of great importance to have available enzymes showing optimal activities at extreme values of pH, temperature. Enzyme immobilization is also an approach with added advantages to enhance value of enzymes for industrial applications by increase in stability under extreme conditions of temperature and pH, organic solvents, recovery and reuse of enzymes. Immobilization by entrapment using suitable matrix is a simple method under milder conditions and results into minimum denaturation of enzyme during process. Alginate, a polysaccharide consisting of glucuronic acid and mannuronic acid moieties has been found to be matrix of priority due to its biocompatibility and processivity (Chan *et al.,* 2002). It is a reversibly soluble insoluble polymer which changes solubility in the presence of Calcium (Smidsrød and Skjaok-Brk 1990). To date,

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cross linked alginate has been successfully used for encapsulation of many biological molecules (Chi *et al.,* 2008; Mittal *et al.,* 2005).

In the present study, we screen and identified a bacterial isolate from rhizospheric soil of crop research centre, G. B. Pant University of Agriculture and Technology, Pantnagar, for extracellular alkaline protease. Kinetic characterization of partially purified unimmobilized and calcium alginate immobilized enzyme was done.

MATERIAL AND METHODS

Material, bacterial strain and growth conditions

All chemicals, media and their components used were of analytical grade and obtained from Sigma chemicals Ltd., HiMedia Laboratories Ltd., SRL, Glaxo, Merck Pvt. Ltd. Previously isolated endosulfan degrading bacterial strains in our lab were taken for following study (Pathak, 2010). The strains were cultivated in Luria Broth (LB) media. Skim milk agar media (Adinarayana *et al.,* 2003) was used to screen bacteria for protease production.

Proteases activity screening and production assay

Five bacterial isolates from soil were screened for production of protease enzyme. For the observation of protease production, bacteria were inoculated on skim milk agar plates containing 10% (w/v) skim milk, 2% agar and 5% NaCl. Plates were incubated at 32°C for 48 h. Clear zones of skim milk hydrolysis around the colonies gave an indication of proteolytic activity.

Protease production was carried out in media (pH 7) with the following composition (%): Casein 1, peptone 0.1, maltose 0.4, NaCl 0.5, $CaCl_2$ 0.005 and $MgSO_4$ 0.005. Cultures were incubated on a shaker (150 rpm) for 36 h at a temperature of 37°C (Venugopal and Saramma 2006). The proteolytic activity was determined by the modified Kunitz method using casein as a substrate (Kunitz 1947). The supernatant of overnight grown culture broth served as the crude enzyme source. For the enzymatic catalysis assay 1ml of casein (2% in 0.1M phosphate buffer, pH 8.0) was incubated with 0.5ml of crude enzyme preparation at 37°C for 30 min. The reaction was stopped by adding 3ml of 1% trichloroacetic acid. The reaction mixture was centrifuged at 8000g for 10 min at room temperature. The absorbance of supernatant was measured at 280 nm. Amount of tyrosine produced is calculated from tyrosine standard curve. One unit of protease activity is defined as the amount of enzyme that released 1µg tyrosine/ml/min at assay condition. All experiments were carried out in triplicate. Protein concentration was determined as per protocol of Bradford (1976).

Partial Purification of alkaline protease

Protease production was achieved by cultivation of strain in media described above. All the purification steps were performed at 4°C. Crude alkaline protease in the cell free supernatant was precipitated by adding ammonium sulphate upto 70%. The precipitates were separated by centrifugation at 10,000 rpm for 15 min and resuspended in 0.1M phosphate buffer (pH 8) and dialyzed against the same buffer for 12 h with regular buffer change. The dialyzed enzyme preparation was applied to a Sephadex G-100 column (1.6 cm x 20 cm) equilibrated with buffer at which the enzyme shown maximum stability. Fractions were collected at the flow rate of 1ml/min and protein content in each fraction was measured at 280 nm. The protease activity in each fraction was measured and the fractions which gave maximum protease activities were pooled and lyophilized for further study.

