Abstract

In the present study *Phanerochaete chrysosporium* and *Aspergillus fumigatus* were screened for polygalacturonase production. The strains were grown in submerged fermentation and the most important physical parameters such as temperature and pH were optimized. The optimum temperature for maximum enzyme production was found to be 50 and 40 °C for *P. chrysosporium* and *A. fumigatus*, respectively. An optimum pH of 4.5 and 5.0 was recorded for *P. chrysosporium* and *A. fumigatus*, respectively. Under optimum condition the strain *P. chrysosporium* and *A. fumigatus* produced 1850 and 3218 units of enzyme per litre of culture broth, respectively. The polygalacturonase enzyme from the culture filtrates of both *P. chrysosporium* and *A. fumigatus* was purified to homogeneity. The purified fraction of polygalacturonase from *P. chrysosporium* and *A. fumigatus* was observed as single band. The molecular weights of purified enzyme from *P. chrysosporium* and *A. fumigatus* were 53 and 63 KDa, respectively. Overall the specific activities of polygalacturonases from *P. chrysosporium* and *A. fumigatus* were increased to 23.49 and 20.94 with a yield of 24.34 and 25.35, respectively.

Key words: *Aspergillus fumigatus*, Phanerochaete chrysosporium, polygalacturonase, optimization, purification.

Introduction

Polygalacturonases are one among the most important hydrolytic enzymes. Currently Polygalacturonases find application in industrial processes such as food, textile, paper and waste water treatment. In food industries, they are used for the extraction, filtrations and clarification of fruit juices (1) and they are also used to stabilize the cloud of citrus juices, purees and nectars. Pectinases are used to remove fibers containing gum in textile making. Bio technological degumming using pectinas in combination with xylanases present an ecofriendly and economic alternative to the chemical degumming treatment (2). In paper making, pectinases are employed in depolymerizing polymers of galacturonic acids, which subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (3). In the treatment of pectic waste water, pectinolytic enzymes, facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (2). Though polygalacturonase originate from different sources including plants, animals and microorganisms, industrially, they are mainly produced by employing microorganisms (4). The advantage of using micro-organism is due to their increased yield and stability. Though pectinas are produced from a wide variety of microbial source such as bacteria, fungi, yeast and actinomycetes (5, 6), among them the major producer is fungi. There are many reports available on polygalacturonase production from fungi belonging to genus *Phanerochaete* and Aspergillus (7, 8).
In view of the above, the present investigation is aimed for the production and purification of polygalacturonase using *P. chrysosporium* and *A. fumigatus*.

**Materials and Methods**

**Microorganism and culture maintenance:** *Phanerochaete chrysosporium* and *Aspergillus fumigatus* used in the present study were obtained from Mycology unit, Dept. of Botany, CAS (Centre for Advanced Studies), University of Madras. Both the strains were maintained at room temperature. The *P. chrysosporium* culture was grown on 2% (w/v) malt agar slants, whereas *A. fumigatus* was grown in yeast soluble starch agar medium containing (g/l): starch, 1.5; yeast extract, 0.4; K,HPO₄, 0.23; KH₂PO₄, 0.2; MgSO₄·7H₂O, 0.05; citric acid, 0.052 at pH 5.6.

**Inoculum preparation:** The fungal cultures were stored on potato dextrose agar (PDA) slants and incubated at 30 °C for 5 days. The sporulated slants were stored at 4 °C. The spores were harvested by adding 2 ml sterile saline containing 0.01% (w/v) Tween 20 to the sporulated slant and the spores were dislodged using the inoculation needle under aseptic condition. This suspension was then collected in a sterile container and used as inoculum source.

**Cultivation of the strains:** The *P. chrysosporium* and *A. fumigatus* strains were grown individually in 500 ml Erlenmeyer flasks containing 100 ml of sterile Hankins medium composed of (g/l): mineral salt solution, 1000 ml; yeast extract, 2; pectin, 10; agar agar, 30. Mineral salt solution contains (g/l): (NH₄)₂SO₄, 4; KH₂PO₄, 8; Na₂HPO₄, 12; FeSO₄·7H₂O, 0.4; CaCl₂, 0.02; H₂BO₃, 0.2; MnSO₄·0.2; ZnSO₄, 0.14; MoO₃, 0.2; 0.5% (v/v) of respective spore suspensions were used as a source of inoculum. The strains were incubated at 30 °C for 5 days.

**Screening of fungi for polygalacturonase production:** The organisms which produce polygalacturonase enzyme were screened by growing them on pectin (1.5% w/v) containing agar plates. At the end of incubation (5 days at 28 °C), the plates were flooded with 1% (w/v) cetrimide solution. Colonies producing polygalacturonase were exhibited with a clear zone around them.

**Submerged fermentation:** The submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing fermentation medium ( Hankin’s medium). The medium was sterilized by autoclaving at 121 °C for 15 min. The fermentation medium was inoculated with 0.5% (v/v) of respective spore suspension. The strains were incubated at 30 °C on rotatory shaker at 240 rpm for 10 days.

