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Article

Decolorization of Triphenylmethane Dye by White-rot Fungi

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ABSTRACT

Decolorization of triphenylmethane dye (crystal violet) by white-rot fungi – *Stereum ostrea* and *Phanerochaete chrysosporium* was investigated in liquid medium for a period of 10 days under shaking conditions. *S. ostrea* exhibited maximum decolorization crystal violet 90% in comparison with *P. chrysosporium* that it was 67% at 0.01% concentration on the 8th day of incubation. *S. ostrea* dominates *P. chrysosporium* on the production of biomass and protein content. The fungal cultures were tested for the production of ligninolytic enzymes – laccase (Lcc), lignin peroxidase (LiP), and manganese peroxidase (MnP). Fungal cultures grew well in dye-containing medium and exhibited decolorization of crystal violet confirming the presence of ligninolytic enzymes. *S. ostrea* displayed maximum Lcc (49 U/ml), LiP (0.34 U/ml), and MnP (48 U/ml) against *P. chrysosporium*.

Key words: Decolorization, Triphenylmethane dye, Laccase, Manganese peroxidase, Lignin peroxidase, Biomass, Protein content.

1. INTRODUCTION

Nowadays, due to rapid industrialization and urbanization, the textile industry and its wastewaters increased proportionally making it one of the main sources of severe pollution problems. India's dye industry produces every type of dyes and pigments. Production of dyestuff and pigments is close to 80,000 tonnes per annum [1] (Mathur *et al.,* 2005). India is the second-largest exporter of dyestuffs and intermediates after China [1] (Mathur *et al*., 2005). Dyes have complex chemical structures, which make them persistent against microbial attack, chemicals, water, and light [2] (Saratale *et al*., 2011). This persistence makes them harmful in the environment. Crystal violet a triphenylmethane recalcitrant dye has been extensively used in veterinary and human medicine as a biological stain and as a textile dye in textile processing industry and remains in the environment for longer period [3]. It is toxic to aquatic and terrestrial life [4]. Crystal violet is a potent clastogenes, which is responsible for promoting tumor growth in some species of fish and also known as potent carcinogenic [4].

Due to the widespread use and potential carcinogenicity of certain dyes, there has been a growing interest in assessing the hazards associated with dyes available in local markets, and hence, decolorization of the dye-bearing effluents is of great importance [5]. Besides, conventional physicochemical methods including coagulation, flocculation, ion exchange, irradiation, precipitation, ozonation, and adsorption or a combination of these methods has been used for dye removal from wastewaters [4]. However, comparing to physicochemical methods, the enzymatic treatment of dyes has low-energy cost and is a more eco-friendly process not yet commonly used in the textile industries [6]. Therefore, interest is now focused on bacteria, fungi, yeasts, and algae for biodegradation and biosorption in aerobic, anaerobic, or combined treatment processes [5]. To overcome these drawbacks, over the past few decades, ligninolytic basidiomycetous fungi focused on the production of different combinations of extracellular oxidoreductive enzymes (laccases [Lcc], lignin peroxidases [LiP], manganese peroxidases (MnP), and versatile peroxidases), which can depolymerize lignin and recalcitrant xenobiotic synthetic compounds [7,8]. Some of the basidiomycetous fungi produce all these enzymes, while others produce only one or two of them [9]. In most cases, the capability of decolorizing the dyes is due to the activities of LiP [10], MnP [11], and Lcc [9,12]. Enzyme-based decolorization treatments are potentially advantageous to bioremediation technologists since the enzyme is produced in large amounts and is often produced constitutively. The aim of the study was to study the ability of fungi to grow in the presence of dyes for the process of dye decolorization and production of ligninolytic enzymes by the fungal cultures.

2. MATERIALS AND METHODS

2.1. Organisms

Stereum ostrea was kindly supplied by Prof. M.A. Singaracharya, Department of Microbiology, Kakatiya University, Andhra Pradesh, India, and was isolated from wood logs. *Phanerochaete chrysosporium* was obtained from IMTECH, India.