Kinetic characterization

The calculation of Km and Vmax was done on the basis of Lineweaver-Burk Plot constructed by plotting the reciprocals of substrate (Casein) concentration on X-axis, and reciprocals of enzyme activity on Y-axis**.**

Enzyme immobilization

Partially purified protease was immobilized by dropping a 3% Sodium alginate solution (in which a suitable amount of enzyme had been diluted in 1:2 ratio) into a 0.25M $CaCl_2$ solution with continuous stirring. Curing of the beads were done for 4 hr in $CaCl_2$, washed several times with a 0.03M $CaCl_2$ solution until no protease activity was observed in the final washing and stored at 4°C in the same solution prior to use. A similar method was followed for the preparation of control beads without enzyme (Betigeri and Neau 2002).

Effect of pH and temperature on stability of extracellular free protease

The stability of the enzyme was

determined by pre-incubating the enzyme for 1 hr at 37°C with various buffers having pH range of 3 to 12. Citrate buffer (pH 3-5), Phosphate buffer (pH 6-8), and Glycine-NaOH buffer (pH 9-12) were used to determine protease activity. After incubation, the residual enzyme activity (%) was measured by using buffered substrate of pH ranging from 3 to 12. The thermal stability of the enzyme was studied by pre-incubating the enzyme at different temperature ranging from 30-80°C for 1 hr at optimum pH.

Effect of pH and temperature on free and immobilized protease activity

The optimum pH for protease activity was studied over a pH range of 3 to 12 as mentioned earlier to determine the protease activity of free form of enzyme as well as immobilized. The protease activity at different pH was determined. The optimum temperature for protease activity was determined by incubating the reaction mixture over the temperature range of 30-80°C at optimum pH.

Identification of bacterial isolates

The genomic DNA from bacterial isolate EN4, having highest protease productivity, was extracted and purified using method of Newman *et al.,* (1992). Isolate was identified using 16S rDNA gene sequencing. Two primers; RDNA 1A (5'-AGAGTTTGATCCTGGCTCAG-3') and RDNA 1B (5'-AAGGAGGTGATCCAGCCGCA-3') were used to amplify the 16S rDNA gene. PCR reaction was performed in a final reaction volume of 50 µl. Amplifications were carried out using the temperature profiles: 94°C for 2 min, followed by 35 cycles of 94°C for 1.0 min, 54°C for 1.0 min, 72°C for 1.5 min & final extension for 10 min at 72°C. Amplified PCR product was separated by gel electrophoresis on 1.2% (w/v) agarose gel.

The amplified PCR product was gel purified using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced by automated DNA sequencer at DNA Sequencing Facility, University of Delhi, South Campus, New Delhi, India. The sequence analysis was done using NCBI database by employing BLASTN algorithm (Altschul *et al.,* 1997). The sequence obtained was deposited in NCBI GenBank (www.ncbi.nlm.nih.gov/GenBank/submit.html). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distance was computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and evolutionary analysis were conducted in MEGA5 (Tamura *et al.,* 2011).

RESULTS AND DISCUSSION

Bacterium with protease activity

Bacterial isolate from rhizospheric soil of CRC, Pantnagar, was found to be gram positive and rod shaped. Halo zone formation around bacterial colony indicates protease production potential of bacterium (Fig. 1). It was also confirmed by extracellular protease activity (140.4 U/ml) by bacterial isolate in production media. Analysis of 16S rDNA sequence of isolate EN4 with available sequences in the NCBI database reveals 99% sequence similarity with the published 16S rDNA sequences of *Bacillus subtilis* and assigned GenBank accession number JN642550.1 (Fig. 2).

