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Effect of Ethidium Bromide on Extracellular Laccase Production by *Pleurotus citrinopileatus*

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Abstract

In the present study, we used chemical mutagen ethidium bromide (EtBr) to enhance/induce the production of extracellular laccase enzyme by *Pleurotus citrinopileatus*. Initially grown on the malt extract agar (MEA) media treated with different concentration of EtBr, the extracellular laccase production was tested in artificial media under static condition. Maximum extracellular laccase production (19.67 U/ml) was recorded when *Pleurotus citrinopileatus* was grown on MEA treated with 1.5 µg/ml ethidium bromide as compared to other concentrations and control. As the concentration increases the production of extracellular laccase activity was decreased. In another experiment when ethidium bromide pre-treated fungal culture was grown in media devoid of EtBr, it produced a fourfold increase in extracellular laccase production. In third attempt, interesting results were found; when pre-treated culture was grown of EtBr supplemented media with 1.5 µg/ml, there was a twofold increase in extracellular laccase production. The enhanced production of extracellular laccase enzyme in economically cheap formula can have multiple applications.

Keywords: *Pleurotus citrinopileatus*; Extracellular laccase; Ethidium bromide

Abbreviations: EtBr: Ethidium Bromide; U/ml-µm/min/ml, DMP-2,6-Dimethoxy Phenol

Introduction

Laccases (benezene-diol: oxygen oxidoreductase, EC1.10.3.2) are copper-containing enzymes that catalyse the oxidation of various phenolic and inorganic compounds, with the concomitant reduction of oxygen in water [1]. They are present in bacteria, fungi as well as in plant [2]. Many enzymes are important but laccases find more important industrial applications such as in paper and pulp industry [3,4] dye bleaching, polymer synthesis, beverage stabilization, anticancer drug development, food industry [5]. Currently, laccase is used as a biocatalyst in organic synthesis [6], in pharmaceutical sector by the synthesis of certain compounds such as anaesthetics, anti-inflammatory, antibiotics, sedatives etc. [6] and in bioremediation. Fungal laccases are eco-friendly in nature and reduce the toxicity of chemical pollutant by oxidation /reduction mode of action [7].

White rot fungi degrade lignin because they secrete oxidoreductases including lignin peroxidase (1,2-bis(3,4-dimethoxyphenyl) propene-1,3-diol: hydrogen-peroxide-Lip EC 1.1.1.14), manganese peroxidase (Mn(II): hydrogen peroxide oxidoreductase EC 1.11.1.13), and laccase (benzenediol: oxygen reductase EC 1.10.3.2). These enzymes oxidise in a nonspecific way both phenolic and non-phenolic lignin derivatives and thus are promising candidates for the degradation of environmental pollutants, for example, phenols, anilines, dyes, and lignocelluloses [8].

Phenanthridium dye ethidium bromide (EtBr) is a nitrogen heterocyclic compound and is the interacting agent. Ethidium bromide is thought to act as a mutagen because it intercalates double stranded DNA (i.e., inserts itself between the strands), deforming the DNA [9]. This could affect DNA biological processes, like DNA replication and transcription. Ethidium bromide has been shown to be mutagenic to bacteria via the Ames test, but only after treatment with liver homogenate, which simulates the metabolic breakdown of the molecule being tested [10].

The present work was carried out to enhance extracellular laccase enzyme secreted in white rot fungus *Pleurotus citrinopileatus*.

Materials and Methods

Chemicals

DMP (2, 6-dimethoxy phenol) was from Sigma Chemical Company, St. Louis USA. All the other chemicals were from Hi media laboratory Ltd, Mumbai.

Microorganism and culture conditions

The culture of *Pleurotus citrinopileatus* was procured from Directorate of Mushroom Research (DMR), Chambaghat, Solan (H.P.) It was maintained on malt extract agar (MEA) consisted of malt extract 20.0 g and agar 20.0 g in 1.0 L double distilled water at 25°C and pH 6.0 and was subculture at regular interval of one month and stored at 4°C [11].

Ethidium Bromide (EtBr) treatment

In plate: In first attempt, the MEA medium treated with ethidium bromide (EtBr) at a final concentration of 1.5 µg/ml, 3.0 µg/ml, 4.5 µg/ml, 6.0 µg/ml, 7.5 µg/ml, 9.0 µg/ml and 10.5 µg/ml were inoculated with one disc each of *P. citrinopileatus* mycelium on MEA medium and were incubated at 25°C for 7 days. In second attempt, pre-treated EtBr culture of *P. citrinopileatus* was again regrown on MEA plates devoid of EtBr.

