Production, Purification of Exo-Polygalacturonase from Soil Isolate *Paecilomyces variotii* NFCCI 1769 and Its Application

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Abstract The aim of the present study was to produce exo-polygalacturonase from potent soil isolate by submerged fermentation and its application for fruit juice treatment. Pectinase producing strains were selectively isolated from pectin industry waste. A selected isolate C2 was found to produce significant amount of exo-polygalacturonase. The isolate was identified as *Paecilomyces variotii* on the basis of morphological characteristics and 18S rRNA gene sequence analysis. The exo-polygalacturonase produced by the isolate was purified by ammonium sulphate precipitation, size exclusion chromatography and ion exchange chromatography. The purified enzyme had MW of 39.4 kD based on SDS PAGE. Under partially optimized conditions, purified exo-polygalacturonase showed specific activity of 98.49 U/mg protein at pH 6.0 and 30°C. The enzyme was comparatively stable from 10 to 30°C and the activity decreased with increasing temperature. Purified enzyme brought about considerable reduction in viscosity of fruit juice samples.

Keywords Pectinase · Exo-polygalacturonase · *Paecilomyces variotii* NFCCI 1769

Introduction

Pectic substances occur as structural polysaccharides in the middle lamella and primary cell wall of higher plants [1]. Pectins are high molecular weight acid polysaccharides, primarily made up of α (1–4) linked D-galacturonic acid residues, which are partially esterified with a small number of rhamnose residues in the main chain. On hydrolysis, pectin yields 60–90% galacturonic acid, some pentoses, galactose, methanol and acetic acid. Pectins are soluble carbohydrates of colloidal nature. Wide range of organisms including fungi, yeast, bacteria, protozoa, insects and nematodes are reported for production of pectinolytic enzymes [2]. World wide interest in pectinolytic enzymes has increased markedly because of its high industrial value particularly in fruit juice treatment [3]. On the basis of its mode of action, pectinases have been classified into seven different classes as Pectinesterase (E.C.3.1.1.11), Polygalacturonase (E.C.3.2.1.15), Galacturan 1,4 α galactouronidase (E.C.3.2.1.67), Exo-poly α galactouronosidase (E.C.3.2.1.82), Endo-pectatelyase (E.C.4.2.2.2), Exo-pectatelyase (E.C.4.2.2.9) and Endopectinylase (E.C.4.2.2.10) [4].

Pectin is present in certain amount in fruits and vegetables, which during extraction remains in the pulp and causes cloudiness in the juice. The use of pectinolytic enzymes is the solution for removal of haze and cloudiness leading to reduction of viscosity and increase yield of juice. In industries, acidic pectinases mainly polygalacturonase (PG) from fungi is used during extraction and clarification of fruit juices. Whereas alkaliphilic pectinases are used immensely in the degumming of fibers and treatment of effluents discharged from fruit processing units [3]. In the present study, an efficient exo-polygalacturonase producing strain was isolated from potent sites of pectin waste and

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identified as *Paecilomyces variotii*. In addition, the conditions for growth and enzyme production were partially optimized followed by enzyme purification and its application.

Materials and Methods

Isolation of Exo-Polygalacturonase Producing Cultures

Cultures were selectively isolated from untreated non rhizospheric soil mainly from fruit processing area and pectin producing industrial wastes by using pectin enrichment method. In brief 1% w/v of each sample was inoculated in 100 ml of pectin broth (5% w/v yeast extract, 1 ml of 0.1% v/v bromothymol blue, 0.6 ml of 10% v/v $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 6.0) supplemented with 0.2% w/v pectin (Himedia) and incubated on rotary shaker at 120 rpm (Remi CIS 24 BL, India) at 30°C for 7 days. A 1 ml of previously enriched culture was inoculated in 100 ml of freshly prepared pectin broth supplemented with 0.5% w/v of pectin for next 7 days of enrichment. After several serial transfers in pectin broth, each time with increasing pectin concentration, the soil sample was finally subjected for isolation of pectinase producers. The efficient strains were isolated by spreading 0.1 ml of enriched broth on the surface of pectin gel containing basal agar medium plate [5]. The enzyme activity in liquid culture was measured, where one unit of polygalacturonase was defined as the amount of enzyme required to release 1 μM of galacturonic acid per minute [6].