Partial purification of alkaline protease

The enzyme was partially purified using ammonium sulphate fractionation followed by dialysis and gel filteration chromatography using Sephadex G-100 column. Purification fold of 1.62 was achieved using ammonium sulphate saturation at 70% dialysis gave enzyme yield of 37.51%. Gel

Table 1. The purification chart of extracellular alkaline protease of EN-4 bacterial isolates

Purification step	Total quantity of enzyme solution (ml)	Total protein (mg)	Total enzyme activity (U)	Total specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	50	4.875	7000	1435.89	1	100
Ammonium sulphate fractionation	15	1.125	2625.7	2333.95	1.62	37.51
Sephadex G-100 column chromatography	1	0.482	1470.07	3051.24	2.12	21.01

Table 2. Yield of immobilized alkaline protease at different concentration of sodium alginate

Sodium alginate conc.	1%	2%	3%	4%
Yield (%)	3.3	47.7	59	53

filteration chromatography of the dialyzed sample resulted into protease yield and fold purification of 21.01 % and 2.12 respectively (Table 1).

Kinetic characterization partially purified alkaline protease

Enzyme kinetics analysis of partially purified enzyme shows K_m and V_{max} values of 0.55 M and 22.2 U/ml, respectively. Calculated turnover number per minute was 296, with a catalytic efficiency of 538.1 M/min.

Immobilization of protease

By varying the concentration of sodium alginate solution (1–4%) and using 15 mg enzyme/ml alginate solution the immobilization process of alkaline protease within calcium alginate beads was carried. High yields of immobilization defined as the ratio of the activity of immobilized enzyme to the activity of the free enzyme used. The percent entrapment activity yield was highest (~59%) at 3% sodium alginate concentration (Table 2). Leakage of enzyme occurs at lower concentration of sodium alginate due to longer pores of less tightly cross linked gel (Figure 3). An approximate 40-60 percent loss of enzyme activity was noticed in all cases due to denaturation of enzyme during gel formation. 3% sodium alginate concentration was found to be optimum. Although a good consolidation of the beads was obtained at 4% (w/w) sodium alginate, but a lower activity was observed. This decrease in immobilization yield with increasing sodium alginate concentration may be due to a higher density of sodium alginate at higher % and it did not allow the trapped enzyme to come out easily. Sodium alginate in concentration of 2-3 percent was used by several workers for enzyme immobilization (Farag and Hassan 2004; Mittal *et al.,* 2005).

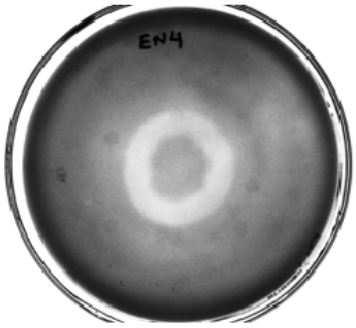

Fig. 1. Halo zone formation by bacterial isolates EN-4 due to casein hydrolysis

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

The effect of pH on extracellular enzyme activity was determined in the pH range of 3 to 12. The maximum protease activity of free enzyme (140 U/ml) was obtained at pH 10 (Figure 4). Incubation of extracted enzyme for 60 min in different buffers before enzyme assay indicates stability of enzyme upto pH 11 (Figure 5). The effect of varying temperature on the enzyme activity was determined in the range of 30°C to 80°C and optimum temperature for protease activity (127.8 U/ml) was found to be 50°C (Figure 6). The enzyme was stable at temperatures between 50°C to 70°C (Figure 7).

