Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production

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Abstract Pectin rich wastes and waste dump yard soils were screened and eighty pectinolytic fungal isolates were obtained by enrichment culturing and ruthenium red plate assay. Eight isolates with higher zones of pectin hydrolysis were selected and tested for polygalacturonase production. One isolate identified as *Aspergillus awamori* MTCC 9166 with highest polygalacturonase activity was tested for utilization of raw pectins for enzyme production. Polygalacturonase production was high in raw pectin sources like Orange peel (16.8 U/ml) Jack fruit rind (38 U/ml) Carrot peel (36U/ml) and Beet root peel (24U/ml). Selected *Aspergillus awamori* MTCC 9166 was found to be having good polygalacturonase, xylanase, cellulase and weak amylase and protease activities. This isolate with multi-enzyme production could have application for enzymes production and degradation of fruit and vegetable waste in the process of urban waste disposal.

Keywords *Aspergillus awamori* · Polygalacturonase · Pectin · Multienzyme production

Introduction

Pectinases are depolymerizing enzymes that degrade pectic substances present in middle lamella and primary cell walls of plant tissues [1]. They have significant commercial value with a share of 25% in global sales of food enzymes [2]. They are also industrially very significant with their all embracing applications in clarification of fruit juices, wines [3, 4], extraction of essential oils, flavors and pigments from plant materials [5], coffee and tea fermentations, retting of jute fibers, degumming of plant bast fibers, waste water treatment [3] and purification of plant viruses [5]. Diverse sources for pectinolytic isolates in nature are peels or rinds of fruits, rotten vegetables and their dumpyards [6]. Pectinases produced by different microbes are divided into depolymerizing enzymes and saponifying enzymes. Depolymerizing enzymes are poly-methylgalacturonases, pectin lyases, polygalacturonases and pectate lyases and saponifying enzymes are pectinesterases [7]. Polygalacturonases are mostly obtained from fungal isolates, and have significant role in fruit processing and juice industry. Fungal polygalacturonases are very significant for clarification of fruit juices as their optimal pH is closer to that of many fruit juices. Fungal enzymes with high stability at mesophilic or thermophilic temperature are preferred as they are both cost effective and recyclable [8]. In the present study, polygalacturonase producer having high enzyme activity and stability with cellulase and xylanase activities is reported.

Materials and methods

Screening and selection of pectinolytic organism: Diverse pectin rich sources and soils like vegetable market soil,

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vegetable waste dump yard, fruit market soil, fruit waste dump yards, sugar cane bagasse dump soil, various fruit and vegetable peels were screened for pectinolytic isolates. These were collected in sterile polythene bags. Serial diluted samples were inoculated on Czapek agar plates enriched with pectin and a pH of 5.2. Plates were incubated at room temperature for 72 h. Plates with fungal colonies were screened for pectinolytic isolates using ruthenium red plate assay [9]. Colonies were flooded with 0.5 ml of 0.02% ruthenium red solution, incubated for 1 h at room temperature and washed to remove unbound ruthenium. Positive pectinolytic isolates were detected based on clear zones of hydrolysis of pectin as indicated by colourless zones around the colonies. Eight colonies with larger zones of pectin hydrolysis were subcultured, identified morphologically and studied for polygalacturonase production in liquid medium using commercial pectin as substrate (Citrus peel pectin, SD fine chemicals).

Enzyme production: Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml Czapek broth with 1% commercial pectin. The flasks were inoculated with 1×10^6 spores/ml. The spore number was estimated by direct microscopic count using haemocytometer. Flasks were incubated for 5 days at 27°C in orbital shaker incubator at 200 rpm. Broth samples were collected at every 12 h and assayed for the enzyme activity. One isolate identified as *Aspergillus awamori* MTCC 9166 having more enzyme activity was selected and studied for enzyme production in pectin medium for a period of 120 h.

Substrates for pectinase enzyme production: Submerged fermentation studies were done with *Aspergillus awamori* MTCC 9166 for production of polygalacturonase using raw pectin substrates in Czapek broth under similar conditions as explained earlier.