**Enzyme preparation:** At the end of incubation period, the broth was centrifuged at 10,000 rpm for 20 min in cooling centrifuge. The supernatant was used as a source of extra cellular enzyme.

**Enzyme activity:** Polygalacturonase activity was determined by measuring the release of reducing groups in the reaction mixture. The reaction mixture (3ml) consists of 0.8 ml of citrus pectin (1.0 % w/v) and 0.2 ml of appropriately diluted enzyme source in 2ml of sodium acetate buffer (0.1M, pH 5). The reaction mixture was incubated at 40 °C for 10 min (9), followed by addition of 1 ml of 1N NaOH and 1 ml of DNS to the tubes and the reaction was stopped by boiling the tubes in boiling water bath for 10 min, the reducing sugars released by enzymatic hydrolysis were determined (10). A separate blank was set up to correct the non-enzymatic release of sugar. One unit of polygalacturonase activity was defined as the amount of enzyme required to release one micromole of galacturonic acid per minute under the standard assay conditions.

**Protein estimation:** Protein content was determined by the method of Lowry et al., (11) using bovine serum albumin (BSA) as standard.

**Effect of temperature:** Broth was inoculated and incubated at different temperatures ranging from 20 to 60 °C for *P. chrysosporium* and 30 to 70 °C for *A. fumigatus*. 

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Effect of pH: The effect of initial pH of the medium and polygalacturonase production was studied by growing the culture at different pH range from 3.5 to 5.5 for *P. chrysosporium* and 4.0 to 6.0 for *A. fumigatus*. The pH of the medium was adjusted by using 0.1N H₂SO₄ or NaOH.

Purification of enzymes: The organisms were grown separately in culture medium at 30 ºC for 5 days. At the end of incubation, the broths from 2 different strains were centrifuged at 6000 rpm for 15 min at 4 ºC to obtain cell free filtrates. The cell free filtrate containing polygalacturonase from two strains were collected and their activities and protein content were determined as described above. 1000 ml of each of the above prepared cell free filtrate from the strains were transferred into two different flasks. Analytical grade solid ammonium sulphate was then added to the flasks at a saturation level of 40%. The flasks were incubated at 4 °C overnight. The precipitations formed from the two different flasks were collected by centrifugation (15,000 rpm, 20 min at 4 °C) dissolved in acetate buffer (50 mM, pH 5.0). The suspension was dialyzed overnight against the same buffer. The dialyzed enzyme was applied onto DEAE cellulose column (24×7 cm), previously equilibrated with acetate buffer (50 mM, pH 5.0) and eluted with gradient salt solution (0 to 0.5 M NaCl). The concentrations of protein in the fractions were measured by the absorbance at 280 nm and enzyme assay was carried out. The active fractions were pooled, and subjected to SDS-PAGE to determine the number of bands.

Results

Screening of polygalacturonase production: Cultivation of *P. chrysosporium* and *A. fumigatus* strains on pectin agar plates supplemented with 1.5 % (w/v) pectin showed clear cut halo forming zone confirms the pectinase production after flooding with cetrimide solution.

Optimization of process parameters

Temperature: *P. chrysosporium* and *A. fumigatus* produced enzyme at all temperature ranges (i.e. 20 to 60 ºC for *P. chrysosporium* and 30 to 70 ºC for *A. fumigatus*) and the optimum temperature for maximum enzyme production was found to be 50 and 40 ºC, respectively (Fig. 1 and 2).

![Fig. 1. Effect of temperature on polygalacturonase production by *P. chrysosporium*](image1.png)

![Fig. 2. Effect of temperature on polygalacturonase production by *A. fumigatus*](image2.png)

![Fig. 3. Effect of pH on polygalacturonase production by *P. chrysosporium*](image3.png)
pH. The effect of initial pH on polygalacturonase production is shown in Fig. 3 and 4. The optimum pH for maximum production of enzyme from *P. chrysosporium* and *A. fumigatus* was found to be 4.5 and 5, respectively (Fig. 3 and 4).

![Effect of pH on polygalacturonase production](image)

**Fig. 4.** Effect of pH on polygalacturonase production by *A. fumigatus*

**Purification:** When the organisms were grown individually in Hankin’s broth supplemented with 1.5 % (w/v) pectin the strain *P. chrysosporium* and *A. fumigatus* produced 1850 and 3218 units of enzyme per litre culture broth, respectively. After salting out the enzymes with ammonium sulphate, the centrifuged culture supernatant showed approximately 918 and 2109 units of polygalacturonase from *P. chrysosporium* and *A. fumigatus* per liter, respectively. There was an increase of specific activities from 5.92 to 21.29 and 7.64 to 37.39, respectively for enzymes from *P. chrysosporium* and *A. fumigatus*.

The enzyme from *P. chrysosporium* was purified about 23.49 fold with a specific activity of 139.09 IU/mg giving a yield of 24.34% after ion exchange chromatography which resulted in almost a single peak when absorbance was recorded at 280 nm. (Table 1 and Fig. 5). The purified polygalacturonase showed a single band on 12 % SDS-PAGE (Fig. 6). The molecular weight was found to be 53 kDa which indicated that it was novel enzyme from *P. chrysosporium*.

