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Size exclusion chromatography for the removal of pigments from extracellular ligninolytic enzyme extracts from decayed wheat straw

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Abstract

Solid-state fermentation of wheat straw was carried out by a native white rot basidiomycete *Daedaleopsis flavida* strain 5A. Extract prepared from the 12-day decayed wheat straw contained extracellular ligninolytic enzymes like manganese peroxidase (MnP), manganese-independent peroxidase (MIP), lignin peroxidase (LiP) and laccase along with straw-degraded products and pigments. Sephacryl S-200 size exclusion chromatography in 16/100 column was used for the separation of these ligninolytic enzymes and straw-degraded products and pigments. Recovery of pigment-free ligninolytic enzyme activities as protein was 40% of the total proteins loaded and specific LiP activity increased 34 fold after size exclusion chromatography. Thus accurate estimation of LiP by veratryl alcohol oxidation assay was possible only after the removal of interfering pigments. The reproducibility of size exclusion chromatography is adjudged satisfactory from the consistent results obtained after seven repetitive uses of matrices.

Key words

Daedaleopsis flavida, Size exclusion chromatography, Extracellular ligninolytic enzymes, Lignin peroxidase, Pigments, Solid-state fermentation

Introduction

White rot basidiomycetes (WRB) form the only group of microorganisms known to mineralize lignin completely (Zabel and Morell, 1992) by their ligninolytic enzyme system (Datta *et al*., 1992; Eriksson *et al*., 1990; Glenn *et al*., 1983; Hammel and Moen, 1991; Hatakka, 1994; Hofrichter *et al*., 1999; Tien and Kirk, 1983; Wariishi *et al*., 1991). Most of these studies were carried out growing WRB in liquid media for the production of ligninolytic enzymes (Hatakka, 1994; Martinez *et al*., 1995; Waldner *et al*., 1988). There are few reports of production of ligninolytic enzymes by WRB during their growth on lignocellulose substrates. White rot basidiomycetes like *Ceriporiopsis subvermispora, Coriolopsis polyzona, Lentinus edodes, Nematoloma frowardii, Phanerochaete chrysosporium, Phlebia radiata, Pleurotus eryngii, Pleurotus ostreatus,* and *Trametes versicolor* are known to produce manganese peroxidases (MnP), manganese-independent peroxidases (MIP), lignin peroxidases (LiP), and /or laccases along with other ligninolytic enzymes during solid-state fermentation (SSF) of wheat straw (Burlat *et al*., 1998; Datta *et al*., 1992; Forrester *et al*., 1990; Hofrichter *et al*., 1998; Lang *et al*., 1996; Lobos *et al*., 1994; Vares *et al*., 1995; Vyas and Molitoris, 1995; Vyas *et al*., 1994).

Some of the obvious disadvantages in the development of liquid culture systems are the huge volume of the effluent generated daily and it does not reflect the natural living conditions of white rot fungi. Solid-state fermentation is defined as the fermentation of solids in the absence of free water, has the advantage of supporting the growth and metabolism of microorganisms under moisture conditions (Pandey, 2003). Solid-state fermentation of agro industry wastes has gained much attention in biotechnology, *e.g.* production of enzymes, as its use offers higher productivities at low production costs (Pandey *et al*., 2000). Ligninolytic enzymes produced from SSF system using agro-wastes

has been reported for its effective role in the degradation of synthetic dye (G´omez *et al.*, 2005; Shrivastava *et al.*, 2005). The advantages of SSF over liquid cultures are also reported (Murugesan *et al.*, 2007).

Directed approach in the researches towards biotechnological applications of ligninolytic enzymes produced by SSF and their involvement in the degradation of organopollutants is interesting as WRB exhibit different patterns of growth and enzyme production secreting complex enzymatic mixture. For instance, Ramazol brilliant blue R (RBBR) decolorizing peroxidase was purified from the culture filtrate of *Pleurotus ostreatus* growing in a liquid medium (Shin *et al.*, 1997) whereas Vyas and Molitoris (1995), have reported production of another extracellular H_2O_2-dependent RBBR decolorizing activity by *Pl. ostreatus* during SSF of wheat straw. Subdued interest shown in studying ligninolytic enzymes produced under solid-state conditions may be attributed to: (i) Simplicity in the experimental set-up of liquid cultures with easy purification steps, (ii) unpredictable nature of fungal physiology and secretion of enzymes in multiple forms (iso-enzymes?) during SSF and (iii) the fact that extracted straw pigments and lignocellulose-degradation products interfere strongly in isolation, analyses and characterization of enzyme(s) produced. The hurdles in studying enzymes from SSF are not limited to the above facts as conventional practice of concentrating proteins by ammonium sulphate, acetone, polyethylene glycol, polyethyleneimine, etc. inevitably concentrates pigments resulting in a dark brown enzyme-pigment cocktail. Accurate estimation of LiP using such cocktail by veratryl alcohol oxidation assay is nearly impossible (Hofrichter *et al.*, 1999; Vares *et al.*, 1995).

