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PRODUCTION AND PURIFICATION OF PECTINASE BY SOIL ISOLATE *PENICILLIUM* SP AND SEARCH FOR BETTER AGRO-RESIDUE FOR ITS SSF

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Abstract

Pectinase producing microorganisms were isolated from pectin industry waste using selective isolation technique. Among them, a potential culture was identified on morphological basis as *Penicillium* sp. It was found to produce significant amount of extracellular pectinase under submerged fermentation process. The type of enzyme produced was subsequently identified as polygalacturonase (PG). On partial optimization, culture showed maximum enzyme production at 35 ^{o}C in a medium having pH 6.0 containing 1.5% pectin on 72 h of incubation. The PG produced by the isolate was further purified by ammonium sulphate precipitation, size exclusion and ion exchange chromatography. The molecular weight of purified enzyme was determined to be 35 kDa by SDS-PAGE. Under optimized conditions, purified PG showed 98.66 U/ml activity which was almost 12 fold higher than crude. In view of cost effective pectinase production, substrate optimization using solid state fermentation was carried out amongst which orange bagasse gave 64.50 U/gm PG activity which was higher than other natural substrates.

Keywords: *Penicillium* sp., Pectinase, Polygalacturonase, Orange bagasse

Introduction

Pectin is important component of middle lamella and primary cell wall of higher plants. These are high molecular weight acid polysaccharide primary made up of α (1−4) linked D-galacturonic acid residues (Torres-Fanela et al, 2003).

The undesirable problem of spoilage and decay of the processed foods can be solved by treatment or use of efficient pectinases which can degrade homogalacturonan and rhamnogalacturonic acid of pectin to convert it in to sugar and other useful compounds. These pectinolytic enzymes catalyse the degradation of pectic substances which is of great industrial importance. Based on mode of action, pectinases were classified in to seven different classes as Pectinesterase (EC 3.1.1.11), Polygalacturonase (EC 3.2.1.15), Galacturan 1, 4 α galactouronidase (EC 3.2.1.67), Exo-poly α galactouronosidase (EC 3.2.1.82), Endo-pectatelyase (EC 4.2.2.2), Exo-pectatelyase (EC 4.2.2.9) and Endopectinylase (EC 4.2.2.10) (Koponen et al, 2008).

In industries, acidic pectinases are used during extraction and clarification of fruit juices. Whereas alkaliphilic pectinases are used immensely in the degumming of fibres, treatment of effluents discharged from fruit processing units. Although, the major sources of acidic pectinases are fungi; some reports have shown that the pectinases were also produced from some alkaliphilic bacteria (Patil and Dayanand, 2006). In the present study; an efficient PG enzyme producing strain was isolated and identified as *Penicillium* sp. on morphological basis. The conditions for submerged production of PG and its purification were partially optimized. Owing to the expensive medium ingredients, solid state fermentation (SSF) was tried using banana peel, wheat bran, sugar cane bagasse, orange bagasse as a carbon source separately and in different combinations in order to find out cheap and suitable natural source for production of this industrially important pectinase enzyme.

Materials and Methods

Isolation: In view of getting efficient pectin degrading cultures, soils rich in pectic waste and fruit waste samples were scrutinized including fruit processing area, sewage of juice centres of different locations, mud and pieces of long stored crop waste from pectin producing industry. For isolation of pectinase producing microorganisms pectin (Hi-Media, Mumbai) containing agar medium was used (Panda et al, 2004). Isolation was carried out in two steps comprising i) *enrichment* and ii) *isolation* as follows;

i) *Enrichment* - The 1gm of sample was inoculated in 100 ml of pectin broth (5% w/v yeast

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extract, 1 ml of 0.1% v/v bromothymol blue, 0.6 ml of 10% v/v $CaCl_2 \cdot 2H_2O$, pH 6.0) supplemented with 0.2% w/v pectin (Hi-media, Mumbai) and incubated on rotary shaker (120 rpm) (CIS 24 BL Remi, Mumbai) at 30 °C for 7 days. A 1ml 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 up to 2.0%, the soil sample was finally subjected for isolation of pectinase producers

ii) *Isolation*- After fifth week of enrichment, 0.1 ml of broth was spread on the surface of pectin gel containing basal agar medium plate and incubated at 30 ^oC.

Qualitative test for pectinase

Isolates were allowed to grow on a pectin plate to which, 1% cetyl trimethyl ammonium bromide (CTAB) was added to observe the zone of clearance which was indication of pectinase.

Screening of isolates

Quantitative estimation for pectinase production was carried out using all isolates obtained through selective enrichment as well as conventional isolation approach to draw comparative account.

