BIOCHEMICAL CHARACTERIZATION OF PECTINASE IN BACILLUS PUMILUS ISOLATED FROM FRUIT WASTE

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ABSTRACT

The purified enzyme exhibited maximal activity at a temperature of 60°C and pectinase was incubated in different pH from 3 to 9 and the highest enzyme activity was achieved at pH 7. The crude enzyme was first precipitated with 20 to 40% ammonium sulfate saturation and column chromatography was conducted to purify the enzyme. Purification step i.e. Gel filtration chromatography (Sephadex G-75) yielded pure protease enzyme and Purity was checked by 10% SDS-PAGE Electrophoretic technique. This pectinase has tremendous applications in textile industry, plant tissue maceration, fruit juice industry and wastewater treatments.

Key words: Bacillus pumilus, Pectinase activity, optimum pH, thermal stability, SDS-PAGE.

INTRODUCTION

Pectinases are one of the upcoming enzymes of fruit and textile industries. These enzymes break down complex polysaccharides of plant tissues into simpler molecules like galacturonic acids. Pectinases constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls (Fogarty & Kelly, 1982). Pectinases are produced by many organisms such as bacteria (Horikoshi, 1972), fungi (Aguilar, 1990) and yeasts (Gainvors, 1993). In the industrial sector, acidic pectinases are used in the extraction and clarification of fruit juices (Rombouts et al., 1986), whereas alkalophilic pectinases are finding immense use in the degumming of ramie fibers (Cao et al., 1992), retting of flax (Sharma, 1987), plant protoplast formation and treatment of effluents discharged from fruit processing units (Tanabe et al., 1987). Pectinase is a general term for enzymes that break down pectin, a polysaccharide substrate that is found in the cell walls of plants. One of the most studied and widely used commercial pectinases is polygalacturonase. Pectinases have also been used in wine production since the 1960s. They can be extracted from fungi such as Aspergillus niger. Like all enzymes, pectinases have an optimum temperature and pH at which they are most active. For example, a commercial pectinase might typically be activated at 45 to 55°C and work well at a pH of 4.8 to 5. If it is hotter it will make the reaction go more quickly. However, the higher temperature will eventually denature the enzyme, eventually stopping it from working.

MATERIALS AND METHODS

Microorganism and culture conditions

portions of 5g of samples of soil, from agricultural and vegetable wastes were pooled and homogenized in sterile medium with pH 6.0 containing 1% citrus pectin with 67% of metoxilation, 0.14% \((\text{NH}_4)_2\text{SO}_4\), 0.20% \(\text{K}_2\text{HPO}_4\), 0.02% \(\text{MgSO}_4\ 7\text{H}_2\text{O}\) and 0.10% nutrient solution (5 mg/L \(\text{FeSO}_4\cdot7\text{H}_2\text{O}\); 1.6 mg/L \(\text{MnSO}_4\), \(\text{H}_2\text{O}\); 1.4 mg/L \(\text{ZnSO}_4\), \(\text{H}_2\text{O}\); 2.0 mg/L \(\text{CoCl}_2\)). A loop of the homogenate was then streaked onto nutrient medium and incubated at 30°C for 24 to 72 h. All morphological contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured onto slants media and maintained for identification and enzymes studies. Identification of the genus was done on morphological and biochemical characteristics and pectin degrading organism was identified as Bacillus pumilus.
Plate assay of depolimerized pectin: The medium was the same used for isolation of cultures, supplemented with 2% agar. Pure culture was inoculated by puncture in the medium and incubated for 48h at 30ºC. After the colonies reached around 3 mm, iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H2O). The liquid medium containing 1% citrus pectin, 0.14% (NH4)2SO4, 0.6% K2HPO4, 0.20% KH2PO4 and 0.01% MgSO4 7H2O, pH 6.0 was inoculated with a suspension containing 10^6 cells/ ml.

Production of pectic enzyme

Cultures were grown in 125ml Erlenmeyers flasks with 25 ml of medium in a rotary shaker (150rpm) at 30ºC. After 48h the biomass was separated by centrifugation at 1000xg for 20 min and the supernatant was used to evaluate pectinase activity.

Assay of pectinase activity: Activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent DNS assay described by Miller. The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture supernatant, was incubated at 40ºC for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of glucose released per minute.

Enzyme purification: Pectinase was purified by the following methods

Ammonium sulphate precipitation and Dialysis:

Crude enzyme was brought to 20% saturation with ammonium sulphate and centrifuged at 6000 rpm for 15 minutes. The resulting precipitate was collected and dissolved in the smallest possible volume of distilled water and dialyzed against distilled water.

Enzyme characterization

Effect of pH on pectinase activity was studied in the range of 3, 5, 7 and 9 (Gummadi SN, et al., 2003) and The effect of temperature on the enzyme activity was determined by performing the standard assay procedure as mentioned earlier for 10 min at pH 6.5 within a temperature range of 40 to 100ºC. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 40-100ºC for 2h in a constant-temperature water bath by Lia, et al., 2008 . After treatment the residual enzyme activities were assayed.