In the present study, we have demonstrated that using size exclusion chromatography (SEC), ligninolytic enzymes are separated from the interfering pigments and straw-degraded products present in the extracts prepared from the wheat straw decayed by native isolate *Daedaleopsis flavida* strain 5A. Additionally SEC allowed fractionation of the enzyme extracts resulting in increased specific activities of pigment-free MnP, LiP and MIP enzymes.

Materials and Methods

Microorganism: Fruit body of *Daedaleopsis flavida* growing on a dead stem, designated as strain 5A was collected from the wild of Dangs, India. Pure culture of *D. flavida* strain 5A was obtained by transferring a surface sterilized (distilled water, 0.01% $HgCl_2$, distilled water, 70% alcohol and distilled water) piece of fruit body in water agar plates and transferring mycelia sprouting on the fruit body to malt extract agar (MEA) plates with or without dye fast green (0.01%) serially. It was taxonomically identified as *Daedaleopsis flavida* in our laboratory (Roy and Mitra, 1983; Bakshi, 1971). Strain 5A was maintained on MEA slants at 5°C and was cultivated on MEA plates at 28°C prior to use.

Solid-state fermentation (SSF) and preparation of enzyme extract: SSF of wheat straw by strain 5A (pre-sterilized 5 g chopped wheat straw moistened with 10 ml of distilled water was inoculated with 10 culture disk of 8 mm diameter) till 18 days and the extraction of ligninolytic enzymes by buffer (0.1 M Na-phosphate (pH 6.5) + 1 M NaCl) were carried out as per the methods described earlier (Vyas *et al.*, 1994). Total proteins from harvest were precipitated by adding ammonium sulphate (80% saturation) to clear supernatant with constant stirring. Precipitates were collected by centrifugation and re-dissolved in a minimum volume of 50 mM Na-acetate buffer, pH 5.5 (buffer A). It was desalted and concentrated in a stirred cell concentrator (Pall Life Sciences, USA) using 10 kDa cut-off membrane with repeated addition of buffer A.

Enzyme activity: Ligninolytic activities like MnP, MIP and laccase were assayed (Vyas *et al.*,1994) with spectrophotometric measurement (Multispec 1501 PDA detector, Shimadzu, Japan) of oxidized blue colored product produced upon oxidation of 3-dimethylaminobenzoic acid and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (Lancaster, USA) as chromogen at 590 nm (ε_{590} = 32,900 $M^{-1}.cm^{-1}$). Lignin peroxidase activity was estimated as described earlier (Tien and Kirk, 1984) following veratryl alcohol (Lancaster, USA) oxidation at 310 nm (ε_{310} = 9,100 $M^{-1}cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that will produce 1 μmol of product min^{-1} upon oxidation of the substrates in reaction mixture.

Size exclusion chromatography (SEC): Ligninolytic enzymes were separated from the interfering pigments by SEC using Akta Prime protein purifying system (Amersham Pharmacia, Sweden). One ml of protein (2.37 mg)-pigment concentrate was loaded to Sephacryl S-200 column (XK 16 x 100; Amersham Pharmacia, Sweden) previously equilibrated with buffer A. Proteins were eluted with buffer A at the flow rate of 1 ml min^{-1} for 200 min monitoring A_{280} (protein) and A_{406} (heme). Fractions of 1 ml were collected, and active fractions were pooled, concentrated (10 kDa microconcentrator, Viva Science, UK) and stored at -20°C after enzyme and protein analyses. The peaks eluted from SEC were analyzed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described (Laemmli, 1970) and gel was stained by silver staining protocol (Dunn, 2002). Medium range protein molecular weight marker from Hi-media, India, was used for the calibration of gel. Protein was estimated according to Bradford, 1976.