Identification of isolate

On the basis of morphological identification, the culture was identified to genus level from National Fungal Culture Collection of India, Pune, India.

Confirmation of enzyme type

The polygalacturonase (PG) type of enzyme was determined by using DNSA method (Miller, 1959), pectin lyase (PL) by measuring the increase in A_{235} of the substrate solution (Albersheim & Killias, 1962) and pectin esterase (PE) using method described earlier by Wicker et al. (1987).

Partial optimization of culture conditions for enzyme production

The operating variables for fermentation were tried to optimize which included pH, temperature, time of incubation and substrate concentration. Optimal temperature and pH were obtained by varying the temperature range from 10 ^oC to 90 ^oC and pH 1.0 to pH 9.0. In addition, enzyme production was monitored from 24 to 120 h of incubation and substrate concentration was checked from 0.5 to 4.5%. The optimized culture conditions were used for *in vitro* enzyme production.

Enzyme production

The PG production was achieved by inoculating 10^4 spores/ml in fermentation medium supplemented with 1.5% of pectin followed by incubation at 35 ^oC for 72 h under submerged cultivation (Kashyap et al, 2000). After incubation, mycelial mass was separated by filtration and resulting supernatant was ultrafiltrated through polysulfone fibre cartridge (AMICON, Ireland) having cut-off of 10 kDa.

Purification and characterization of exo-polygalaturonase

The retenate was subjected to precipitation by ammonium sulphate starting from 30% up to 90% with 5% increase each time. The precipitate was removed and supernatant was again subjected with further addition of ammonium sulphate in view to remove other proteinaceous material. The resulting precipitate bearing high pectinase activity was dissolved in the small quantity of 0.01M Tris-HCl buffer (pH 6.0) and dialyzed against same buffer with constant stirring (Afifi and Foaad et al, 2002) and was subjected to further purification.

Gel filtration chromatography

The dialyzed enzyme fraction was further purified as per the standard method (Keller et al, 2006) with certain modifications. It was loaded on sephadex G-100 column (35×1.5 cm, bed volume 60 ml) and eluted with 0.01M Tris-HCl buffer (pH 6.0) with the flow rate of 20 ml/h. Total 88 fractions of 3 ml each were subsequently collected and its protein content was measured by taking A_{280} on spectrophotometer (UV-VIS 1601 Shimadzu, Japan). The fractions bearing high A_{280} were collected and evaluated for its PG activity. The fractions showing higher enzyme activity were pulled together for further characterization.

Ion exchange chromatography

The carboxymethyl cellulose (CMC) was packed in to a glass column (15× 0.55 cm, 10 ml bed volume) and equilibrated with 0.01 mM Tris-HCl buffer (pH 6.0). The concentration gradient of 0.01 to 0.05% sodium chloride was used for elution of protein on a cation exchanger resin with a flow rate of 20 ml/h using 0.01 mM Tris-HCl buffer (pH 6.0). The significant 5 fractions were collected and its protein content and enzyme activity were determined as per the method described earlier. The significant fractions were pooled together and dialyzed against 0.01 mM Tris-HCl buffer (pH 6.0).

The purified enzyme was lyophilized (3.3 L VirTis, USA) and stored in deep freezer at –20 °C for further characterization.

SDS-PAGE

The purity of enzyme was checked by using SDS-PAGE. The standard protein marker and purified enzyme were allowed to run simultaneously for determination of molecular weight. The protein bands on gel were visualized by staining it with coomassie brilliant blue.

Search for cheaper substrate for PG production by SSF

Different agro-residues comprising waste and by-products such as banana peel, wheat bran, sugar cane bagasse, orange bagasse were used as a carbon source separately or in combinations for SSF (Silva et al., 2002).Before using these substrates for enzyme production, pre-treatment was given considering the type of materials and complexity of carbohydrate present in it. All the dried material was sieved through 2 mm mesh to get uniformity. Those materials were as follows;

a) Banana peel - dried and ground
b) Wheat bran- dried and used untreated
c) Sugarcane bagasse - ground, sieved and dried at 80 °C
d) Orange bagasse- pressed pulp and peel was ground, dried at 80 °C

In addition, combinations (1:1) used were;

e) Sugarcane bagasse and wheat bran
f) Sugar cane bagasse and orange bagasse
g) Sugar cane bagasse and banana peel
h) Orange bagasse and wheat bran

