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Purification and Kinetic Studies of Polygalacturonase from *Aspergillus niger* RBF96

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Polygalacturonases (PG) are enzymes that degrade pectic substances and are widely used in juice and fruit beverages to improve the quality of the process. The PG isolated from *Aspergillus niger* RBF96 was partially purified and characterized. It was purified upto 7 folds with an yield of 14.04 % and specific activity of 83.06 U/ig, by gel filtration using sephadex G100. The SDS-PAGE revealed the presence of multiple bands in the crude enzyme, while a single band in the purified fractions. The molecular weight of the PG protein was 37 kDa. The Km and Vmax of the enzyme were 0.155 U/mL and 38.57 U/mL respectively. The PG was found to be relatively more stable to temperature and pH changes. The optimum temperature and optimum pH of the enzyme were 50° C and 4.5 respectively.

Key words: *Aspergillus niger* RBF96, Polygalacturonase, SDS-PAGE, Purification, Km and Vmax.

Polygalacturonase (PG) comprises a heterogeneous group of enzymes that catalyse the breakdown of pectin containing substrates. These are used in the food industry to improve cloud stability in fruits and vegetable nectars. Production of pectinases can be done by both solid state cultures and submerged fermentation techniques[1]. However, production of pectinase by *Aspergillus* strains was observed to be higher in solid state fermentation than in sub-merged process[2]. It has been reported that microbial pectinases account for 25 per cent of the global food enzymes sales. Most of the commercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes which are generally regarded as safe (GRAS) by United States Food and Drugs Administration (USFDA)[3].

During enzyme production process, in addition to the target enzyme, growth medium may have some undesirable metabolites of the micro-organisms. The purified enzymes exhibit higher activity, lesser risk of harmful substances and, thus, better affectivity for the specific product. Moreover, characterization of enzyme is imperative, to achieve better performance in a particular application; as it is a prerequisite to get information regarding the suitable conditions of enzyme action. *Aspergillus niger* RBF96 has been identified as the most potent strain which produced the highest PG activities which effectively clarified orange fruit juice[4]. Hence, this enzyme was purified and characterized.

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MATERIALS AND METHODS

Chemicals, media and standard enzyme

All the chemicals used in the present investigation were of analytical grade (AR grade) and procured from HiMedia Laboratories Limited, Mumbai and from Sigma-Aldrich Corp, MO, USA. All the experiments for isolation, screening and pectinolytic enzyme production were conducted under sterile conditions, and adequate safety measures were undertaken.

Pectinolytic fungus and Inoculum preparation

Aspergillus niger RBF96 strain, which was isolated from rotten ber fruit and characterized using molecular approach in our laboratory, was used in this experiment. The culture was maintained on Czapek Yeast Extract Agar (CYA) medium and sub-cultured periodically throughout the duration of the research. The potato dextrose broth medium was prepared, adjusted to pH 5.5 and autoclaved for 15 minutes at 121°C and cooled. A loop-full culture from sporulation medium of the fungus raised on Czapecdox yeast extract agar (CYA) slants, was transferred aseptically in a laminar air flow into the conical flask (250 ml) containing 100 ml sterilized inoculum medium. It was incubated on an orbital shaker (130 rpm) at 37 °C. After 72 h, the spore count in the inoculum was adjusted to 1×10^7 spores/ml with the help of a haemocytometer[5].

Solid state fermentation (SSF) studies using citrus peel as the substrate

Citrus peels were collected from different fruit juice centers, located in Hubli and Dharwad city and used for SSF studies. Ten g of citrus peel in a flask was mixed with adequate quantity of sterile water to maintain the moisture content along with the mixture of salt solution but without pectin. The pH of substrates was adjusted accordingly with either 0.01 M HCl or 0.01 M NaOH and autoclaved for 15 minutes at 121°C and cooled. The spore concentration was adjusted to $1x10^7$ spores/ml. From this suspension, 1ml was withdrawn and inoculated into each flask and incubated at different temperature (°C). Four fermentation periods of 2nd, 4th, 6th and 8th day were given to each treatment to arrive at the best period of fermentation under pre-optimized conditions. For proper aeration, flasks were intermittently shaken.

Enzyme harvesting

After 6 days of incubation, the experimental trays were harvested. The extract from SSF trays was prepared by mixing a known quantity of fermented material with distilled water (1: 3, w/v) for 30 min on a rotary shaker (180 rpm). The fermented biomass sample was filtered using a muslin cloth and the filterate was centrifuged at 10,000 rpm for 10 minutes at 4° C temperature to remove the spores of the organism. The supernatant was filtrated again on Whatman no.1 filter paper and stored aseptically under refrigerated conditions to prevent contamination. The crude enzyme extract, thus, obtained was subjected to purification after enzyme assay.