Results and Discussion

Daedaleopsis flavida Strain 5A produced thick cottony white fluffy mycelia covering the entire wheat straw within 15 days. It produced MnP, MIP, LiP, laccase and other ligninolytic enzymes under given conditions. MnP activity was detectable even on the 2nd day of incubation and is secreted continuously during the growth. Pattern of MnP secretion by strain 5A is similar to that observed in most of the WRB (Vyas *et al.*, 1994). MIP and laccase appeared after sixth day when MnP activity subsides. LiP, the first enzyme shown to be involved in ligninolysis (Tien and Kirk, 1984) unlike MnP, was not detected in the cultures younger than 9 days of incubation. The amount of proteins present in the cultures of strain

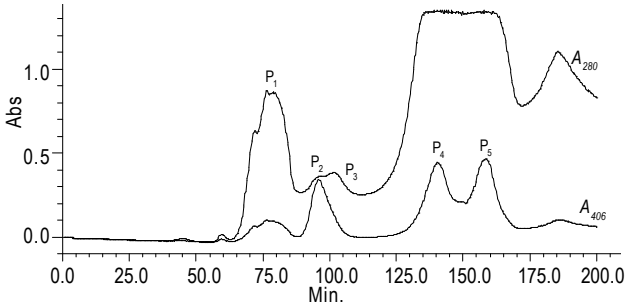

Fig. 1: Elution profile of size exclusion chromatography using Sephacryl S-200 matrices in XK 16 100^{-1} column (Flow rate: 1 ml min^{-1}; sample volume: 1 ml) showing separation of ligninolytic enzymes and pigments (P_1-P_5) from enzyme extract prepared from 12-day old cultures of *Daedaleopsis flavida* strain 5A grown on wheat straw

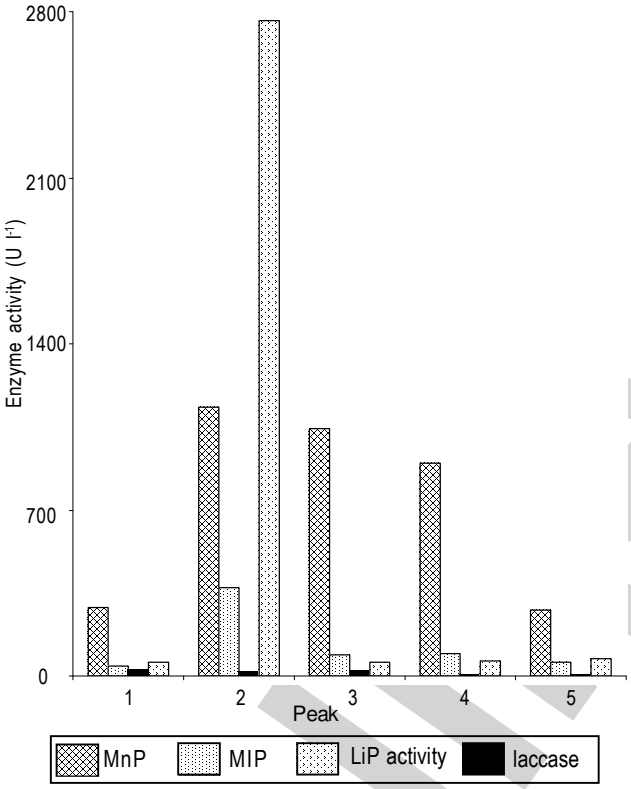

Fig. 2: Ligninolytic enzyme activity of peaks (P_1-P_5) eluted from size exclusion chromatography (SEC)

5A was observed to be more on 12th day (harvesting time) than at any other time during experiment. Straw extracted enzyme cocktail also contained mixture of pigments and straw degraded products that interfered in the detection and accurate estimation of LiP activity.

The concentrate obtained after ultrafiltration was dark brown in color as straw degraded products and pigments that precipitated with proteins also got concentrated. Use of polyethylenimine (Supelco, USA) in a final concentration of 0.4% (w/v) for the removal of pigments from enzyme extracts (Forrester *et al*., 1990) was not successful. Our results are similar with the study of Vares *et al*., 1995, where they did not find any significant effect of adding polyethylenimine for the removal of colored compounds from the liquid extracted from the straw infested by *Phlebia radiata*.

Size exclusion chromatography resolved 1 ml of concentrated dark brown enzyme extract into five distinct peaks showing absorbance at 280 and 406 nm (Fig. 1). The elution profile obtained was as a result of the column dimensions having narrow diameter-increased height and flow rate maintained during the process. Major activities of MnP, MIP, laccase and LiP were recovered as pigment free ligninolytic enzymes (proteins of 66 to 20 kDa) in peaks 2 and 3 (Fig. 2) whereas most of the pigments and straw degraded products having lower molecular mass were eluted in peaks 4 and 5 as observed in SDS-PAGE (Fig. 3). The bed length of matrices influenced the retention time of low molecular weight substances; with a bed length of 100 cms, the retention of interfering pigments was sufficiently long leading to its separation from ligninolytic enzymes which have higher molecular weight. Recovered proteins as pigment-free ligninolytic activities was equal to 40% of the total protein loaded. LiP activity eluted in peak 2, was estimated accurately by veratryl alcohol oxidation assay which could not be estimated appropriately before SEC. The purity of enzymes and effectiveness of SEC was unambiguous, evident by the increase in specific activities of ligninolytic enzymes after SEC (Table 1). Increase in specific LiP activity (ca. 34 fold) from 0.3 to 10.2 was due to the accurate measurement of LiP after the fractionation of enzyme extracts by SEC.

