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Research Article

EFFECT OF ANTIBIOTICS ON LIGNINOLYTIC ENZYMES PRODUCTION FROM *STEREUM OSTREA* AND *PHANEROCHAETE CHRYSOSPORIUM* UNDER SUBMERGED FERMENTATION

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ABSTRACT

The effect of different antibiotics (chloramphenicol, ampicillin, neomycin, erythromycin and tetracycline) on production of ligninolytic enzymes- laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) from *Stereum ostrea* (*S. ostrea*) and *Phanerochaete chrysosporium* (*P. chrysosporium*) have been studied. All the antibiotics tested at a concentration of 200 ppm affected the fungal growth, release of extracellular protein content and ligninolytic enzymes production to different extent. Though growth and secretion of extracellular protein by *S. ostrea* were comparable to those of *P. chrysosporium* in presence of tetracycline, but yields of laccase enzyme by *S. ostrea* were higher than laccase titres of *P. chrysosporium* by more than 18 folds on the peak production. *S. ostrea* yielded titres of 55 units of laccase/ml as against 3.0 units of laccase/ml on the VIth day of incubation. *S. ostrea* also exhibited activities of other ligninolytic enzymes – LiP and MnP higher than the reference culture. Increasing the concentrations of tetracycline enhanced ligninolytic enzymes production from *S. ostrea* and *P. chrysosporium*.

Keywords: Ligninolytic enzymes, *S. Ostrea, P. Chrysosporium*

INTRODUCTION

Lignin is an abundant natural aromatic polymer occurring in the woody tissue of higher plants. Due to its hydrophobicity and complex random structure lacking regular hydrolysable bonds, lignin is poorly biodegraded by microorganisms. The best degraders of lignin are white-rot fungi that produce laccase (Lac) (EC. 1.10.3.2), and peroxidases- lignin peroxidase (LiP) (1.11.1.4) and manganese peroxidase (MnP) (EC. 1.11.1.13) [1]. Lignin degrading white-rot fungi and their ligninolytic enzymes find potential applications in the pulp and paper industry, detoxification of environmental pollutants, decolorization of dyes, cosmetics (including hair dyes), food and beverages, clarification/stabilization of fruit juices, clinical diagnostics, enzymatic conversion of chemical intermediates and upgradation of animal feeds (Minussi et al., 2002). Some wood-degrading fungi contain all three major ligninolytic enzymes such as Lac, LiP and MnP, while others may contain only one or two of these enzymes (Hatakka, 1994; Pelaez et al., 1995). Certain organisms of white-rot fungi – *Phanerochaete chrysosporium*, *Trametes versicolor, Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus, Lentinus tigrinus, Phanerochaete sordida, Phlebia radiata, Pleurotus eryngii, and Poliporus cialatus* have drawn more attention for lignolytic enzymes [3]. *S. ostrea* known as false turkey-tail fungus is a white-rot fungus belonging to family Stereaceae and most common in hard wood forests and is relatively characterized for lignolytic enzymes. *S. ostrea* has been recently shown to secrete laccase into extracellular medium [4]. There was confusion prevailing about ability of *S. ostrea* to secrete MnP in extracellular medium in view of contradictory results of two studies [5, 6]. According to our recent study [3], *S. ostrea* has been recognized to produce all three main lignolytic enzymes – Lac, LiP and MnP. Low levels of extracellular ligninolytic enzymes production by white-rot fungi are not sufficient for their commercial applications. Therefore, in order to enhance production of ligninolytic enzymes various conventional and modern experimental approaches like mutagenesis and recombinant DNA technology are being targeted. Many workers have reported the use of different antibiotics such as ampicillin, tetracycline (Garber and Yoder, 1983), streptomycin sulfate and penicillin G (Rogers et al., 1989) for successful prevention of bacterial proliferation to get luxuriant fungal growth. In the present study, attempts have been made to understand the growth, secretion of extracellular protein content and production of Lac, LiP and MnP from *S. ostrea* and *P. chrysosporium* in the liquid medium in the presence of different antibiotics.