Screening and Identification of Isolates

Seven different strains were isolated and screened for pectinase production. In short crude extract of isolates were treated with 50 mM iodine and titrated against 100 mM $\text{Na}_2\text{S}_2\text{O}_3$ to detect oxidation product [7]. The selected potential isolate was then characterized by using morphological and 18S rRNA gene sequence analysis.

Confirmation of Enzyme Type

Enzyme produced by isolate was subjected to determine its exo or endo category for which cell-free supernatant and cell lysate were checked for enzyme activity. Further, the type of pectinase was determined using following assays.

Polygalacturonase (PG)

PG activity was determined by quantifying monomers of galacturonic acid liberated from the pectin solution by DNSA method. In brief, 2.5 ml 0.5% (w/v) of citric pectin

in 0.025 M acetate buffer (pH 6.0) to which 2.5 ml of culture extract was added. The blank was prepared by taking buffer solution instead of the crude enzyme. On incubation at 50°C for 10 min, 0.5 ml of DNSA solution was added to it and boiled for 5 min. After cooling, absorbance at 575 nm was measured and compared with standard curve of monogalacturonic acid [8, 9].

Pectin Esterase (PE)

PE activity was evaluated by the pH decrease due to release of carboxylic groups. The culture extract of 1.0 ml was added to 2.0 ml of citric pectin solution 1% (w/v) prepared in 0.025 M Tris acetate buffer (pH 6.0). The blank was prepared by substituting culture extract by buffer solution. Then it was incubated at 50°C for 2 h and then kept in boiling water bath for 3 min. Finally, samples were cooled and titrated against 0.01 M NaOH solution [9].

Pectin Lyase (PL)

The PL activity was assayed spectrophotometrically. Briefly, 1 ml of culture extract was added to 5.0 ml of pectin solution 1% (w/v) and volume was adjusted to 10.0 ml with distilled water. The samples were incubated at 40°C for 2 h followed by addition of 0.6 ml of zinc sulphate 9.0% (w/v) and 0.6 ml of 0.5 M sodium hydroxide. The samples were centrifuged ($3000 \times g$, 10 min) and 5.0 ml of the clear supernatant was added to a mixture of 3.0 ml of 0.04 M thiobarbituric acid, 2.5 ml of 0.1 M HCl and distilled water (0.5 ml). The mixture was heated in a boiling water bath for 30 min, cooled to room temperature and the absorbance of the coloured solution was measured at 550 nm [10].

Partial Optimization of Culture Conditions for Enzyme Production

Operating variables viz. pH, temperature, time of incubation and substrate concentration were independently optimized for fermentation [11]. The temperature ranged from 10 to 90°C while pH ranged from 1.0 to 9.0. In addition to pH and temperature, enzyme production was monitored for 24 to 120 h of incubation and substrate concentration was checked from 0.5 to 4.5% w/v [8]. These partially optimized culture conditions were then used for production of enzyme.

Enzyme Production

The exo-polygalacturonase production was achieved by inoculating 10^4 spores/ml in fermentation medium. In brief, culture was supplemented with 1.5% w/v of pectin

and allowed to incubate at 30°C for 72 h under submerged cultivation [12]. After incubation, mycelial mass was separated by means of filtration and resulting supernatant was ultrafiltrated through polysulfone fiber cartridge (Amicon, Ireland) having 10 kD cutoff size.

Purification and Characterization of Exo-Polygalacturonase

The retentate was subjected to ammonium sulphate precipitation up to 90% w/v. The resulting precipitate was dissolved in a small quantity of 0.01 M Tris–HCl buffer (pH 6.0) and dialyzed against the same buffer with constant stirring [13].