In third attempt, pre-treated EtBr (1.5 µg/ml, 3.0 µg/ml, 4.5 µg/ml, 6.0 µg/ml, 7.5 µg/ml, 9.0 µg/ml and 10.5 µg/ml) culture of *P. citrinopileatus* were regrown on MEA plates treated with EtBr (1.5 µg/ml, 3.0 µg/ml, 4.5 µg/ml, 6.0 µg/ml, 7.5 µg/ml, 9.0 µg/ml and 10.5 µg/ml).

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In broth: 250 ml Erlenmeyer flask containing 50 ml artificial media [4] composed of Glucose 10 g, asparagine's 1.0 g, yeast extract, 0.5 g $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ and double distilled water 1 litre and amended with natural lignin substrates such as wheat bran (1 gm/100 ml) was sterilized by autoclaving at 121 psi for 15 minutes and then after autoclaving media was inoculated with culture treated EtBr with different concentration were incubated at 30°C for 7 days. After 7 days, for laccase production, protein estimation and fungal biomass in artificial liquid culture medium.

Enzyme assay method

The filtered extract was analyzed for extracellular laccase activity using DMP as a substrate. The 1.0 ml assay solution containing, 1.0 mM DMP in 50 mM sodium malonate buffer (Ph-4.5) at 37°C, λ=468 nm and molar extinction coefficient of 49.6 mM/cm [12]. The UV/Vis spectrophotometer of Thermo Scientific Evolution 201 model was used for absorbance measurement. One enzyme unit produced 1μmol of the product per minute under the specified assay conditions.

Protein estimation

The extracellular Protein estimation was done by following the methodology of Lowry et al. [13].

Biomass

The fungal biomass was determined by oven drying the mycelium at 80°C for 24 h. All assays were performed in triplicates and results are reported as average of three replicates.

Statistical analysis

All the experiments were carried out in triplicate and data were expressed in mean, standard deviation (SD) and SEM.

Results and Discussion

Figure 1 shows the extracellular laccase production from *Pleurotus citrinopileatus* when initially grown with different concentration of EtBr. Laccase activity (19.67 U/ml) was higher in 1.5 μg/ml as compared to control (8.85 U/ml). But according to Perlman and Mahler [14], they reported that ethidium bromide which acts as an effective mutagen they bring about quantitative conversion of facultative anaerobic yeast cells cytoplasmic petites mutant that could be resulted into the characteristic respiratory deficiency. They also reported that in case of aerobic yeast

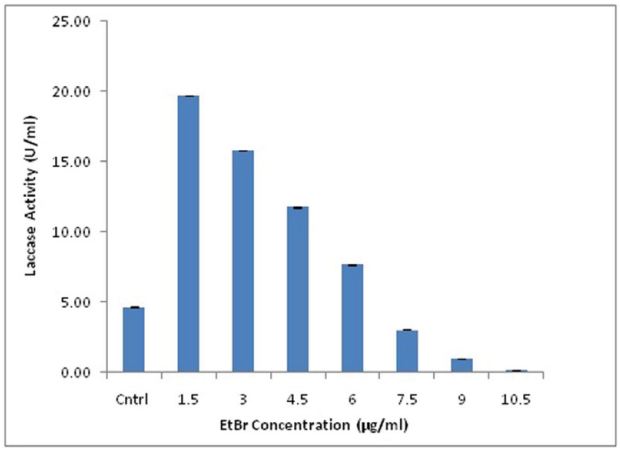

Figure 1: Extracellular laccase production from *P. citrinopileatus* culture pre-treated with different concentration of EtBr in artificial media at 30°C, pH 6.0.

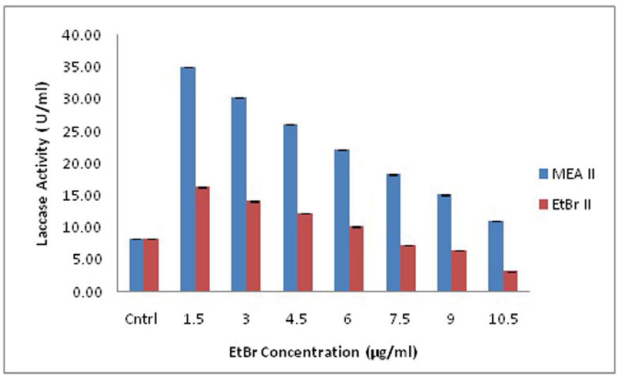

Figure 2: Extracellular laccase activity: Growth of EtBr pre-treated *P. citrinopileatus* on MEA devoid of EtBr as well as repeated treatment with EtBr on MEA media supplemented with different concentration of EtBr.