Purification of pectinase by Gel filtration on Sephadex G–75 column

The dialysed and concentrated fraction was subjected to gel filtration on Sephadex G-75 column. Five grams of Sephadex G-75 (Sigma, USA) was suspended in 50 ml distilled water and kept for swelling in overnight with intermittent stirring at shorter intervals to prevent formation of lumps. The swelling gel bead solution was poured into a column tube (2.5 X 50cm) which was previously inserted with glass wool at the bottom. The gel beads were allowed to settle gently without trapping of air bubbles. In this fashion, the prepared column was pre-equilibrated with 0.05 M sodium citrate buffer (pH 5.0). The loaded column was eluted with the same buffer. 1.5 mL fractions of eluate were collected at a flow rate of 1 ml/min in test tubes in an automatic fraction collector (Biorad, Model-2110) at 4 °C and absorbance of 1.5 mL fractions at 280 nm were measured in a UV-visible spectrophotometer for protein content. Fractions with high protein activity were pooled, concentrated and dialysed against the same buffer for 6 hr. The purified enzyme thus obtained was stored at -22°C.

Confirmation of Purification by sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli 1970, is a frequently applied method for the determination of the purity of the enzyme. The enzyme preparation of endoglucanase was denatured by boiling in the presence of 1% SDS and 1% 2-mercaptoethanol and subjected to SDS-PAGE on slab gel with 1% stacking gel (pH 6.8) overlaided on 10% separating gel (pH 8.8). The separating gel consisted of 10% (W/V) acrylamide, N-methylene-bis-acrylamide (sigma, USA) at a concentration such that the ratio of monomers to bis was 30:08; 0.375 M Tris-HCl (pH 8.8) and 0.1% sodium dodecyl sulphate. It was chemically polymerized with 0.05% (V/V) TEMED (Merck, FRG). The solution was cast...
into slabs and was overlayered with butanol to exclude contact with air. The stacking gel containing 4% (W/V) acrylamide, 0.12 M Tris – HCl (pH 6.8), 0.1% SDS, 0.05% (W/V) ammonium persulphate, 0.05% (V/V) TEMED was overlayered on separating gel. Samples (50-200 µg) were mixed with an equal volume of sample buffer having 0.0625 M Tris-HCl (pH 6.8), 10% (V/V) glycerol, 5% 2-mercapto ethanol, 2% SDS and 0.002% bromophenol blue and heated in a boiling water bath for 5 min. After cooling samples were loaded into the slots. The samples (crude fraction and purified endoglucanase) were stacked at 50V and run at 100 V about 6 hours using 0.025 M Tris, 0.192 M glycine buffer (pH 8.3) containing 0.1% SDS as the electrode buffer.

**Visualization of proteins separated by electrophoresis**

The protein bands separated on acrylamide gel were visualized by staining with Coomassie Brilliant blue R-250, 0.25% in 40% methanol and 7% acetic acid, followed by destaining in methanol: acetic acid: water (40:5:55) (V/V).

**RESULTS AND DISCUSSION**

The effect of pH on pectinase activity produced by the *Bacillus* sp was determined by using reaction mixtures at pH values ranging between 3.0 and 9.0. Pectinolytic activity was the highest at pH 7.0 which presented optimal pH between 5.0 and 7.0. Assay for determination of the optimal temperature for Pectinase activity indicated maximal activities at 40–90ºC. To determine the effect of temperature on the stability of pectinase, enzyme solutions were incubated for 60 min at temperatures between 40ºC and 90ºC and the residual activity was assayed after cooling. Practically no changes in pectinolytic activities were observed when the incubation temperature was lower than 50ºC. The pectinolytic activity of the crude solution has specific properties which may offer advantages over currently available pectinase preparations. The enzyme solution can be applied directly to vegetables without the need for pH modification. Furthermore, because of the temperature stability of the enzyme, pectinases are thermotolerant enzymes.
The Crude enzyme was first precipitated with 20 to 40% ammonium sulfate saturation and column chromatography was conducted to purify the enzyme. Purification step i.e. Gel filtration chromatography (Sephadex G-75) yielded pure protease enzyme and Purity was checked by 10% SDS-PAGE Electrophoretic technique.

CONCLUSIONS

In the present study, characterization of plant pectinase from fruit waste was investigated and reported. Mango peel, being a by-product of the mango processing industry, has been found to be a source of pectinase with interesting properties. The high pectinase activity and stability of the enzyme at elevated temperatures, alkaline pH of 7.0 and it can be a useful enzyme for the food industry and biotechnological applications. These properties thus make pectinase from mango peel an enzyme of great importance in industrial applications.

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CONFLICT OF INTEREST:

Authors declare that there is no conflict of interest

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