Gel Filtration Chromatography

The dialyzed enzyme was further purified [14] with certain modifications. The dialyzed enzyme was loaded in Sephadex G-100 column (35 × 1.5 cm, bed volume 60 ml) and eluted with 0.01 M Tris–HCl buffer (pH 6.0) with the flow rate of 20 ml/h. Fractions of 3 ml each were subsequently collected and its protein content was measured by taking absorption at 280 nm on UV spectrophotometer (Shimadzu, Japan). The fractions showing maximum absorption at 280 nm were collected and evaluated for its polygalacturonase activity. The fractions showing higher enzyme activity were pulled together for further characterization.

Ion Exchange Chromatography

Ion exchange chromatography was performed to purify the enzyme further. Carboxymethylcellulose (CMC) was packed into a glass column (15 × 0.55, 10 ml bed volume) and equilibrated with 0.01 mM Tris–HCl buffer (pH 6.0). The concentration gradient of 0.01 to 0.5% w/v of sodium chloride was eluted for elution of protein on a cation exchange resin at a flow rate of 10 ml/h [15]. The fractions were collected and its protein content and enzyme activity were determined as per the method described earlier. The relevant fractions were pooled together and dialyzed against 0.01 mM Tris–HCl buffer (pH 6.0). The purified enzyme was lyophilized (VirTis, USA) and stored at –20°C for further characterization.

SDS-PAGE

The purity of enzyme at each stage was checked by using SDS-PAGE. The standard denatured protein markers and purified enzyme were allowed to run simultaneously for determination of molecular weight of sample. Purified denatured enzyme was loaded on the polyacrylamide gel along with the standard marker proteins and the

electrophoresis was run with supply of 40 mA current. Then the gel was stained overnight with Coomassie Brilliant Blue R-250 staining solution. Finally, the gel was destained and bands were visualized [16].

Temperature Stability

The effect of temperature on enzyme was examined by incubating the enzyme at different temperatures ranging from 10 to 80°C. The enzyme activity was determined at 20 min interval for 240 min of incubation [17]. Maximum activity of fresh enzyme at optimum temperature was considered as 100%. Relative enzyme activities were expressed as % residual activity at specific time and temperature.

Fruit Juice Treatment of Enzyme

Paecilomyces variotii NFCCI 1769, showed predominance of polygalacturonase type of enzyme which has wide scope of application in fruit juice treatment [18]. Seven different fruit juices, orange, banana, grape, apple, pomegranate, guava, and sapota were prepared in a mixer and strained through cheese cloth and treated with the purified lyophilized enzyme. Finally, the fruit juice clarification was evaluated by determining density and viscosity of samples [19]. Density of fruit juices was determined by specific gravity method using specific gravity bottle.

Enzyme treatment 98.49 U of purified enzyme was allowed to react with 100 ml of juice for 30 min at 40°C. Time of flow for each juice sample before and after enzyme treatment with respect to water was determined using Ostwald's viscometer. Viscosity was calculated using the following formula;

$$\% \text{Viscosity } \eta_s = \frac{\eta_w \rho_s t_s}{\rho_w t_w}$$

where η_s , viscosity of sample; η_w , viscosity of water; ρ_s , density of sample; ρ_w , density of water; t_s , flow time of sample; t_w , flow time of water. Commercially available polygalacturonase (Sigma, USA) was used as a positive control.