MATERIALS AND METHODS

S. ostrea was kindly supplied by Prof. M.A. Singaracharya, Department of Microbiology, Kakatiya University, Andhra Pradesh, India and was isolated from wood logs. The reference culture, *Phanerochaete chrysosporium* was obtained from IMTECH, India. The fungal cultures were maintained at 30^0 C on 2% Koroljova-Skorobogat'ko medium [8] because of good growth [3]. The maintenance medium was prepared according to Koroljova et al., [8] containing the following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH 6.0).The stock solutions of antibiotics (chloramphenicol, ampicillin, neomycin, erythromycin and tetracycline) were filter sterilized and added to the sterilized medium at a final concentration of 200 ppm Sterile Koroljova-Skorobogat'Ko medium was dispersed into sterile 250 ml Erlenmeyer flasks at a rate of 50 ml of broth supplemented with different antibiotics per flask. The flasks were inoculated with homogenized mycelial suspension and incubated in an orbital shaker (Orbitek, Chennai, India) at 30^{0}C and speed of 150 rpm. All the flasks with growing cultures of *S. ostrea* and *P. chrysosporium* were withdrawn for processing with analytical methods.

Analytical methods

Fungal biomass estimation

The cultures - *S. ostrea* and *P. chrysosporium* in the flasks at regular intervals were aseptically filtered through pre-weighed Whatman No-1 filter paper to separate mycelial mat and culture filtrate. The filter paper along with mycelial mat was dried at 70°C in an oven until constant weight and this weight was recorded. Difference between the weight of the filter paper bearing mycelial mat and weight of only filter paper represented biomass of fungal mat. Fungal growth was expressed in terms of g/flask. Filtrate collected was used as a source of MnP.

Protein estimation

An aliquot of culture filtrates of *S. ostrea* and *P. chrysosporium* with appropriate dilution was used for estimation of soluble protein content according to Lowry *et al.,* [9]. Bovine serum albumin was used as protein standard.

Enzyme Assay

Activities of lignolytic enzymes in the cultural filtrate of both fungal cultures were estimated following the standard protocols. Laccase activity was assayed using 10mM guaiacol in 100mM acetate buffer

(pH 5.0) containing 10% (V/V) acetone. The change in absorbance of the reaction mixture containing guaiacol was monitored at 470 nm (ε = 6740 M^{-1} cm^{-1}) for five minutes of incubation (12) Laccase activity was expressed in International Units (IU) where one unit corresponded to the amount of enzyme that oxidized one micromole of guaiacol per minute. Lignin peroxidase activity was determined by oxidation of veratryl alcohol at 310 nm (ε = 9,300 M^{-1} cm^{-1}) (39). The reaction mixture was composed of 0.5 ml culture filtrate, 0.4 mM H_2O_2 and 50 mM tartaric acid (pH 2.5) and 2 mM veratryl alcohol. The enzyme activity was expressed in IU where one unit of LiP corresponded to the amount of enzyme that oxidized one micromole of veratryl alcohol per min. MnP activity was determined by oxidation of phenol red at 610 nm (28). The assay mixture includes 0.5 ml culture filtrate, 0.25 M sodium lactate (pH 4.5), 0.5% bovine albumin, 200 mM $MnSO_4$, 2.0 mM H_2O_2 (prepared in 0.2 mM sodium succinate buffer pH 4.5) and 0.1% phenol red. The changes in absorbance of reaction mixture was monitored at 610 nm (ε = 22,000 M^{-1} cm^{-1}) for 5 min. MnP activity was expressed in IU where one unit of MnP was defined as the amount of enzyme that oxidized one micromole of phenol red per min.