Sephacryl S-200 was repeatedly used for seven cycles after adequate washing with 1 M NaOH and equilibrating with buffer A prior to next cycle. Bound colored materials were removed completely during washing with 1 M NaOH. The reproducibility of SEC in the present study is adjudged satisfactory from the consistent results obtained from seven repeated cycles. The reproducibility of SEC is also reported by Nuber *et al.* 1990 for extraction of serum bile acids. They found that recovery of bile acids was 75-104% and reproducibility was satisfactory for SEC than the standard methods of adsorption and reversed-phase chromatography that yielded incomplete recovery (33-93%) with poor reproducibility. Reproducibility and repeatability of the results by SEC is also established by the work of Berek *et al.* 2001.

Estimation and purification of ligninolytic enzymes especially for lignin peroxidase was hindered by decayed wheat straw pigments and polyphenols while studying ligninolytic enzymes produced under SSF conditions (Forrester *et al*., 1990; Hofrichter *et al*., 1999). Vares *et al.,* 1995, have reported non-suitability of veratryl alcohol oxidation assay for the estimation of LiP activity from colored straw extract and have used Azure B dye assay (Archibald, 1992). They concluded that LiP activity in concentrates was undetectable by both the assay methods. They further reported that while purifying enzymes with anion chromatography, most of the colored compounds bound tightly with Q-Sepharose and were eluted from the column with increase in salt gradient over 0.16 M interfering the monitoring *of* A_{280} (protein) and A_{409} (heme). Similar conclusions were drawn by Hofrichter *et al*., 1999, for detecting and purifying LiP from wheat

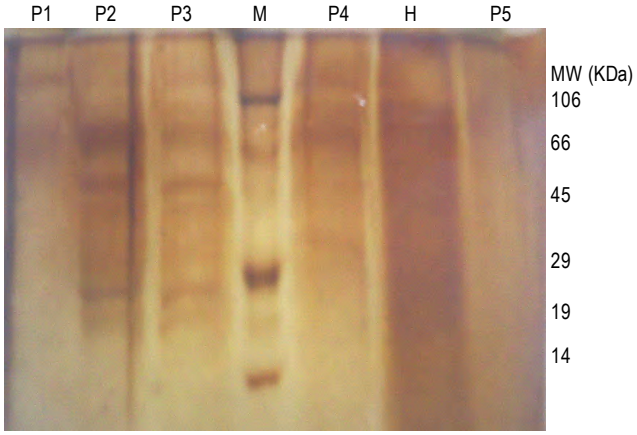

Fig. 3: SDS-PAGE analysis of peaks (P_1-P_5) harvest (H) and mwm (M) eluted by size exclusion chromatography (SEC) from enzyme extract prepared from 12-day old *Daedaleopsis flavida* strain 5A infested wheat straw. Molecular weight (kDa) of standard protein markers is indicated

Table 1: Differences in the specific activities of ligninolytic enzymes achieved after size exclusion chromatography (SEC) of wheat straw extract prepared from 12th day old *Daedaleopsis flavida* strain 5A infested wheat straw

Enzymes	Specific activity (U mg^{-1})	
	Before SEC	After SEC
MnP	13.2	17.9
LiP	0.3	10.2
MIP	2.8	4.2
Laccase	0.1	0.1

MnP = Manganese peroxidase, LiP = Lignin peroxidase, MIP = Manganese-independent proxidase, SEC = Size exclusion chromatograpgy

straw extract in their study of mineralizing ^{14}C-DHP by *Nematoloma frowardii*. To the best of our knowledge, this is the first report of using SEC in the chromatographic separation of pigments and ligninolytic enzymes prepared from decayed wheat straw extracts. Removal of interfering pigments using anion exchange matrices has been reported. Vyas *et al.,* (1993), presented a protocol for the removal of interfering pigments from wheat straw extract after binding them to Q-Sepharose in 0.2 M NaCl concentration at which enzymes remained unbound. But, the limitation of this protocol is one time use of expensive matrices due to the incomplete removal of bound colored pigments.

In the present study, we have shown that SEC repeated for seven times consistently offers better removal of interfering pigments for the preparation of pigment free ligninolytic enzymes and accurate estimation of lignin peroxidase.

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