NOTE

The pectin lyases (PNLs) are the only known pectinases capable of degrading highly esterified pectin (like those found in fruits) into small molecules without previous action of other pectinases. On the other hand, polygalacturonase and pectinesterase act together to degrade the pectin molecule completely and liberate methanol as a by-product. Therefore, the PNLs are preferred in juice and wine industries, as they decrease the viscosity of fruit juice, without damaging the volatile ester content responsible of specific aroma of various fruit juices. These enzymes are also extensively used in the textile industry in degumming and retting of natural fibres (ramie, hemp, flax and jute), in treatment of pectic waste water produced by the citrus processing industries, in oil extraction and coffee and tea leaf fermentation. The major source of PNLs on industrial scale is the fungi belonging to the genus *Aspergillus*, *Penicillium* and *Fusarium* spp, though few bacterial PNLs are also reported.

Solid-state fermentation (SSF) has emerged as a preferred technology in industrial fermentation, as it offers several practical and economical advantages. Agro-wastes can be successfully utilized in the SSF. Nagpur is well known for mandarin orange (*Citrus reticulata* Blanco) cultivation and processing and agro-waste such as deoiled mandarin peel meal is readily available. Being rich in pectin, it has potential to be a good SSF medium for the pectic enzyme production. In the present study, we report the purification and characterization of PNL produced by the *Penicillium oxalicum* strain in solid-state culture medium containing mandarin peel meal.

**Materials and Methods**

Citrus pectin, DEAE-Sephadex A-50, Sephadex G-100 and molecular weight markers were purchased from Sigma Chemical Co., USA. Ampholines and ief markers were from Amersham Pharmacia, Sweden. Other chemicals and reagents were of AR grade. Deoiled mandarin peel meal was a gift from Nagpur Orange Growers Association (NOGA), Nagpur.

**Organism and inoculum**

*Penicillium oxalicum*, kindly provided by Plant Biochemistry Laboratory of the Department of Biochemistry, RTM Nagpur University, Nagpur.
found to produce significant amount of PNL in SSF, thus used in the present investigation. The culture was maintained on Czapek-Dox agar slants and sub-cultured every fortnight. Three days old culture was used for seeding the SSF medium. Inoculum was prepared by suspending spores in the sterile water and 1 ml of it containing about $1 \times 10^8$ spores was inoculated in each 250 ml conical flask containing 10 g of the solid medium.

**Solid-state fermentation**

The 10 g of solid medium containing 21% deoiled mandarin peel meal, 7.7% wheat bran, 0.3% ammonium sulfate and 71% moisture was inoculated with spores of *P. oxalicum* and was incubated at 26 ± 2°C for 5 days. The moldy bran was then homogenized with cold distilled water and the volume of the extract was made to 100 ml. The homogenate was filtered through nylon cloth and the filtrate was centrifuged at 6,000 × g for 15 min. The supernatant constituted the crude enzyme and was used for purification.

**Purification of pectin lyase**

All operations were carried out at 0-4°C, unless stated otherwise. The crude enzyme was saturated 75% with ammonium sulfate and after 30 min of standing centrifuged at 6,000 × g for 15 min. The pellet was dissolved in minimum volume of water and the resultant solution was dialyzed overnight against distilled water. The dialysate was again centrifuged at 6,000 × g for 15 min to remove any precipitate formed. The supernatant constituted the 75% ammonium sulfate fraction and was loaded on to a DEAE- Sephadex A-50 column (21 × 1.6 cm), equilibrated with 40 mM Tris-HCl buffer, pH 8.0. Proteins were eluted with a stepwise gradient of NaCl (0-1.0 M) in the Tris-HCl buffer (pH 8.0) at the flow rate of 0.4 ml/min. The 5 ml fractions were collected and assayed for PNL activity. A minor and a major peak of the enzyme activity were separated, suggesting a possible microheterogeneity (isoforms). Fractions belonging to the major peak were pooled, concentrated and loaded on to a column of Sephadex-G-100 (30 × 1.6 cm), equilibrated with 40 mM Tris-HCl buffer, pH 8.0. Elution was carried out with the same buffer and 3 ml fractions were collected at the rate of 0.2 ml/min. Again, the fractions showing the highest activity were pooled and concentrated. This constituted the purified enzyme.

**Enzyme assay**

PNL activity was assayed by the method described previously. Briefly, 2.7 ml of buffered substrate (0.25%w/v pectin in 0.1 M Tris-HCl buffer) (pH 8.0) was mixed with 0.3 ml enzyme. The mixture was incubated at 37°C in a water bath and absorbance was measured at 235 nm ($A_{235}$) at 0 time and after 1 h. One unit of enzyme activity was defined as that amount of the enzyme that increased the $A_{235}$ by one in 1 h under the given assay conditions. Specific activity was expressed as Units/mg protein. Proteins were determined by the method of Lowry using bovine serum albumin (BSA) as standard.

