Removal of tannin from cross-linked and open chain polymeric tannin substrates using heme peroxidases of *Phanerochaete chrysosporium*

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**Abstract** Removal of tannin from different tannin substrates using heme peroxidases of *Phanerochaete chrysosporium* was studied. Complete removal of tannin components in spent tan liquor was observed after 24 h of incubation with peroxidases. Tannin in aqueous media containing tannic acid and condensed tannin substrates were removed by 65.70±0.97 and 52.43±0.83%. Chemical oxygen demand was reduced by 24.38±0.73, 33.22±0.20, and 58.94±0.07%, respectively, in spent tan liquor, tannic acid and condensed tannin substrates containing aqueous media.

**Introduction**
Tannins, the polyphenolic compounds are used in leather manufacturing for the conversion of putrescible collagen fibers into non-putrescible leather. However, both the forms of tannins i.e., hydrolyzable and condensed tannins are found to be inhibitors to a broad spectrum of biological systems [1]. Amongst the different types of industries, the leather industry alone requires 90% of the global tannin production of which the Indian domestic market requirement was estimated to be about 75,000 million tons of vegetable tannin per annum and was estimated to increase by 0.10 to 0.12 billion tons in 2000 AD [2]. The unabsorbed tannin in spent tan liquor accounts for 50% or more of the chemical oxygen demand (COD) [3, 4, 5]. The residual tannins inhibit the activity of methanogenic bacteria in anaerobic systems used for treatment of wastewater discharged from tanning industries [6, 7]. The accumulated tannin in soil at 1–2% concentration retards the microbial degradation of organic materials discharged on open land for disposal [6, 8].

Catabolism of tannin by ligninolytic enzymes (ligninases; LiP and MnP) has long been left unattended. The structural similarity and the ability of ligninolytic enzymes to oxidize a wide variety of aromatic compounds [9] suggest that mineralization of tannins may be attempted by the application of heme peroxidases of *P. chrysosporium*. Moreover, culture conditions that accelerate lignin degradation by *P. chrysosporium* should also enhance the catabolism of the tannin molecule. Despite the considerable amount of information available on degradation of organic contaminants using ligninolytic enzymes, the literature available on mineralization of tannins is scarce to non-existent. The focal theme of this paper is on the production of heme peroxidases by *P. chrysosporium* and its application for removal of tannin offered in different molecular configurations in the aqueous phase.

**Materials and methods**

**Organism and culture conditions**

*Phanerochaete chrysosporium* NCIM 1197 was maintained on YMPG medium agar slants at 4°C and grown on plates for inoculum preparation using the same medium at 37°C for 6 days. Stationary culture (25 ml; 250-ml flask) was maintained as described previously by Alic et al. [10] and Perie and Gold [11]. The medium described previously (Kirk et al. [12], Perie and Gold [11]) was supplemented with glucose at 1 or 2% with 1.2 mM or 12 mM ammonium tartrate, yielding low carbon-low nitrogen (LCLN), low carbon-high nitrogen (LCHN), high carbon-low nitrogen (HCLN), and high carbon-high nitrogen (HCHN) concentrations to get the maximum ligninolytic activity. All the experiments were carried out in triplicate.

Spores were harvested by suspension in sterile water followed by filtration through sterile glass wool. Spore concentration was determined by measuring the absorbance of the culture fluid at 650 nm using UV-visible spectrophotometry.

**Peroxidases assay (LiP and MnP)**
The mycelial mat from the liquid culture medium was separated manually and the medium was filtered using sterile glass wool to remove the residual mycelial particles. This was followed by centrifugation at 10,000 rpm for 15 min. The crude culture filtrate with partial purification was used in our study. The culture filtrate may have conventional enzymes other than LiP and MnP. Activity of LiP and MnP were determined at different incubation periods. LiP was assayed as described by Tien and Kirk
[13, 14] with veratryl alcohol as substrate. MnP was assayed by the method of Paszczynski et al. [15] using vanillin acetone as substrate. Our study was centered around the action of LiP on the degradation of tannin despite the other conventional enzymes present in the extracellular fluid (filtrate). Activity was expressed in UL⁻¹ (one unit of enzyme oxidizes 1 μmol substrate per minute).