RESULTS AND DISCUSSIONS

Since media composition and growth conditions are important factors affecting the production of extracellular ligninolytic enzymes and consequently lignin degradation [11], the influence of antibiotics on Lac, LiP and MnP production by fungal cultures in the culture medium was examined in the present study. Though cultures were monitored for growth, secretion of extracellular protein and MnP at 2-day interval for period of 10 days in all experiments, only results of 6th-day incubation are presented in the present study because of peak production at this sampling time.

Effect of antibiotics on biomass, extracellular protein content and ligninolytic enzyme production

Cultivation of *S. ostrea* and *P. chrysosporium* on Koroljova medium supplemented with different antibiotics did not improve the growth of both cultures as reflected by biomass in comparison to biomass of the same cultures on unsupplemented medium (control) (Fig. 1). *S. ostrea* produced maximum biomass of 1.114 g/flask on the 10th day of incubation as against 1.000 g/flask in respect of *P. chrysosporium*.

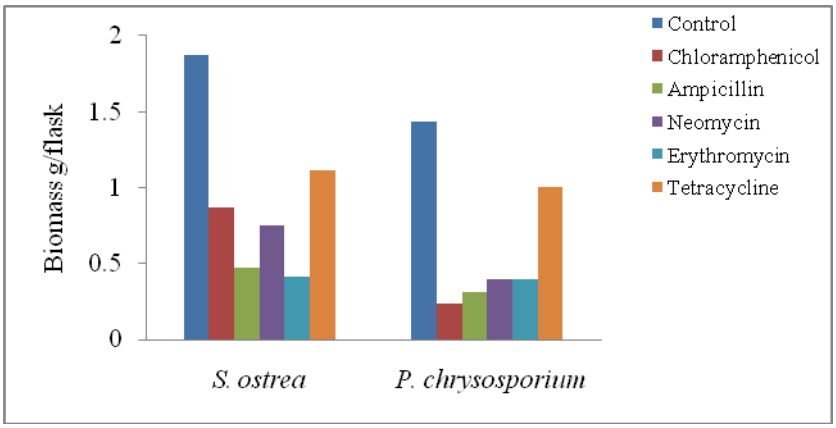

Fig. 1: Biomass of the fungal cultures

Growth of both organisms on the medium with supplementation of antibiotics resulted in secretion of protein into the medium in considerable proportions (Fig. 2). Among antibiotics supplemented in this study, only tetracycline caused more release of extracellular protein of 800 and 710µg/ml into the medium by *S. ostrea* and *P. chrysosporium* on 8th day of incubation (Fig. 2).

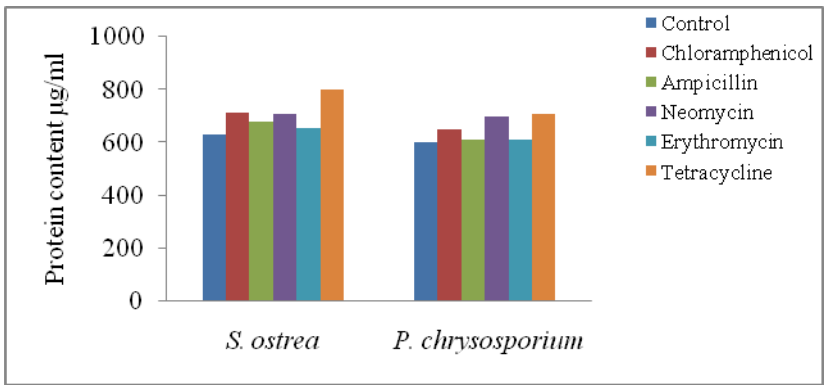

Fig. 2: Extracellular protein of the fungal cultures

Both cultures exhibited Lac, LiP and MnP activity when grown on medium in the presence of antibiotics (Table 1). Production of enzymes by both cultures touched peak on 6th day of incubation and there onwards declined (data not shown). Maximum yields of Lac, LiP and MnP were 55, 0.897, 5.45 U/ml was recorded as against only 3.0, 0.864, 2.251 U/ml in respect of *S. ostrea* and *P. chrysosporium* on 6th day of incubation in presence of tetracycline (Table 1). *S. ostrea* displayed activity of even LiP and MnP on higher side than *P. chrysosporium.* Thus results clearly shows that *S. ostrea* was better than the reference culture on the score of Lac, LiP and MnP production.