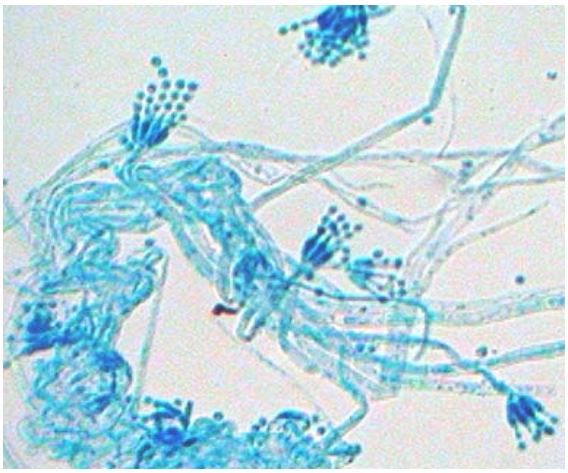
The 5 gm of pre-sterilized substrate was fortified with 10 ml of sterile micronutrients solution to which 10 ml aliquots of 10^7 spores/gm was inoculated and incubated at 35 °C for 7 days. After incubation, extraction was carried out by adding 10 ml sterile distilled water in 1gm of fermented solids followed by vigorous shaking, filtration and centrifugation to get crude enzyme in supernatant. PG activity of crude enzyme extract was determined by using DNSA method (Miller, 1959).

Results and Discussion

Isolation, screening and identification of organisms

Total five cultures were selectively isolated by enrichment method. Out of five three were fungi and other two were bacterium and yeast species. On the basis of prominent zone of clearance on pectin agar plate strain C1 was selected for further study. The morphological studies identified the strain as *Penicillium* sp., as shown in Figure 1.

Figure 1: Microscopic structure of *Penicillium* sp.



The culture filtrate was used as crude enzyme extract of isolate C1 that showed maximum of 8.39 U/ml of crude pectinase activity which was the highest (Table 1) when compared with others; hence used throughout this study.

Table 1: Enzyme activities of crude enzyme extract of isolates

Sr. No.	Code No.	Name of isolate	Enzyme activity (U/ml)
1	C1	*Penicillium* sp	8.39
2	D2	*Fusarium* sp.	2.11
3	A3	*Rhizopus* sp.	0.73
4	A9	Bacterial isolate	0.60
5	B6	Yeast isolate	0.74

Confirmation of enzyme type

Penicillium sp. showed predominance of PG type of extracellular enzyme while other types were minor.

Determination of culture condition for enzyme production

The PG production ability of the isolate was further searched for better conditions giving maximum production. This showed maximum activity at pH 6.0 in 1.5% of pectin containing liquid medium at 72 h of incubation at 35 °C as shown in Figure 2, 3, 4, and 5