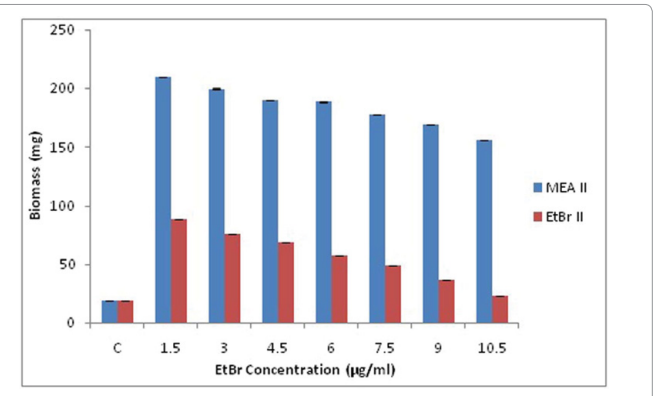

Figure 3: Biomass production: Growth of EtBr pre-treated *P. citrinopileatus* on MEA devoid of EtBr as well as repeated treatment with EtBr on MEA media supplemented with different concentration of EtBr.

cells when treated with EtBr, it blocks the formation of cytochromes a, a_3 and did not showed the induced petite mutation.

In case of animal cell lines, Gomez-Diaz et al. [15] reported that when treated with EtBr animal cell line HL -60 leads to the degradation of mitochondrial DNA and as well as mitochondrial respiratory chain that in turn resulted into the increased glycolysis as well as various other enzymes that would provide activation energy to overcome the oxidative stress threshold.

Effect of EtBr on the extracellular production of laccase, which is an oxidoreductase enzyme they enhanced the production of laccase to a specific concentration i.e., at 1.5 μg/ml (Figure 1) due to the oxidative/respiratory stress in the EtBr treated culture. As the concentration increases the extracellular laccase production decreases in agreement with Dhawan et al. [16] and as shown in Figure 1 that could be the detrimental effect of EtBr on the cell growth. Similar results were found by Pinto et al. [17]. They reported the EtBr that induces mutation from wild type to the cytoplasmic respiratory deficient petite in yeast cells. Interestingly, higher concentration of EtBr causes toxic effect on the yeast cells.

In second attempt, pre-treated EtBr culture of *P. citrinopileatus* were again regrown on MEA plates devoid of EtBr, laccase activity (35.05 U/ml), biomass(210 mg) and protein concentration (2.5 mg/ml) were found to be highest in 1.5 μg/ml as compared to control (Figures 2-4). In the above attempt, drastic fourfold increase in extracellular laccase production in artificial media.

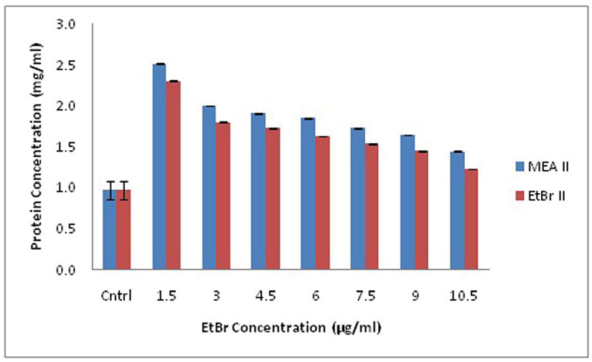

Figure 4: Protein concentration: Growth of EtBr pre-treated *P. citrinopileatus* on MEA devoid of EtBr as well as repeated treatment with EtBr on MEA media supplemented with different concentration of EtBr.

In the third attempt, pre-treated EtBr culture of *P. citrinopileatus* were again regrown on MEA plates supplemented with different concentration of EtBr, laccase activity (16.29 U/ml), biomass (88.1 mg) and protein concentration (2.3 mg/ml) were found to be highest in 1.5 µg/ml as compared to control (Figures 2-4). Due to the detrimental effect of repeated treatment with EtBr, according to Desjardins et al. [18].

The induction or increase in laccase activity by EtBr confirms a regulatory role of this aromatic compound in laccase production. Previously, various research groups have reported that elevated production of laccase by EtBr could possibly be due to respiratory stress induced in the cell. However, more work is needed to elucidate the molecular mechanism underlying regulation of laccase gene transcription by EtBr.

Conclusion

EtBr treated culture of *P. citrinopileatus* stimulates the extracellular laccase production which in turn can bioremediate the harmful existing pollutants such as xenobiotic compounds, dyes, and heavy metals. The extracellular laccase enzymes are more efficient and effective in bioremediation than the intracellular enzymatic system**.** As it the green solution, it is economically very important. Besides laccase have many applications such as in cosmetics, bleaching of pulp in paper industry, food, beverages, and various other biotechnological applications.

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