Results and Discussion

Isolation, Screening and Identification of Pectinase Producing Organisms

Total seven cultures were selectively isolated by enrichment method. Out of the seven different isolates such as bacterial species, yeast and five strains of fungi, the fungal strain C2 showed highest enzyme activity hence it was used throughout this study. The C2 was identified by

Table 1 Screening of isolates for pectinase production

Sr. no.	Code number	Isolates	Enzyme activity of culture extract (U/ml)
1.	B3	<i>Aspergillus niger</i>	63.66
2.	C2	<i>Paecilomyces variotii</i> NFCCI 1769	47.83
3.	C1	<i>Penicillium citrinum</i> Thom 996	30.33
4.	D2	<i>Fusarium</i> spp.	17.83
5.	A3	<i>Rhizopus</i> spp.	15.33
6.	A9	Bacterial isolate	9.50
7.	B6	Yeast isolate	14.50

morphological studies and 18S rRNA gene sequence analysis which showed it to be *Paecilomyces variotii*. The strain was deposited under accession number NFCCI 1769 in National Fungal Culture Collection of India, Pune (India). The crude enzyme extract of the isolate B3 (*Aspergillus niger*) and C2 (*Paecilomyces variotii*) showed 63.66 and 47.83 U/ml of polygalacturonase activity which were comparatively better than other strains (Table 1). *Aspergillus niger* is very well reported for pectinase production [12, 17, 20] while there are very few reports on the study of polygalacturonase production from *Paecilomyces variotii*.

Comparative Enzyme activity of different types of pectinase **Fig. 1** Comparative enzyme activity of different types of pectinases

Confirmation of Enzyme Type

Cell-free supernatant of selected strain *Paecilomyces variotii* NFCCI 1769 showed higher pectinolytic activity than that of cell lysate which indicates that the enzyme activity was mainly contributed by exo-cellular type of enzyme. On

Fig. 2 **a** Effect of pH on Polygalacturonase production. **b** Effect of temperature on Polygalacturonase production. **c** Effect of substrate concentration on Polygalacturonase production. **d** Effect of incubation time on Polygalacturonase production

Fig. 3 Elution profile of polygalacturonase on Sephadex G-100 column

Fig. 4 SDS-PAGE of polygalacturonase at various stages of purification. Samples in wells: 1 culture extract (Cell free culture supernatant), 2 after ultrafiltration, 3 precipitated on ammonium sulphate, 4 dialyzed, 5 Sephadex G-100 gel filtrated, 6 standard protein marker (97400, phosphorylase b; 66000, bovine serum albumin, 43000, ovalbumin; 29000, carbonic anhydrase; 20100, soybean trypsin inhibitor, 14300, lysozyme)

further determination, it was found to be predominantly PG type of pectinase with fewer amounts of PE and PL (Fig. 1).

Optimization of Culture Condition for Enzyme Activity

The exo-polygalacturonase activity of the isolate was further partially optimized which showed maximum activity

Fig. 5 Temperature stability profile of the purified enzyme

at pH 6.0 in 1.5% w/v of pectin for 3 days of incubation at 40°C (Fig. 2a–d).

Purification of Enzyme

Crude enzyme was obtained by saturating culture supernatant with 70% ammonium sulphate. Elution profile of the crude enzyme subjected to gel filtration on Sephadex G-100 column chromatography showed distinct peak with maximum enzyme activity at fraction number 18 (Fig. 3). Gradient of 0.05 M NaCl on CMC column gave precise separation of polygalacturonase. The purified PG exhibited a single band of protein showing ~39.4 kD mol wt when compared with standard mol wt marker proteins run on SDS-PAGE (Fig. 4).

The isolated strain *Paecilomyces variotii* NFCCI 1769 particularly showed presence of PG type of pectinase enzyme [15]. The activity of PG at different stages of purification was determined (Table 2). The overall purification was about 41.91 fold with a recovery of 26.90%.

Temperature Stability of Purified Enzyme

The thermal stability result of PG indicated that it was relatively unstable at high temperature. On 4 h incubation, it retained about 85% of its original activity at 10, 20 and 30°C while at 40°C only 50% of activity was retained.