Figure 2: Effect of pH on PG production

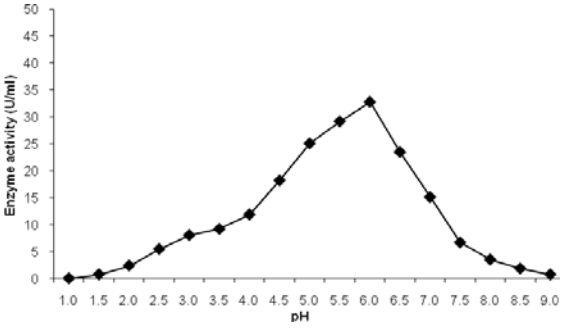


Figure 3: Effect of substrate concentration on PG production

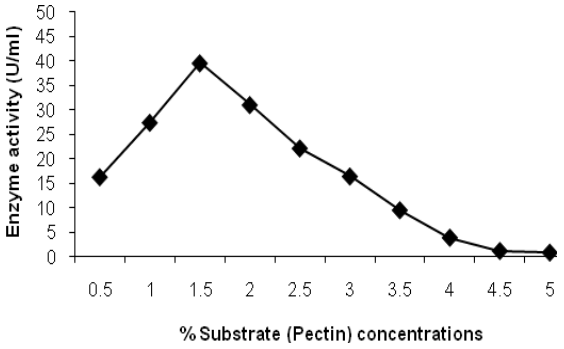


Figure 4: Effect of incubation time on PG production

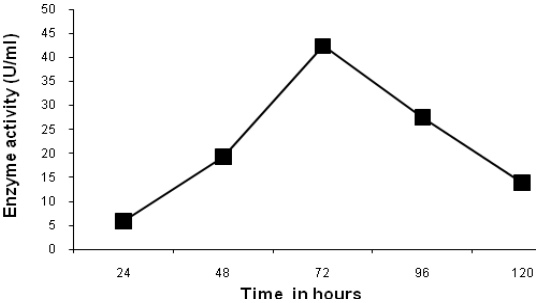
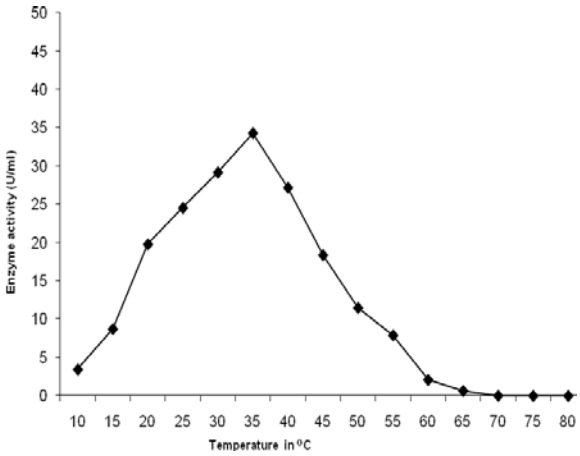


Figure 5: Effect of temperature on PG production



Purification of enzyme

Cell supernatant saturated with 60% ammonium sulphate, obtained for crude enzyme as shown in Figure 6. Elution profile of the crude enzyme subjected to gel filtration on sephadex G-100 column chromatography is shown in Figure 7. The fractions from 10 to 22 exhibited high PG activity. This removed many of the other contaminating proteinaceous impurities. Gradient of 0.05 M NaCl on CMC column using 0.01 mM Tris-HCl buffer (pH 6.0) gave more precise separation of PG enzyme.

Figure 6: Ammonium sulphate precipitation of crude PG

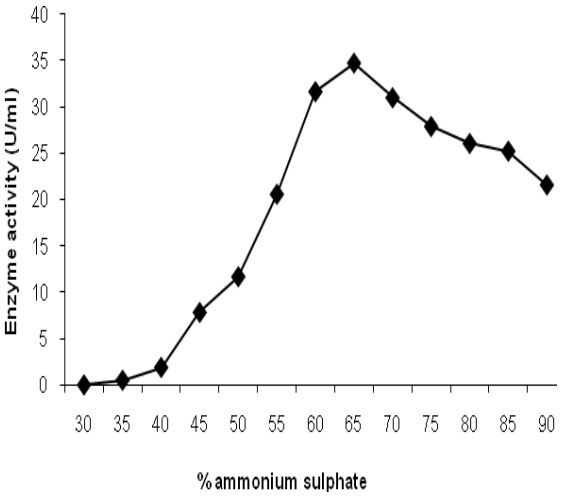


Figure 7: A typical elution profile of PG on Sephadex G-100 chromatography

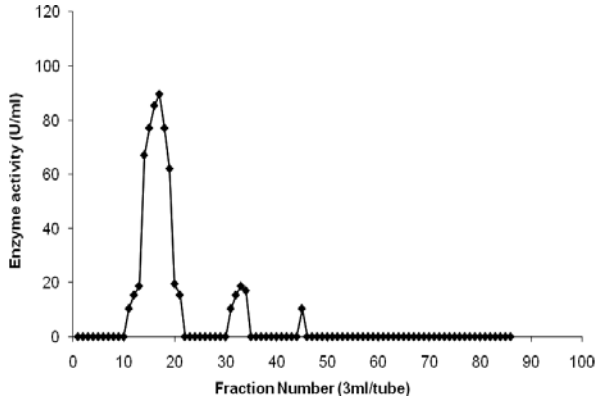
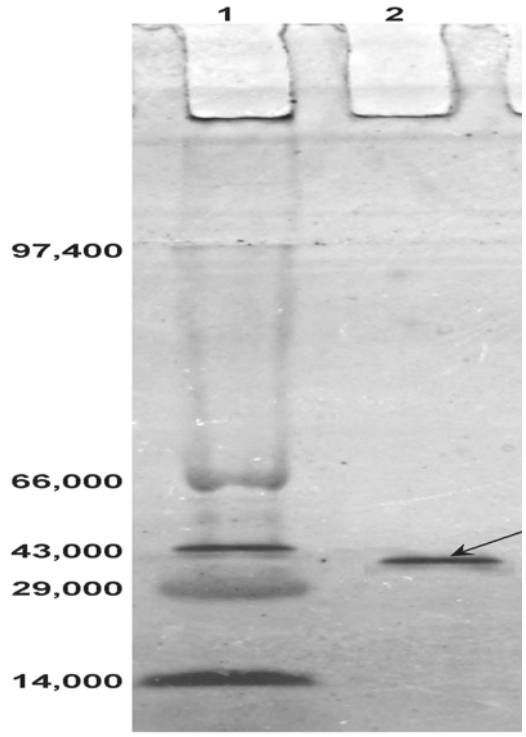


Figure 8: SDS-PAGE of purified PG of *Penicillium* sp.