Pectin substrates were prepared from four vegetable sources and four fruit sources. The vegetable sources were peels of carrot, beetroot, bottle-gourd and tomato pulp. The fruit sources were peels of orange, pineapple, jack fruit rind and pomace from apple preparation. These were thoroughly washed in tap water to remove soluble sugars and dried in hot air oven at 60°C to constant weight. Dried materials were powdered and used as source of pectin. The pectin content of these was determined by carbazole method [10]. The selected isolate was subjected to submerged fermentation studies using raw pectin sources for polygalacturonase production for a period of 5 days [11].

Other enzymes production: The isolate *Aspergillus awamori* MTCC 9166 was further tested for its multi-enzyme production property. The enzymes studied were xylanase, cellulase, amylase and protease using appropriate substrate at 1% concentration in fermentation medium.

Enzyme assays: One ml of culture broth was cold centrifuged at 4°C, 5000 rpm for 10 min. Supernatant was taken as enzyme source.

Polygalacturonase: The enzyme was assayed by measuring the D-galacturonic acid released from polygalacturonic acid as substrate [11, 12]. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μ M of galacturonic acid per minute at 50°C [11, 13].

Xylanase: The enzyme was assayed by measuring the xylose liberated from xylan by Miller's method [14]. One unit of xylanase activity is defined as the amount of enzyme required to liberate 1 μ M of xylose per minute.

Amylase: The enzyme was assayed using 1% soluble starch as substrate. The reducing sugars liberated on incubation of enzyme with substrate were determined by Miller's method [14]. One unit of amylase activity is defined as the amount of enzyme required to liberate 1 μ M of reducing sugars (glucose equivalents) per minute.

Cellulase: The enzyme was assayed using filter paper (Whatman No. 1) as substrate [15]. The activity measures the capacity of an enzyme preparation to hydrolyze filter paper strip into reducing sugars. The reducing sugars (glucose equivalents) are measured by Miller's method [14]. One unit of activity is defined as the amount of enzyme required to liberate 1 μ M of reducing sugars per minute. Xylanase, amylase and cellulase were assayed at 50°C.

Protease: The enzyme was assayed using casein as a substrate by Kuinz method [16]. The activity measures free tyrosine liberated on incubation of enzyme with substrate casein. One unit of enzyme which hydrolyzes casein to produce colour equivalent to 1 μ M of tyrosine per minute at pH 7.5 at 37°C.

Results and discussion

Eighty isolates were obtained by enrichment culture technique using 1% pectin. Pectin being predominant component of fruit and vegetable cell walls [17] promotes the growth of pectinolytic isolates hence good pectinolytic isolates were obtained from such samples like vegetable and fruit dump yards. Peels of fruits and vegetables were used as pectin source for screening. Pectinolytic isolates that depolymerized pectin produced colourless hydrolytic zones around colonies in ruthenium red plate assay. Fungal pectinolytic isolates having 1–1.5 cm zones of hydrolysis were considered as potential pectinase producers (Fig. 1).

Use of a chromogen like ruthenium red that specifically binds to free carboxyl groups in pectin giving a red colour and a colourless halo in absence of pectin was an efficient and significant screening strategy for pectinolytic isolates

[9]. With interest in only polygalacturonase production, the isolates were further subjected to screening for this enzyme. Among the primary isolates, three *Aspergillus* sp., two *Penicillium* sp., one *Fusarium* sp. and two *Saccharomyces* sp. identified morphologically were tested for polygalacturonase production by submerged fermentation (Fig. 2). Among the polygalacturonase producers, one fungal isolate further identified as *Aspergillus awamori* MTCC 9166 from fruit market soil showed high enzyme activity and was selected for further study as its enzyme production was comparatively higher and growth in broth demonstrated pellet like appearance.

Polygalacturonase enzyme production of the selected *Aspergillus awamori* MTCC 9166 was studied in submerged fermentation for five days at 200 rpm and 27°C and the results are presented in Fig. 3. Highest enzyme production was observed after 60 h. Fungal products are generally produced after 72 h [18], but the present enzyme was produced by 60 h. Shorter fermentation cycle is beneficial for any commercial strain hence the isolate could have potential application.