Table 2 Polygalacturonase activity of *Paecilomyces variotii* NFCCI 1769 at different stages of purification

Sr. no.	Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
1.	Crude enzyme	59337.33	25147	2.35	1.0	100
2.	Ultra filtration	46124	13164	3.50	1.48	77.73
3.	Ammonium sulphate precipitation & dialysis	18542	813	22.79	9.69	31.24
4.	Sephadex G-100	15966	162	98.49	41.91	26.90

Table 3 Fruit juice treatment using pure polygalacturonase from *Paecilomyces variotii* NFCCI 1769

Sr. no.	Fruit juice sample	Viscosity before treatment	Viscosity after treatment		Percent reduction in viscosity (%)	
			Purified enzyme	Commercial enzyme (positive control)	Purified enzyme	Commercial enzyme (positive control)
1.	Orange	2.46×10^{-2}	1.04×10^{-2}	0.73×10^{-2}	42.49	70.23
2.	Apple	2.07×10^{-2}	1.03×10^{-2}	0.58×10^{-2}	50.14	71.67
3.	Pomegranate	0.77×10^{-2}	0.59×10^{-2}	0.33×10^{-2}	22.72	56.19
4.	Sapota	2.41×10^{-2}	1.05×10^{-2}	0.68×10^{-2}	43.77	71.63
5.	Grape	1.12×10^{-2}	0.81×10^{-2}	0.43×10^{-2}	27.93	56.26
6.	Banana	1.23×10^{-2}	0.58×10^{-2}	0.26×10^{-2}	47.68	78.07
7.	Guava	1.50×10^{-2}	1.23×10^{-2}	0.41×10^{-2}	17.67	58.33

However, at 50°C after 3 h and 20 min, activity was completely lost. No activity was detected after heating the enzyme at $\geq 60^\circ\text{C}$ beyond 1 h incubation (Fig. 5).

Fruit Juice Treatment of Enzyme

On fruit juice treatment PG of *Paecilomyces variotii* NFCCI 1769 showed comparatively significant reduction in viscosity for orange, apple, banana and sapota juice sample among the different juice samples tested. Though the reduction in viscosity by the commercially available polygalacturonase is more, the results obtained by polygalacturonase from *Paecilomyces variotii* NFCCI 1769 were found to be significant (Table 3). Apple, orange, grape, banana and sapota are rich in pectin, hence showed high reduction in viscosity of its juice while pomegranate and guava juice showed less reduction in viscosity since it contains less amount of pectin [21].

Paecilomyces variotii NFCCI 1769 is a mesophilic culture, however it showed enzyme activity at broad range of temperature from 30 to 45°C with optimum at 30°C. The optimum pH range for the production of PG was found to be between pH 5.0–6.0, which is in accordance with earlier report [20]. Substrate concentration of 1.5% (w/v) yielded highest polygalacturonase activity (43.08 U/ml) which decreased with increasing substrate concentrations may be because of substrate inhibition. The thermal inactivation of enzyme is always due to denaturation of enzyme proteins [17]. Pectinase from the *Paecilomyces variotii* strain has been earlier described to be susceptible to the denaturation at temperature $>50^\circ\text{C}$ [13]. In fruit juice treatment of apple, stone fruits and berries; temperature 30–35°C for about 15–90 min is required for effective treatment of enzyme [21]. The purified PG of *Paecilomyces variotii* NFCCI 1769 showed very good stability at 40°C up to 120 min of incubation. In view of commercial viability, PG of the isolate can be used at 40°C for the specific duration up to 120 min appropriate for enzymatic clarification of different fruit juices [17, 19, 21].

The application of pectinases mainly exo-polygalacturonase is in variety of fruit juices and wine processing industries to increase juice yield, clarification, promoting antioxidant formation and juice concentrate production. The addition of such exogenous enzyme also allows more specific degradation which is necessary to give a characteristic smooth texture, colour and increased level of reducing sugar [22].

The stability of enzyme at higher temperature carries high importance since in industrial processes it is a crucial character. The pectin renders thickness and cloudy appearance to juice which is reduced by the treatment with pectinases [23]. In addition to fruit juice treatment, thermostable pectinase in combination with xylanase may have application in bleaching of paper pulp [24].

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