Lane No. 1: Standard protein molecular weight marker;
Lane No 2: Purified enzyme of *Penicillium* sp.

SDS-PAGE

The purified PG exhibited a single band on SDS-PAGE. On comparing it with electrophoretic mobilities of standard molecular weight markers, it showed molecular weight of 35.022 kDa as shown in Figure 8.

The polygalacturorase activities of isolate C1 at different stages of purification was summarized in Table2

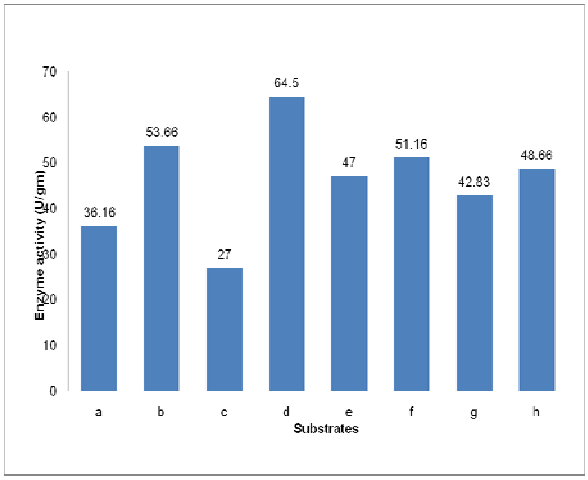
Table 2: Activities of the pectinase at different purification steps

Sr. No	Purification steps	Enzyme activity (U/ml)
1.	Crude enzyme extract (culture free supernatant)	8.39
2.	Gel filtration	89.50
3.	Ion-exchange chromatography	98.66

SSF

Since agricultural residues are attractive due to its low cost and abundant availability, the ability of isolate *Penicillium* sp. was tested for the production of the enzyme PG and utilization of agro-waste. Results of use of different agricultural waste and by-products as a sole carbon source for the production of industrially important enzyme are summarized in the Figure 9 given below;

Figure 9: Production of PG using agricultural wastes and by-products by SSF



a) Banana peel, b) Wheat bran, c) Sugar cane bagasse, d) Orange bagasse, e) Sugar cane bagasse and wheat bran (1:1), f) Sugar cane bagasse and orange bagasse (1:1), g) Sugar cane bagasse and banana peel (1:1), h) Orange bagasse and wheat bran

The various parameters for enzyme production by submerge fermentation were studied *in vitro* (Pereira et al, 2002). The optimum pH for PG production was found to be 6.0 while 1.5% was the substrate concentration which yielded 98.66 U/ml activity.

In concern with the use of various agricultural waste and agro-industrial by-products, in the present study suggested that orange bagasse found to be the best substrate for PG production by *Penicillium* sp. The Similar studies were also carried out by Silva et al., (2002) where orange bagasse and wheat bran gave higher yields of PG by the culture *P. viridicatum* RFC3. In order to use enzyme from the isolates for commercial application, it must have desirable biochemical, physio-chemical characteristics and low cost of production. Orange bagasse is very cheap, abundantly available and could be easily stored after sun drying. This waste is generated after the extraction of juice and available in high quantity from fruit processing industries, but has a limitation of availability in only particular season. Its dumping in nature causes pollution problems; hence its eco-friendly utilization is essential which tempted to use agro waste for pectinase production by solid state fermentation (Afifi and Foaad et al, 2002).

The production and purification of industrially important enzyme was the prerequisites for its successful biotechnological applications. The exploitation of pectinases, mainly exo-PG have been well established in variety of fruit juice and wine processing industries to increase the 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 increases level of reducing sugar (Vlugt et al, 2000). In this context, viscometric studies as described earlier (Gusakov et al, 2002) may be carried out for enzymatic fruit juice treatment which may justify effective application of enzyme preparation. Further application of enzyme alone or in combination with other enzyme like cellulase, hemicellulase and amylase may increase its applicability in many other applications.

Conclusion

In present study, attempt was made to isolate potential pectinase producing strains from the potent sites. Among all isolates, strain *Penicillium* sp. showed highest activity and predominance of exo-PG enzyme having molecular weight 35.022 kDa. The strain showed maximum pectinase activity at pH 6.0, temperature 35 ^{o}C, and substrate concentration of 1.5% for 72 h. These optimized conditions were moderate and can easily be applied for the treatment of juice without altering its quality. Furthermore, *Penicillium* sp. was found to utilize agricultural waste and by-products for enzyme production. Among the tested substrates orange bagasse was found to be the best substrate for PG production. This study has potential of utilizing agricultural waste provides cost effective and eco-friendly method for pectinase production on large scale.

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