![Elution profile of polygalacturonase](image)

**Fig. 5.** Elution profile of polygalacturonase produced by *P.chrysosporium*.

**Table 1.** Purification of polygalacturonase produced extracellularly by *P.chrysosporium*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total Activity</th>
<th>Total Protein (mg)</th>
<th>Specific activity (IU/mg)</th>
<th>Fold (IU)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1000</td>
<td>1850</td>
<td>312</td>
<td>5.92</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>56</td>
<td>918</td>
<td>43.1</td>
<td>21.29</td>
<td>3.59</td>
<td>49.62</td>
</tr>
<tr>
<td>Ion chromatography</td>
<td>32</td>
<td>459</td>
<td>3.3</td>
<td>139.09</td>
<td>23.49</td>
<td>24.34</td>
</tr>
</tbody>
</table>

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The enzyme from *A. fumigatus* was purified about 20.94 fold with a specific activity of 160 IU/mg giving a yield of 25.35% after the chromatography which resulted in almost a single peak when absorbance was recorded at 280 nm (Table 2 and Fig. 6). The purified polygalacturonase showed a single band on 12% SDS PAGE (Fig. 7). The molecular weight was found to be 63kDa.

Lane 1 indicates Standard marker, Lane 2 and 3 indicates Crude extract, Lane 4 and 5 indicates partially purified enzyme by salt fractionation, Lane 6 and 7 indicates Purified polygalacturonase enzyme by ion exchange chromatography.

**Discussion**

The influence of temperature on polygalacturonase production is related to the growth of the organism. Most polygalacturonase production studies have been carried out with fungi. Optimum yields of polygalacturonase were achieved at 42 °C for *Mucor circinelloides* (12), 50 °C for *Penicillium chrysogenum* (13) and 37 °C for *Penicillium expansum* (14). In this study, the enzyme synthesis of *P. chrysosporium* and *A. fumigatus* occurred at a temperature range between 20 and 60 °C and 30 and 70 °C with an optimum of 40 and 50 °C, respectively. Fevela-
Tores et al., (15) described about the optimum temperature of polygalacturonase from *A. niger* has been established in the range of 36 to 45 ºC which is close to our finding.

Among the physical parameters tested the pH of the growth medium plays an important role by inducing morphological change observed during the growth of the organism and also affects product stability in the medium. Most of the *Aspergillus* and *Phanerochaete* strains used commercially for the production of polygalacturonase by SmF have an optimum pH of 4.0-5.0. Maximum enzyme production was achieved at pH 4.5 and 5 by *P. chrysosporium* and *A. fumigatus*, respectively in this study. The results are in accordance with polygalacturonase production by *A. niger*, SA6 (16), *P. griseoroseum* (17) and *P. viridicatum* (18). Piccoli-Valee et al., (17) observed high polygalacturonase activity by *P. griseoroseum* at acidic pH of 4.5 and 5. Same result has been given by Silva et al., (9) using *P. viridicatum* for polygalacturonase. In another study the optimum pH for polygalacturonase production by *Penicillium chrysogenum* was found to be 6.5 (13). In the present study, the enzyme from *P. chrysosporium* and *A. fumigatus* was precipitated up to 40 % saturation. Baumann (18) and Berger et al., (19) reported that polygalacturonase can be precipitated up to 90 % of ammonium sulphate depending on the source of the enzyme. Nithin Kumar and Bhushan (20) saturated the cell supernatant with 60 % ammonium sulphate. The purification of enzyme in this study was carried out with ion exchange chromatography. This purification method is similar to Alana et al., (21) who purified the enzyme from *P. italicum* on DEAE cellulose of ion exchange chromatography.

In the present study, the molecular weight of *P. chrysosporium* and *A. fumigatus* was found to be 63 and 53 KDa with 23.49 and 20.94 fold higher than crude respectively. Akhilesh Thakur et al., (12) purified polygalacturonase on Sephacryl S-100 gel filtration chromatography and obtained the molecular weight of 66 kDa on SDS-PAGE. (24) purified the enzyme from *Penicillium* sp. by ion exchange chromatography and obtained the molecular weight of 35 kDa. Jurick et al., (23) reported molecular weight of polygalacturonase from *Penicillium expansum* as 45 kDa.

In the present study, two strains *P. chrysosporium* and *A. fumigatus* were selected and screened for polygalacturonase production. The strains were grown in submerged fermentation and the most important physical parameters such as temperature and pH were optimized. Under optimum conditions, the strain *P. chrysosporium* and *A. fumigatus* produced 1850 and 3218 units of enzyme per litre of culture broth, respectively. The enzyme was purified by ammonium salt precipitation and ion exchange chromatography. The purified enzyme obtained from this study has a wide range of industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries etc.

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**Conflict of interest :** The authors declare that they are no competing interests.

**References**


Production of Polygalacturonase