2.2. Media and Growth Conditions

The fungal cultures were maintained on Koroljova medium [13] (Koroljova-Skorobogat'Ko *et al.,* 1998) due to good growth [9]. The fungal cultures – *S. ostrea* and *P. chrysosporium* were maintained on medium containing the following composition (g/l): 3.0 peptone, 5.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$,

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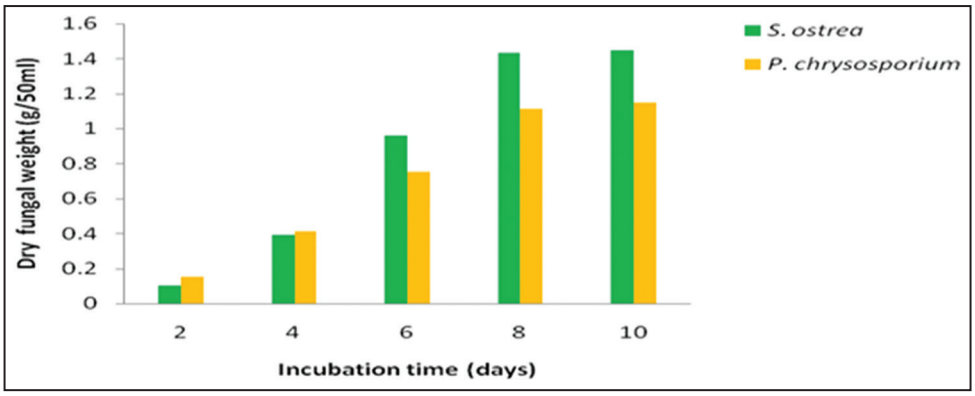

Figure 1: Growth of fungal cultures in crystal violet medium.

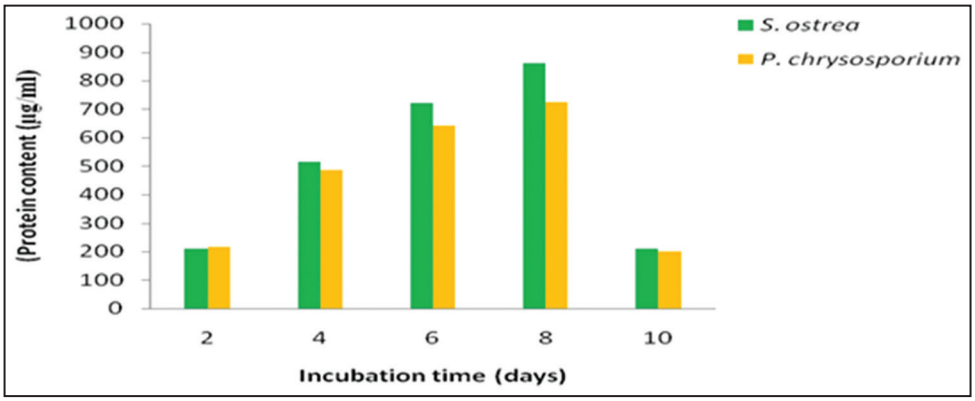

Figure 2: Secretion of extracellular protein content of the fungal cultures.

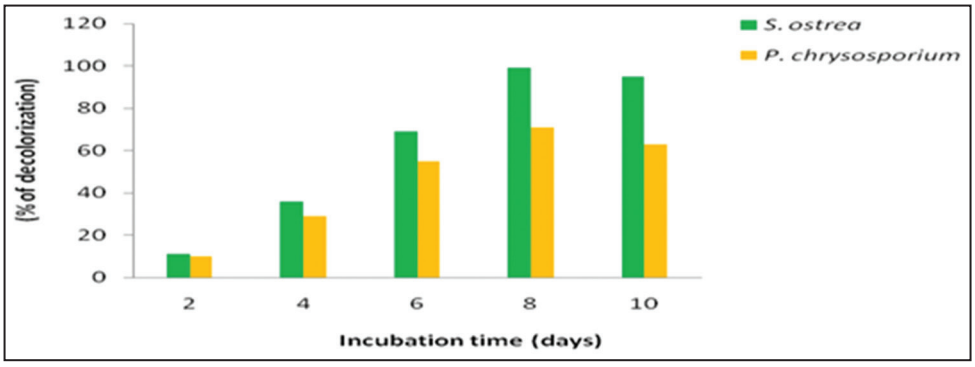

Figure 3: Decolorization of crystal violet (0.01%) by fungal cultures.

0.05 $MnSO_4$, 0.5 $MgSO_4$, and 20.0 agar (pH 6.0) were used for the production of ligninolytic enzymes and decolorization experiment.