Polygalacturonase (PG) assay

Polygalacturonase (PG) assay was measured by determining the amount of reducing groups released and galacturonic acid was estimated by Dinitrosalicylic acid (DNSA) method[6]. One unit of Polygalacturonase (PG) is defined as the amount of enzyme that releases one µ mol of galacturonic acid/ml/min under the assay conditions.

Protein determination

Protein was estimated following the method[7] using bovine serum albumin as the standard and absorbance was measured at 600 nm.

Purification and characterization of PG enzyme

Ammonium sulphate fractionation

The cell free extract of the enzyme produced by the selected efficient fungal isolates was subjected to precipitation using ammonium sulphate starting from 20 per cent up to 90 per cent with 10 per cent increase each time. The precipitated protein was removed by centrifugation at 10,000 rpm for 30 min at 4°C and the supernatant was subjected for purification with further addition of ammonium sulphate to remove other unknown proteinaceous material[8].

Dialysis

After ammonium sulphate precipitation, the enzyme salt solution was dialyzed against the 0.01M Tris-HCl buffer (pH 6.0) for 24 h at 4°C to remove ammonium salt with changes of the buffer at every 4 h interval. The dialysis was carried out using cellulose tubing (dialysis sacks) which retains proteins with Molecular weight higher than 12,000 Da. The tubes measured a length of 30 cm

and flat width of 25 mm with inflated diam. of approx. 16 mm open ended.

Gel filtration chromatography

Sephadex G-100 (5.0 g) (Sigma-Aldrich) was suspended in distilled water placed in a boiling water bath for 5 h and the solution stirred occasionally. After cooling, distilled water was decanted and the swelled gel was equilibrated with 1M Tris-HCl running buffer (pH 6.0). The dialyzed enzyme fraction was further purified as per the method. It was loaded on to Sephadex G- 100 column (50 x 2.0 cm, bed volume 80 mL) and eluted with 1M Tris-HCl running buffer (pH 6.0) at the flow rate of 30 mL/h. Many fractions of 2 mL per tube were collected and the fraction in which protein present was detected. The fractions bearing high protein were collected and evaluated for pectinase activity**.** The fractions showing higher enzyme activity were pooled together for further characterization.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl sulphate Polyacrylamide Gel Electrophoresis was carried out following the protocol[9]. The glass plates were assembled as per the manufacturer's instructions. Ten mL of 10 per cent polyacrylamide gel was poured into the gap between the glass plates leaving sufficient space for the stacking gel. SDS (0.1 %) was poured on the top of the polyacrylamide gel in order to make the surface smooth and level. The gel was left for 30 minutes or until it solidified. After solidification, the SDS layer was removed by washing with water for two to three times. About 4 mL of 5 per cent stacking gel was poured on the resolving gel after inserting the comb. The sample for the SDS-PAGE was prepared freshly and diluted to get the equal concentration of total protein. To this supernatant, equal volume of the loading dye was added and heated upto 95^{o}C for 10 minutes. To each well, about 10 mg of the sample and equal volume of dye (v/v) mixture were loaded. After loading the dye, the apparatus was submerged in the running buffer. Initially, the voltage of the current was kept at 70 mV and when protein entered the resolving gel the voltage was increased upto 120 mV. The gel was run upto the end of the resolving gel, for about four hours. After running the polyacrylamide gel electrophoresis, the gel was carefully removed and placed in the staining solution. The gel was stained for 3 hours in shaking condition. After staining with coomassie brilliant blue, the excess dye was removed by destaining solution under shaking condition. The de-staining solution was replaced for two to three times until the clear blue color bands appeared. The gel was sealed in polyethylene bag and stored at 4^{o}C. The purity of the enzyme was checked by comparing with the standard protein marker bands and purified enzyme and crude enzyme bands for determination of the molecular weight.

K_m values of the purified enzymes

The Michaelis constants (K_M and V_{MAX} values) of PG were determined by measuring the activity reaction rates. The K_m value was determined from Lineweaver-Burk plot by using pectin as substrate at concentrations of 0.2 to1.0 mg/mL.

Optimum Temperature of the pure enzyme

The maximum activity of the pure enzyme was determined at different incubation temperatures which ranged from 4, 10, 20, 30, 40, 50, 60, and 70^{o}C for 30 min.

Optimum pH of the pure enzyme

The pH optimum was determined using 0.1 M sodium acetate buffer (for the pH range 3.0-5.0) phosphate buffer (for the pH range 6.0-7.0) and 0.1 M tris – HCl buffer (for the pH 8.0-9.0). The pure enzyme was incubated at different pH values at 50 ^{o}C for 10 min and the activity measured under the standard conditions.