Table 1: Production of ligninolytic enzymes by fungal cultures

Antibiotics	*S. ostrea*			*P. chrysosporium*		
	Lac	LiP	MnP	Lac	LiP	MnP
Control	27.48	0.156	1.000	1.245	0.100	0.278
Chloramphenicol	30.40	0.361	1.341	1.864	0.105	0.451
Ampicillin	33.40	0.410	1.255	1.742	0.115	0.676
Neomycin	41.78	0.647	2.000	1.898	0.210	0.745
Erythromycin	30.98	0.410	2.100	1.900	0.545	0.550
Tetracycline	55.05	0.897	5.450	3.000	0.864	2.251

Tetracycline at a concentration of 200 ppm maximally enhanced ligninolytic enzymes production from both the fungi. Hence the effect of different concentrations of tetracycline on Lac, LiP and MnP production was also studied. Increasing concentrations of tetracycline enhanced the production of these enzymes from *S. ostrea* and *P. chrysosporium* (Table 2).

Table 2: Effect of tetracycline on production of ligninolytic enzymes by fungal cultures

Tetracycline (ppm)	*S. ostrea*			*P. chrysosporium*		
	Lac	LiP	MnP	Lac	LiP	MnP
Control	**27.10**	**0.150**	**1.000**	**1.300**	**0.105**	**0.280**
100	30.15	0.411	1.678	1.989	0.366	1.870
200	55.05	0.895	5.400	3.000	0.870	2.260
300	56.75	0.944	5.947	3.451	0.945	2.899
400	60.66	1.051	6.110	3.860	1.000	3.125
500	61.10	1.128	6.500	3.991	1.110	3.460

Fungal growth inhibition by antibiotics was observed to be correlated with the enhanced production of ligninolytic enzymes (Dhawan et al. 2005). Froehner and Eriksson (1974) have reported inhibition of extracellular protein synthesis in *Neurospora crassa* stimulated for laccase production in the presence of cycloheximide, owing to normal protein turnover. The derepression of tyrosinase synthesis in *N. crassa* by cycloheximide, actinomycin D, puromycin (Horowitz et al., 1970) and alkaline phosphatase synthesis in *E. coli* by chloramphenicol and canavanine, respectively, has been reported (Gallant and Stapleton, 1963). However such a correlation between total protein content and ligninolytic enzymes production was not observed in *S. ostrea* and *P. chrysosporium*. Sandhu and Arora (1985) observed induction of laccase production in *Polyporus sanguineus* in the presence of different phenolic compounds and protein synthesis inhibitors. They further proposed that laccase thus induced represents a mechanism to eliminate these toxic compounds by enzymatic transformation. Hence the possibility that the white-rot fungi sense the antibiotic as a phenolic substrate to attack and to detoxify it is not ruled out. Similarly, *Phlebia radiata* has been shown to produce lignin-modifying enzymes for detoxification purposes when toxic compounds were present in its environment (Rogalski et al., 1991). Hence, the stimulation of ligninolytic enzymes production by certain antibiotics could be that fungi might be mimicking the antibiotics similar to phenolic substrates. Moreover, by inhibiting fungal growth, the antibiotic might be causing physiological stress which inturn might onset release of proteins like Lac, LiP and MnP.

CONCLUSIONS

The following conclusions can be drawn from the results of the present study. The white-rot fungus *S. ostrea* produces a complete ligninolytic system Lac, LiP and MnP in presence of antibiotics. Lac appears to be a dominant component in the ligninolytic system of *S. ostrea* and *P. chrysosporium*. For production of ligninolytic enzymes, *S. ostrea* culture is more promising and potential culture than the reference culture *P. chrysosporium*

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