2.3. Dye Stuff and Chemicals

Crystal violet and all chemicals were of the highest purity and of an analytical grade, which were purchased from HiMedia Laboratory (Mumbai, India). Spectrophotometric scanning of dye solution was performed in an ultraviolet (UV)–visible spectrophotometer (Chemito, UV-2600) and the absorption maximum of crystal violet was identified as 580 nm.

2.4. Assay for Dye Decolorization

Dye decolorization experiment was carried out in 250 ml Erlenmeyer flasks containing 50 ml of liquid that Koroljova media amended with crystal violet at 0.01% were inoculated with fungal cultures and incubated at 30°C in an incubator under shaking conditions. Separation of culture filtrate and mycelia mat was aseptically filtered using Whatman filter paper. The filter paper along with mycelial mat was dried in an oven until constant weight. Difference between the weight of the filter paper having mycelial mat and weight of only filter paper represented biomass of fungal mat. Fungal growth was expressed in terms of mg/flask.

Fungal cultures were estimated for the secretion of extracellular protein content in culture filtrates according to Lowry *et al*., 1951 [14]. Culture filtrate devoid of fungal biomass was centrifuged in a cooling centrifuge (REMI C24-BL). The clear supernatant was used to determine decolorization by measuring the change in absorbance of culture supernatant at the maximum absorption wavelength (λ_{max}) of crystal violet (580 nm).

2.5. Enzyme Assays

Lcc activity was determined spectrophotometrically with guaiacol as the substrate [12]. LiP activity measurement was based on the oxidation

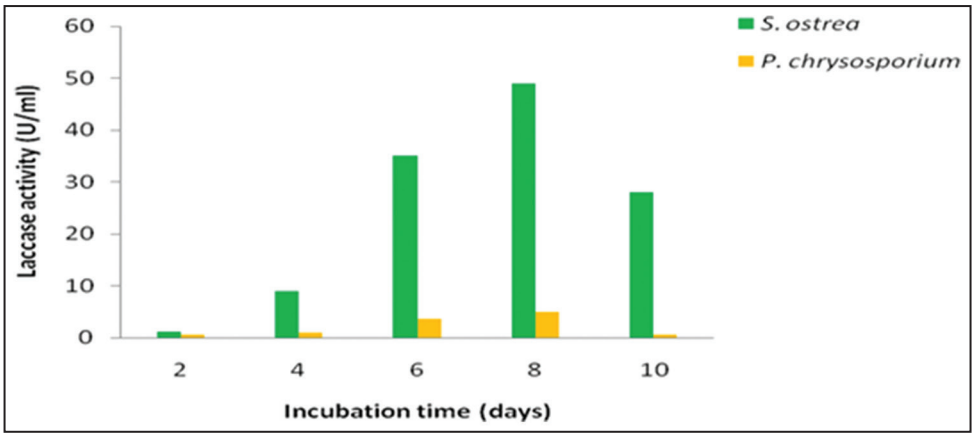

Figure 4: Laccase production by fungal cultures.

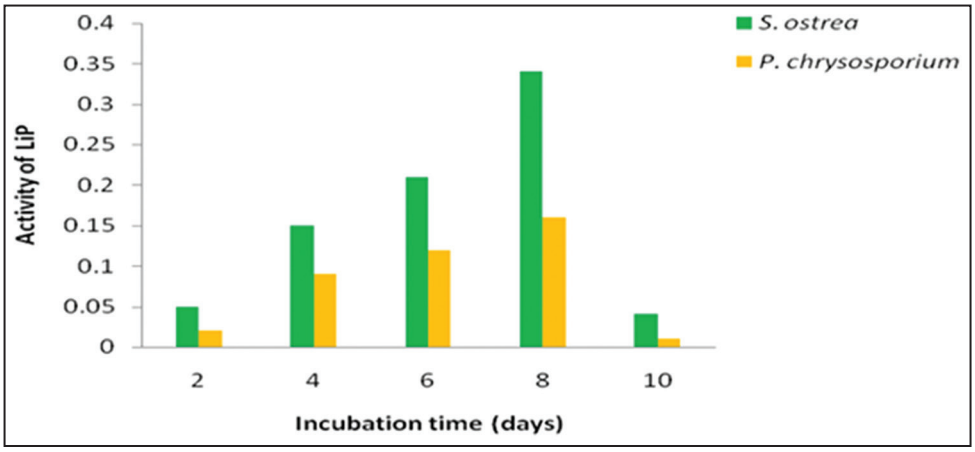

Figure 5: Activity of lignin peroxidase by fungal cultures.