RESULTS AND DISCUSSION

Purification of polygalacturonase produced by *Aspergillus niger* RBF96

The crude PG enzyme produced by *A. niger* RBF96 had a total enzyme activity (27306 U), total protein (2312 µg) and the specific activity of 11.81 U/ìg protein. The purification steps resulted in enhanced specific activity; the ammonium sulphate precipitation resulted in 16515 U of the total enzyme activity with specific activity of 18.85 U/ìg protein. After purification through gel filtration chromatography using Sephadex G-100, the maximum specific activity was 83.06 U/ìg proteins. The details of the results are furnished in Table 1.0. These treatments increased the purification by 1.59 folds after ammonium sulphate precipitation. Whilst, the maximum purification was

Table 1. Purification of polygalacturonase produced by Aspergillus niger RBF96

Strain	Purification step	Total activity (U)	Total protein (µg)	Specific activity (U/ µg)	Yield (%)	Purification folds
Aspergillus niger RBF96	Crude Extract	27306	2312	11.81	100	1.0
	Ammonium sulphate fraction (80%)	16515	876	18.85	37.88	1.59
	Gel filtration using Sephadex G-100	10217	123	83.06	14.04	7.03

observed after gel filtration (7.03 fold). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield was reduced to 37.88 % after ammonium sulphate precipitation which was further reduced to 14.04%, after gel chromatography separation.

The purified pectinases were characterized for molecular mass, Michaelis-Menten kinetics (K_m and V_{max}) and pH and temperature optima. The electrophoresis revealed the presence of multiple bands in the crude enzyme while a single band in the purified fractions. The molecular weight of the PG protein produced by *Aspergillus niger* RBF96 was 37 kDa. The bands revealing molecular weight of crude and purified polygalacturonase proteins by SDS-PAGE are shown in (Fig. 1).

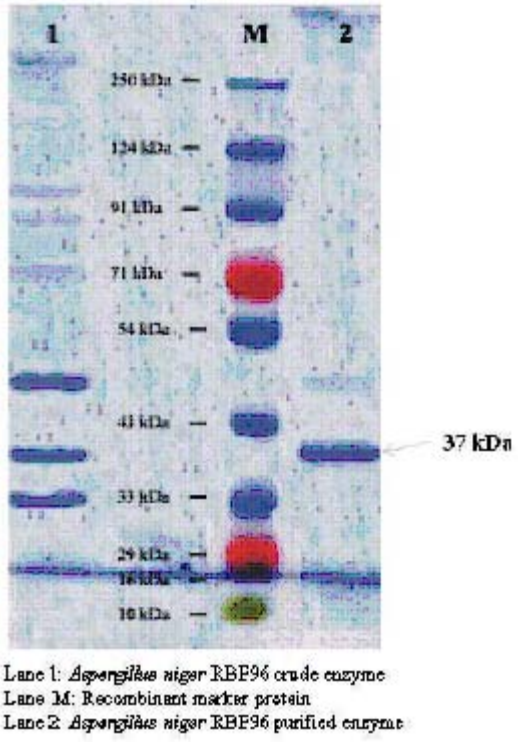

Fig. 1. SDS-PAGE for determination of molecular weight of polygalacturonase from *Aspergillus niger* RBF96

Kinetic studies of the PG enzyme

The kinetic properties of an enzyme include: K_m, V_{max}, thermal stability and pH. K_m, the substrate concentration which gives half of the maximal velocity possible at that enzyme concentration, is a constant characteristic for a particular enzyme, acting on a particular substrate. Michaelis constant (K_m) and V_{max} of the purified pectinases were determined by measuring the reaction velocities at various concentrations of pectin. The apparent K_m and V_{max} values (Fig. 2) for polygalacturonase from *Aspergillus niger* RBF96 were 0.155 U/mL and 38.57 U/mL respectively.

It was found that the enzyme exhibited maximum activity (774 U/mL) at 50°C (Fig. 3). When the temperature was increased above 50°C, the activity of the enzyme was gradually reduced. Similarly, below 50°C, a gradual decline in the PG activity was observed. The highest enzyme activity (712 U/mL) in *A. niger* RBF96 was found at pH 4.5. The enzyme showed maximum stability at pH 4.0, 4.5 and 5.0. When the pH of the enzyme medium

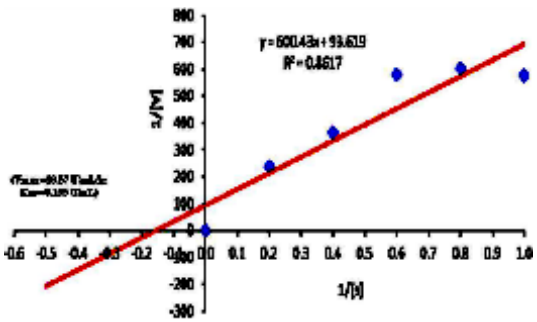

Fig. 2. Lineweaver-Burk plot for polygalacturonase activity from *Aspergillus niger* RBF96 showing Michaelis-Menten kinetics

was increased up to 8.0; it showed a decline in the enzyme activity. Likewise, when the activity of PG was assayed at pH values lower than 4.5, it again showed a decreasing trend with a decrease in pH as shown in fig. 4.