**Characterization of purified enzyme**

Homogeneity of the purified enzyme was determined by Native-PAGE, at pH 8.3 in 7% gel using Tris-HCl buffer as described. About 50 µg protein was loaded per lane at the constant current of 4 mA and staining was done with 2.5% Coomassie blue in 7% acetic acid and 4% methanol. Molecular mass of the enzyme was determined by SDS-PAGE in 7% gel as described. Molecular weight markers used were: BSA (66 kD), egg albumin (45 kD), pepsin (34.7 kD), trypsinogen (24 kD) and lysozyme (14.3 kD).

Isoelectric point of the enzyme was obtained by isoelectric focusing (Amersham-Pharmacia). Standards used were: amylglucosidase (pI 3.5), glucose oxidase (pI 4.3), β-lactoglobulin (pI 5.1), carbonic anhydrase (pI 5.9) and trypsin inhibitor (pI 4.8). Kinetic properties of the enzyme determined were: pH optimum (over pH range of 2.0-10.0), temperature optimum (over 10-80°C), pH stability (16 h at 10°C), temperature stability (10-80°C for 15 min) and $K_m$ (Lineweaver-Burke plot). The effect of phenolic compounds (tannic, caffeic, chlorogenic acid and ferulic acids) and metal ions (Ca$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Mo$^{4+}$, Ba$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Al$^{3+}$, Pb$^{2+}$, Mg$^{2+}$ and Ag$^{+}$) at 2 mM on the enzyme activity was also studied. The effect of phenolic compounds was studied since these compounds are found in the cell wall along with pectins.

**Results and Discussion**

As shown in Table 1, pectin lyase of *P. oxalicum* could be purified 46-fold by the present protocol, with about 3% yield. The purified preparation showed apparent homogeneity as determined by Native-PAGE (Fig. not shown). The purification and yield obtained in the present study was higher than the PNL produced by *Penicillium expansum* and comparable
to that produced by a soft rot fungi *Pythium splendens*\(^{19}\). Multiple forms of the enzyme were reported from several microorganisms\(^{20,21}\), though single PNL was also reported\(^3,18\). The PNL produced by *P. oxalicum* in the present study appeared to consist of more than one isoform, as indicated by DEAE-Sephadex A-50 chromatography (Fig. 1). The molecular mass of the PNL, as determined by SDS-PAGE was approximately 50 kD (Fig. 2), which was comparable to the range of molecular mass (22-52 kD) reported in literature\(^{22-26}\) for other PNLs.

The molecular and kinetic properties of the enzyme are shown in Table 2. The isoelectric point (*pI*) of the PNL was 5.0. Both acidic and basic *pI* values were reported for PNLs from different sources. A PNL showing a *pI* of 5.9 was reported from *Aspergillus niger*\(^{25}\), whereas pectinol R-10 lyase had shown a *pI* between 3.0 to 4.0\(^{27}\). The PNLs showing basic *pIs* included those obtained from *Pythium splendens* (*pI* 8.0)\(^{19}\), *Rhizoctonia solani* (*pI* 10.1)\(^{24}\), *Erwinia carotovora* (*pI* 9.4)\(^{28}\) and *Erwinia chrysanthem* (*pI* 9.4)\(^{29}\).

The enzyme showed the optimum *pH* of 8.0 and the *pH* stability was also highest at *pH* 8.0, although reasonably high enzyme activity was retained over the *pH* range of 5.0-9.0. Earlier, similar *pH* optima (8.0) and *pH* stability (8.0) was reported for the PNL produced by *Pythium splendens*\(^{19}\). However, varying *pH* optima and stability were reported for other PNLs produced by *Penicillium* spp. For example, an

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**Table 1**—Purification of pectin lyase produced by *Penicillium oxalicum* in solid-state fermentation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total (Units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg protein)</th>
<th>Purification fold</th>
<th>Recovery (% yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>2250</td>
<td>280</td>
<td>8.0</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate (0-75%)</td>
<td>1001</td>
<td>30.5</td>
<td>34.6</td>
<td>4.3</td>
<td>44.6</td>
</tr>
<tr>
<td>DEAE Sephadex A-50</td>
<td>171</td>
<td>1.56</td>
<td>109.6</td>
<td>13.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>66.0</td>
<td>0.18</td>
<td>366</td>
<td>45.8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Table 2**—Molecular and kinetic properties of purified pectin lyase produced by *P. oxalicum* in solid-state fermentation