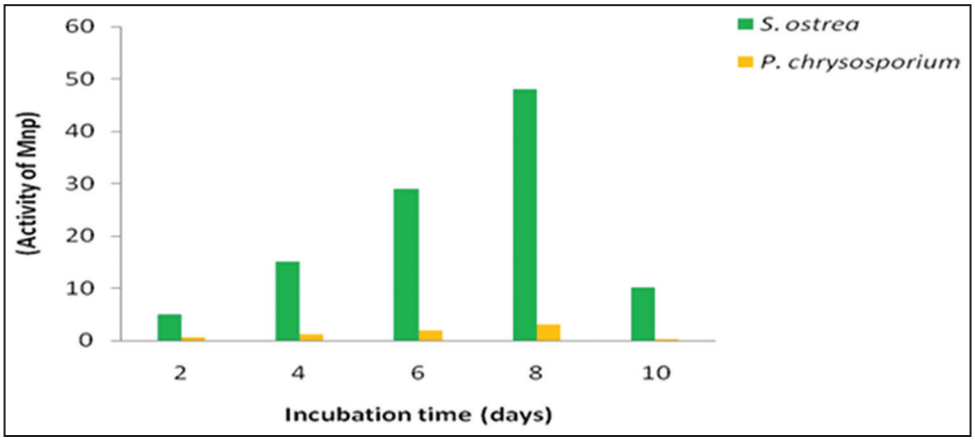

Figure 6: Activity of manganese peroxidase by fungal cultures.

of veratryl alcohol to veratraldehyde in the presence of H_2O_2 [9]. MnP activity was determined spectrophotometrically with $MnSO_4$ as the substrate [9].

3. RESULTS AND DISCUSSION

White-rot fungi are well suited for the treatment of broad range of dyes and effluents due to the versatility of the lignin-degrading enzymes produced by them. Hence, experiments were conducted with unexplored basidiomycetes *S. ostrea* to widen our knowledge in dye decolorization and ligninolytic enzyme production in comparison with explored culture *P. chrysosporium*.

3.1. Biomass of Fungal Cultures in Dye Amended Medium

Fungal cultures were grown in dye amended liquid Koroljova medium under shaking conditions. Growth was initially slow and later on picked up for both fungal cultures. Biomass kept on increasing until the past day (10^{th} day) of incubation for both fungal cultures used in this study. *S. ostrea* exhibited maximum biomass of 1.41 g/flask on the 10^{th} day of incubation, as against 1.20 g/flask in respect to *P. chrysosporium* (Figure 1).

Table 1: Decolorization of crystal violet at different concentrations with fungal cultures.

Incubation period (days)	% decolorization of dye					
	Stereum ostrea at			*Phanerochaete chrysosporium* at		
	0.02%	0.06%	0.10%	0.02%	0.06%	0.10%
II	20.05	18.67	10.00	15.55	11.05	8.50
IV	42.67	40.65	31.64	38.70	26.31	14.67
VI	68.87	59.90	48.66	45.00	48.72	33.74
VIII	87.90	79.89	70.11	55.00	48.30	39.12
X	87.34	79.88	70.00	54.86	48.00	39.00

3.2. Extracellular Protein Content of Fungal Cultures

Both fungal cultures secrete extracellular protein in crystal violet amended liquid medium under shaking conditions. The secretion of extracellular protein into the medium by both cultures increased with increase in incubation time and reached maximum on the 8th day of incubation (Figure 2). *S. ostrea* exhibited maximum protein content of 825 µg/ml into the medium against 700 µg/ml by *P. chrysosporium*.

3.3. Dye Decolorization by Fungal Cultures

Fungal cultures – *S. ostrea* and *P. chrysosporium* decolorized 90% and 67% of crystal violet dye at the 8th day of incubation at 0.01% concentration (Figure 3). The decolorization has occurred without H_2O_2 and $MnSO_4$ indicated that the decolorization was due to the presence of Lcc, LiP, and MnP, respectively [11]. However, Claus and Filip reported that acid blue 193 and acid black 210 were completely decolorized within 24 h, whereas crystal violet took 120 h for 70% of decolorization. Reactive blue B (S) (31%) and reactive blue BL/LPR (26%) were partially decolorized in 120 h [15]. Decolorization of different dyes depends on structure and complexity [5]. Small differences can markedly affect decolorization. These differences are due to electron distribution and charge density, although steric factors may also contribute [5].