Like all other chemical reactions, the velocity of the enzyme catalyzed reactions also increases with an increase in temperature. However, eventually a temperature is reached where the enzyme becomes unstable and begins to denature, at which point the reaction rate again declines. The results of the present investigation showed that the optimum temperature for the maximum activity of PG was 50° C, after words, a sudden drop in enzyme activity was observed. As the denaturation of the enzymic protein occurs at elevated temperatures, the enzyme activity decreased rapidly. Enzyme deactivation and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes. Enhancing the stability and maintaining the desired level of activity over a long period are two important points considered for the selection and design of pectinases. The catalytic activity of many enzymes is markedly dependent on pH. It can exert its effect in different ways: on the ionization of groups in the enzymes active site, either on the ionization of groups in the substrate, or by affecting the conformation of either the enzyme or the substrate. The stability of pectinases is affected by pH and temperature. In this study, it was interesting to note that the PG showed maximum stability in the pH range 4.0 to 5.0.

Changes in pH of a medium have been reported to affect the affinity of the enzyme for substrates. The pH of the medium in which the enzyme is exposed affects the ionization state of its amino acids which dictates the primary and secondary structure of the enzyme thus controlling its activity. The observed reduction in the enzyme

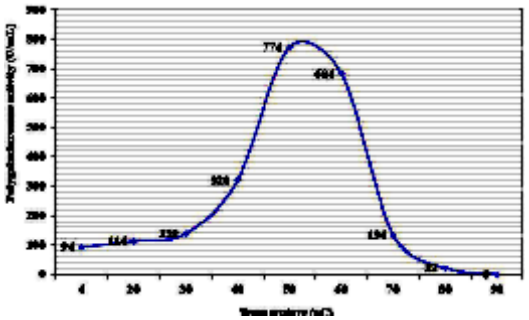

Fig. 3. Optimization of temperature for maximum polygalacturonase activity

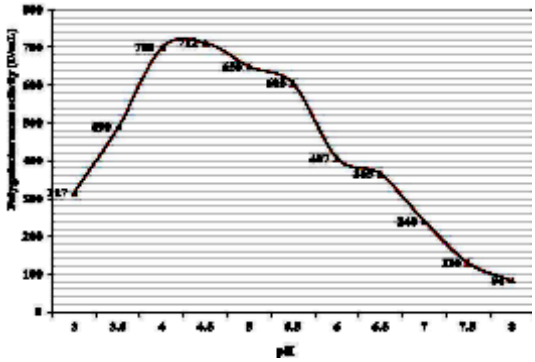

Fig. 4. Optimization of pH for maximum polygalacturonase activity

activities of pectinolytic strains at pH values other than the optimal pH could also be attributed to a probable change in the state of the ionic groups involved in the maintenance of the active conformation of the enzymes. Extreme pH has been reported to initiate chemical reactions that could alter, cross-link or destroy amino residues of the protein molecules resulting in irreversible inactivation[10]. Similarly, the extracellular PG produced from *Pleurotus ostreatus* NRRL-0366 on lemon peel (*Citrus limonium*) was purified to a homogenous form, with about 27 folds purification and specific activity of 158 U/mg proteins by two chromatographic steps in DEAE cellulose and Sephacryl S-300[11]. The enzyme was stable at 30° C and pH (3.0-3.5). It exhibited K_m of 1.33 mg mL^{-1} and V_{max} of 28.6 ìmol min^{-1}.

CONCLUSIONS

The polygalacturonase excreted by *A. niger* RBF96 was partially purified and characterized. The SDS–PAGE analysis of the enzyme revealed that it existed in a monomeric form with a molecular weight of 37 kDa. The Km and Vmax of the enzyme were 0.155 U/mL and 38.57 U/mL respectively. The PG was found to be relatively more stable to temperature and pH changes. An

improved awareness of the kinetic properties of PG is important in commercialization of this enzyme in various fields. The data presented in this article would provide empirical reference for future work with *A. niger* RBF96 strain.

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