The selected isolate was further tested for its utilization of different fruit and vegetable raw pectins for polygalacturonase production. The raw pectin sources were selected based on literature survey [5]. The pectin content in selected sources was determined by carbazole method [10] and results are presented in Table 1. Different pectin substrates

were studied for polygalacturonase (PGU) production by *Aspergillus awamori* MTCC 9166 at 1% pectin concentration in fermentation medium. The results of PGU production with fruit pectins and vegetable pectins in comparison with commercial polygalacturonic acid are presented in Figs. 4 and 5 respectively. Maximum enzyme production was observed with Jack fruit rind (38 U/ml) followed by Orange peel (16.8 U/ml) (Fig. 4). Similarly vegetable pectin sources indicated highest enzyme production with carrot peel (36 U/ml) followed by beet root peel (24 U/ml) (Fig. 5).

The polygalacturonase production by *Aspergillus awamori* MTCC 9166 was compared with various other strains of *Aspergillus* [19–21] (Table 2). It was more than the reported strains and nearly 400 fold more when com-

Table 1 Pectin content in different raw pectin sources

Pectin source material	% of Pectin
Orange Peel	14.8
Pineapple Peel	9
Jack fruit rind	26
Apple pomace	1.8
Carrot Peel	16.5
Beet Root Peel	12
Bottle gourd Peel	6
Tomato pulp	3.5

Fig. 1 Pectinolytic fungal isolates from various pectin rich sources tested by ruthenium plate assay.

Fig. 2 Polygalacturonase activity of selected primary pectinolytic isolates Note: Asp 3 is identified as *Aspergillus awamori* MTCC 9166.

Fig. 3 Production profile of polygalacturonase by *Aspergillus awamori* MTCC 9166.

Table 2 Polygalacturonase production by different *Aspergillus* strains

<i>Aspergillus</i> spp.	Substrate	PGase activity U/ml	Reference
<i>Aspergillus niger</i>	Pectin	0.055	19
<i>Aspergillus giganteus</i>	Pectin	9.5	20
<i>Aspergillus niger</i>	Pectin	14.5	21
<i>Aspergillus awamori</i>	Pectin	0.046	22
<i>Aspergillus awamori</i> MTCC 9166	Pectin	17.8	Present study

pared to the reported strain of *Aspergillus awamori* [22]. Good enzyme production by the selected *Aspergillus awamori* MTCC 9166 was observed in the present study

with three substrates among the eight locally available raw pectin substrates. These are the substrates available locally at no cost as waste materials. Any commercial product like polygalacturonase produced by using such substrates is beneficial.

Multienzyme producing strains have ample application in biodegradation of plant biomass materials that accumulate and cause pollution in soils if not degraded. The plant biomass waste contains cellulose, hemicellulose, starch, pectin and protein substances in different proportions depending on the source material [23]. These strains also have ample application in fruit juice clarification [24]. The present selected isolate *Aspergillus awamori* MTCC 9166 was studied for multienzyme production like cellulase, xylanase, amylase and protease production using appropriate

Fig. 4 Polygalacturonase production by *Aspergillus awamori* MTCC 9166 using fruit pectins.

Fig. 5 Polygalacturonase production by *Aspergillus awamori* MTCC 9166 using raw vegetable pectins.

Fig. 6 Multienzyme production profile of *Aspergillus awamori* MTCC 9166.

substrates (Fig. 6). This isolate has good multienzyme activity with xylanase (20 U/ml), cellulase (15 U/ml) and less amylase (3 U/ml) and protease (4.4 U/ml) in addition to polygalacturonase (17.8 U/ml) as major enzyme.

An efficient pectinolytic organism, *Aspergillus awamori* MTCC 9166 was isolated by screening different source samples. The isolate showed nearly 400 fold more polygalacturonase production than the reported wild type strains [22, 25]. Enzyme production was good with cheap raw pectin substrates like jack fruit rind followed by citrus peel and carrot peel. It also produced other industrial enzymes relatively in good quantity. This isolate could be further exploited for improved polygalacturonase enzyme or multi enzyme production.

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