3.4. Toxicity Studies

Fungal cultures were tested for decolorization at different concentrations of crystal violet (0.02, 0.06, and 0.10%) in liquid Koroljova medium. Decolorization of crystal violet was observed at the highest concentration (0.10%) in both cultures (Table 1). *S. ostrea* exhibited maximum decolorization of dye at three different concentrations – 0.02, 0.06, and 0.10% which was found to be 87.90, 79.89, and 70.11%, respectively, whereas *P. chrysosporium* exhibited 55.00, 48.30, and 39.12 at the 8th day of incubation (Table 1). Crystal violet dye up to 0.10% concentration did not show any effect on growth of the fungal cultures (data not shown). Hence, the higher concentration of crystal violet dye is not toxic to *S. ostrea* and *P. chrysosporium*.

Decolorization of triphenylmethane dyes and biodegradation mechanisms of dyes of several microorganisms mainly depend on the enzyme secreted by them [9]. *Cunninghamella elegans* ATCC 36112 transformed crystal violet through sequential N-demethylation to N,N′,N″-trimethylpararosaniline [16]. Thus, it is hypothesized that the biological degradation of crystal violet was based on the reduction reaction, reductive splitting reaction, and demethylation reaction [17].

3.5. Production of Ligninolytic Enzymes by White-rot Fungi

3.5.1. Lcc production

Many authors have clearly explained the involvement of non-specific fungal oxidative enzymes such as Lcc, LiP, and MnP in decolorization of azo dyes [18]. Some white-rot fungi produce only one or two of these enzymes, while others produce all ligninolytic enzymes [9,12]. However, the participation of these enzymes in dye decolorization varies from fungus to fungus. For this reason, we have studied the enzymes produced from *S. ostrea* for their ability to decolorize the crystal violet dye. The fungal cultures *S. ostrea* and *P. chrysosporium* were cultivated in crystal violet liquid medium in submerged and shaking conditions for assessing Lcc, LiP, and MnP production. Both cultures produced Lcc touched peak on the 8th day of incubation and there onward declined (Figure 4). *S. ostrea* culture yielded about 9-fold higher production of Lcc than *P. chrysosporium* on the 8th day of incubation. Maximum Lcc activity of 49 U/ml was recorded by *S. ostrea* as against 5 U/ml by *P. chrysosporium* on the 8th day of incubation (Figure 4).

3.5.2. LiP and MnP production

S. ostrea and *P. chrysosporium* displayed LiP and MnP throughout the incubation period in submerged and shaking conditions (Figures 5 and 6). *S. ostrea* exhibited maximum activity of LiP 0.34 and 0.16 U/ml at the 8th day of incubation (Figure 5).

Like LiP and MnP peroxidase productions were observed in both fungal cultures on the 8th day of incubation (Figure 5). The highest MnP activity of 48 U/ml was obtained by *S. ostrea* as against 3 U/ml of MnP by *P. chrysosporium* on the 8th day of incubation (Figure 6).

The major mechanism of living cells is producing lignin-modifying enzymes for biodegradation to mineralize synthetic lignin or dyes [19]. However, the relative contributions of Lcc, LiP, and MnP for decolorization of dyes may be different for each fungus [14]. LiP was found to be responsible for the decolorization of the dyes in *P. chrysosporium* [20]. Park *et al*. [21] also reported that LiP played a major role in the decolorization of azo, triphenylmethane, heterocyclic, and polymeric dyes by *P. chrysosporium* and that MnP was not required to start the degradation of these dyes. Young and Yu [11] studied the decolorization of the eight synthetic dyes including azo, anthraquinone, metal complex, and indigo by *Tinea versicolor* and also reported that MnP did not decolorize these dyes while ligninase-catalyzed oxidation removed 80% of the dyes. However, Zhang *et al*. [22] observed that decolorization of cotton bleaching effluent by unidentified white-rot fungus MnP, while there was no involvement of LiP in decolorization. Our results correlated with the previous reports, indicating that the production of ligninolytic enzymes in dye amended medium is responsible for the maximum dye decolorization.

4. CONCLUSION

The present study concluded that, both fungal cultures - *S. ostrea* and *P. chrysosporium* produce an array of ligninolytic enzymes – Lcc, LiP, MnP in dye amended liquid medium. *S. ostrea* is more promising and potential culture in decolorization of dye in different concentrations than the reference culture *P. chrysosporium.*

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***Bibliographical Sketch**

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