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneity (Native-PAGE at <em>pH</em> 8.3)</td>
<td>Apparently homogeneous</td>
</tr>
<tr>
<td>MW (SDS-PAGE)</td>
<td>50 kD</td>
</tr>
<tr>
<td>Isoelectric <em>pH</em> (<em>ief</em>)</td>
<td>5.0</td>
</tr>
<tr>
<td><em>pH</em> Optimum</td>
<td>8.0 (Significantly active at <em>pH</em> 4.0-8.5)</td>
</tr>
<tr>
<td>Temp. optimum</td>
<td>40-50°C</td>
</tr>
<tr>
<td><em>pH</em> Stability (16 h at 10°C)</td>
<td>8.0</td>
</tr>
<tr>
<td>Temp stability (15 min)</td>
<td>Upto 50°C</td>
</tr>
<tr>
<td><em>K</em>(_m)</td>
<td>1.1 mg/ml citrus pectin</td>
</tr>
<tr>
<td><em>V</em>(_{max})</td>
<td>416 U/mg protein</td>
</tr>
<tr>
<td>Effect of metal ions (2 mM)</td>
<td>Completely inhibited by Mo(^{4+}), Pb(^{2+}), Ag(^{+})</td>
</tr>
<tr>
<td></td>
<td>No effect by Ca(^{2+}), Ba(^{2+}), Mn(^{2+}) and Mg(^{2+})</td>
</tr>
<tr>
<td>Effect of phenolics (2 mM)</td>
<td>Inhibition in decreasing order of: tannic, caffeic, ferulic, and chlorogenic acids</td>
</tr>
</tbody>
</table>

Fig. 1—Sephadex A-50 (A) and Sephadex G-100 (B) column chromatography of PNL produced by *Penicillium oxalicum* in solid-state fermentation ([•], PNL activity; and (••), proteins)
optimum pH of 6.0-7.0 and maximum stability at pH 8.0 for PNL from *P. italicum*\(^22\), an optimum pH of 7.0 and instability at alkaline pH for PNL from *P. expansum*\(^18\), and an optimum value of pH as high as 10.5 and maximum stability in a range of 3.5-4.5 for PNL from *P. veridicatum* RFC\(^32\) were reported.

The purified enzyme exhibited optimum activity at temperatures between 40-50°C. Similar temperature optima were reported for other fungal PNLs\(^{19,33}\). The purified enzyme was comparatively stable at temperatures up to 50°C, when exposed to different temperatures for 15 min, but the activity decreased rapidly at temperatures higher than thereafter. PNLs from *P. adamatezii*, *P. citrinum* and *P. janthinellum* were stable for 60 min at 40°C, but were completely inactivated within 15 min at 60°C\(^33\). The PNL from *Pythium splendens* was stable for at least 30 min at temperature up to 50°C\(^19\).

The \(K_m\) of the purified PNL was 1.1 mg/ml of pectin and \(V_{max}\) was found to be 416 U/mg protein. Most of the microbial PNLs had shown higher \(K_m\) values (low affinity), as compared to this enzyme. For example, PNL from *P. italicum* and *P. expansum* showed a \(K_m\) of 3.2\(^22\) and 9 mg/ml\(^18\) respectively; \(V_{max}\) of *P. expansum* 105 U/mg protein. Thus, the PNL from *P. oxalicum* had shown a higher affinity for citrus pectin, as well as a higher catalytic rate constant.

The enzyme was not activated by Ca\(^{2+}\), while Mo\(^{4+}\), Pb\(^{2+}\) and Ag\(^+\) completely inhibited the enzyme activity. Ba\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) did not seem to significantly affect the enzyme activity. Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\) at 5 mM did not affect the PNL activity of *P. italicum* and *P. paixilli*\(^22,34\). On the other hand, although mono and divalent cations including Ca\(^{2+}\) at 2.5 mM enhanced the PNL activity in *P. citrinum* culture, but the activity was inhibited with four-fold increase in Ca\(^{2+}\) concentration.\(^35\) Tannic acid completely inhibited the purified PNL at 2 mM, while chlorogenic, ferulic and caffeic acids were also inhibitory in the decreasing order. Polygalacturonases produced by *Rhizopus arrhizus* were strongly inhibited by tannic acid, as compared to other phenolic compounds.\(^36\)

In conclusion, a high-affinity pectin lyase was purified from *P. oxalicum* in solid-state fermentation utilizing deoiled mandarin peel meal.

Acknowledgements

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References

NOTES

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