Effect of pH and temperature on activity of immobilized protease

Optimum pH and temperature for immobilized alkaline protease from isolate EN-4

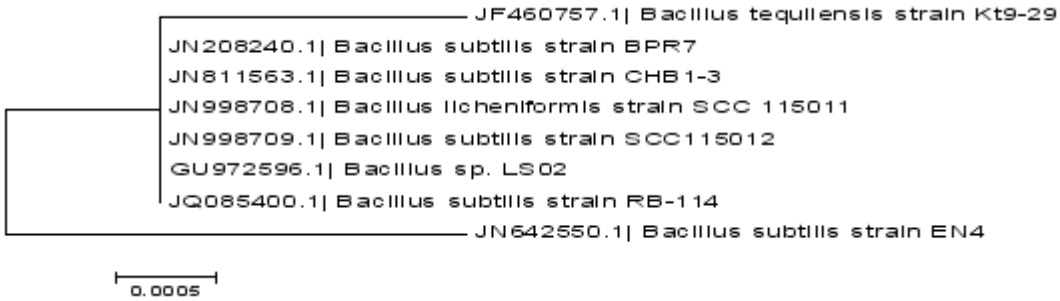

Fig. 2. Neighbor-joining tree based on 16S rDNA sequence of *Bacillus subtilis* EN-4 (JN642550.1) and related sequences

were 9.0 and 60°C, respectively (Figure 4 and 6). It was quite different from the pH of free enzyme. This was due to some change in enzyme stability and substrate availability which was because of its immobilization. The appreciably enzyme activity and stability at different pH and temperatures makes this isolate industrially promising and of special interests for basic and applied research.

1% Alginate 2% Alginate 3% Alginate

Fig. 3. Immobilized alkaline protease in different concentration of alginate

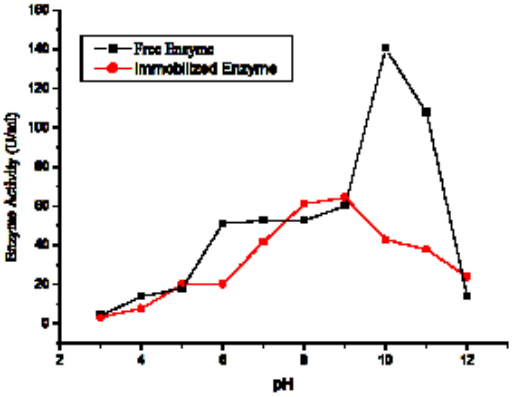

Fig. 4. Effect of pH on enzyme activity

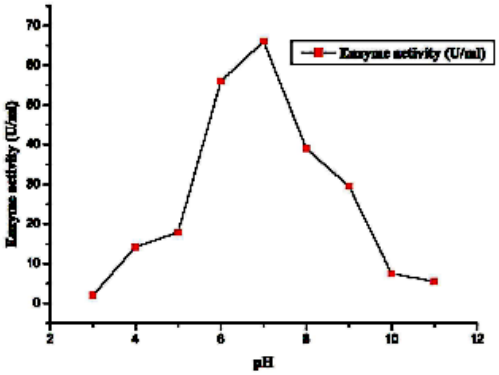

Fig. 5. Effect on pH on the stability of free enzyme

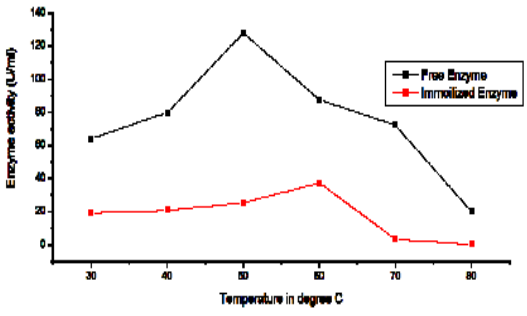

Fig. 6. Effect of temperature on enzyme stability

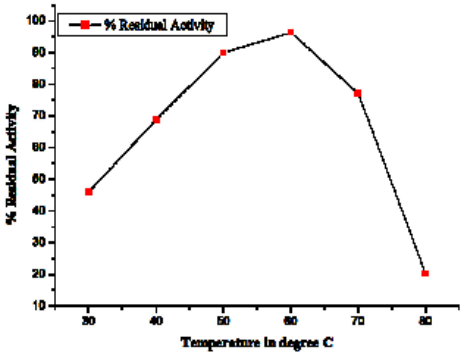

Fig. 7. Effect on temperature on the stability of free enzyme

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