Substrates for degradation study
Tannic acid (open chain), condensed tannin (cross-linked chain) were obtained from commercial traders and the spent tan liquor (wash water) samples were collected from a tannery processing leather using wattle extract as the tanning agent. Initial tannin concentration of the samples was estimated in accordance with standard methods for examination of water and wastewater [16]. The following are the concentrations of the substrates considered for the study.

- Tannic acid: 398.12±5.29, 804.23±9.17, 1196.10±10.40 μg ml⁻¹
- Condensed tannin: 349.45±3.60, 700.67±5.00, 1043.48±7.63 μg ml⁻¹
- Spent tan liquor: 50.43±1.06, 101.84±1.72, 150.67±1.29 μg ml⁻¹

Extracellular enzyme extraction and partial purification were carried out by filtration followed by precipitation with saturated ammonium sulfate (85%–90%) and dialysis using 0.02 M sodium acetate buffer. The dialyzed extract was used in our further studies.

Removal of tannins by partially purified heme peroxidases was carried out by incubating the reaction mixture of enzyme extract and chosen tannin substrates at 37°C. After predetermined time intervals (24, 48 and 72 h), the pH of the samples were recorded. The peroxidase reaction on tannin substrates was arrested by the addition of 0.1 ml of 6 M HCl to the reaction mixture and it was analyzed for tannin and COD.

The efficiency of the enzyme for the removal of tannin in aqueous phase was assessed on the basis of change in pH of the reaction mixture, percentage removal of tannin and COD of the sample under study. The removal of tannin from a solution of predetermined concentration at varied peroxidase activities was carried out. Tannin removal per unit of peroxidase activity (ΔTan/LiP) in terms of LiP was calculated from the difference in concentration of tannin for the applied LiP activity.

Results and discussion
Ligninolytic activity of P. chrysosporium was observed to increase with increase in incubation period (Fig. 1). In this study, both the peroxidase activities (LiP and MnP) were found to be increased from the second day onwards. Maximum activity was attained on the fifth and sixth day respective to MnP and LiP in LCLN-supplemented medium. Both peroxidase activities were decreased after the eighth day. The maximum LiP activity of 799.60±0.56 UL⁻¹ during the sixth day and maximum MnP activity of 402.38±1.36 UL⁻¹ on the fifth day were attained.

Efficacy of peroxidase activity on the removal of tannin
All the three substrates were treated separately with different peroxidase activities obtained on days 4, 5 and 6. Peroxidase activity on day 4 (401.81±1.35 UL⁻¹) recorded only a marginal reduction of tannin in applied substrates compared with the activity of day 5 and day 6. At tannin concentration 398.12±5.29 μg×ml⁻¹ applied as tannic acid, the percentage removal was only 29.8±0.52% for peroxidase activity of 401.81±1.35 UL⁻¹ after 24 h and it was 40.58±0.30% and 47.23±0.36%, respectively, for 600.46±0.45 (day 5) and 799.60±0.56 UL⁻¹ (day 6) of peroxidase activity at the same incubation period (Fig. 2).

Tannin concentration of condensed tannin at 349.45±3.60 was reduced by 3.81±0.02% at day 4 peroxidase activity (401.81±1.35 UL⁻¹) and 52.68±0.27% at day 6 activity (799.60±0.56 UL⁻¹) after 24 h of incubation (Fig. 2).

Spent tan liquor samples have recorded the percentage reduction of 88.39±0.30% for 401.81±1.35 UL⁻¹ and 99.76±0.53% with both 600.46±0.45 and 799.60±0.56 UL⁻¹

![Fig. 1. Peroxidase activity (LiP and MnP) of P. chrysosporium with increasing incubation period with LCLN medium](image-url)