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Enhanced biodegradation and kinetics of anthraquinone dye by laccase from an electron beam irradiated endophytic fungus

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ABSTRACT

In this study, enhancement of biodegradation and detoxification of recalcitrant anthraquinone dye was achieved by laccase from an electron beam irradiated (EBR) endophytic fungus without mediators. The partially purified laccase enzyme (160.8 ± 1.15 U mg^{-1}) from 0.2 kGy irradiated endophytic fungus *Phomopsis* sp. catalyzed more than 85% of Remazol Brilliant Blue R (RBBR) (50 mg l^{-1}) degradation into non-toxic molecules within 5 min. Laccase enzyme production increased to 1.6 fold (27.7 ± 1.06 U mg^{-1}) due to electron beam radiation (0.2 kGy) of *Phomopsis* sp. compared to the non-irradiated fungus (17.28 ± 1.09 U mg^{-1}). Laccase enzyme from electron beam irradiated fungus exhibited extreme tolerance for the metals Zn^{2+}, Cu^{2+}, Cr^{2+} and Ca^{2+} up to 10 mM concentration. The kinetic parameters V_{max} and K_m for enzymatic decolorization (0.2 kGy EBR) were estimated by Lineweaver-Burk plot and were found to be more potent than the laccase from non-irradiated fungus. The laccase enzyme retained activity in broad range of temperature (25–45 °C) and pH (4–8). The COD of the original dye was reduced to 60% after biodegradation by the irradiated fungus. The toxicity studies revealed that the degraded dye was non-toxic on plants and microbial growth. The data from LC-MS analysis clearly indicates partial degradation of dye into non-toxic molecules by laccase (0.2 kGy) following oxidation, hydroxylation, deamination and ring cleavage.

1. Introduction

Synthetic dyes have posed environmental threats due to its recalcitrant nature. Pollution is due to the long durability, stability and resistance of the dyes towards degradation due to the complex chemical structure (Saratale et al., 2009a, 2009b). Many of the synthetic dyes are carcinogenic, mutagenic and cause severe health hazards to the mankind (Nilsson et al., 1993). Conventional chemical methods of textile effluent treatment like adsorption and electrochemical decolorization has the disadvantage of formation of toxic compounds, inefficient removal of Chemical Oxygen Demand (COD)/Biological Oxygen Demand (BOD) and energetically unfeasible process (Correia et al., 1994).

Microbial decolorization and degradation of synthetic dye is often economical alternative in comparison with physical and chemical processes (Ngieng et al., 2013). Bacterial and fungal decolorization of the synthetic dyes has fetched considerable importance in bioremediation programs. Bacterial degradation of dyes has a disadvantage that it relies on the organic pollutants as growth substrates. There is a positive feedback loop between pollutant degradation and bacterial growth. Thus, toxic micro-pollutants which are less bioavailable often escape the degradation cycle and persist in the environment. Fungal degradation system therefore gains the importance, as fungi are capable of sustaining on marginal living conditions and act on micro-pollutants efficiently (Harms et al., 2011). White-rot fungi are usually used in bioremediation of xenobiotics, as they produce unique class of extracellular enzyme called ligninases with broad substrate specificity. The oxidative enzymes degrade lignin by nature and are now exploited for the degradation of complex xenobiotics compounds due to their similar mechanistic action in degrading them (Gianfreda et al., 1999).

Endophytes residing within the healthy plant host do produce a small titer of cell-wall degrading enzymes as their saprobic counterpart. These enzymes help them in colonizing and penetrating the host and later on switching to the saprophytic lifecycle from mutualistic association when the situation permits. Amongst the degrading enzyme, a very few investigators have reported laccase enzyme from endophytic fungi (Kwon and Anderson, 2001; Oses et al., 2006; Petrini et al., 1993; Promputtha et al., 2010; Wang, 2006). However, there are no detailed studies on endophytic fungal laccase and its mechanism of dye degradation. Although, there are reports on optimization of media components for endophytic fungal laccase production using physico-

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chemical methods, to the best of our knowledge, no reports on using electron beam radiation (EBR) for amelioration of laccase has been made (Oses et al., 2006; Wang, 2006). In the present study, the endophytic fungi was irradiated with different sub-lethal doses of EBR and studied for increased laccase production and thereby hastened synthetic dye degradation. The physico-chemical parameters for optimal dye degradation, biotoxicity and mineralization of synthetic dye and its degraded compounds were studied to confirm the detoxification of possible environmental contamination.

2. Materials and methods

Chemicals: Remazol Brilliant Blue R (R8001), 2, 2-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) (A1888) and chemicals (D4545) used for Polymerase chain Reaction were purchased from Sigma Aldrich, India. ITS primers were purchased from Eurofins Genomics (38.1006). Potato Dextrose Agar media (M096), Nutrient Agar media (M001) and Nutrient Broth media (M002) used for microbial growth were purchased from Himedia, India. All other chemicals and solvents used in the study were of analytical grade and are of high purity.

2.1. Isolation, screening for ligninase enzyme and identification of endophytic fungi

Endophytic fungi were isolated from symptomless petiole and leaves of *Simarouba glauca* (L) according to the modified method (Sathish et al., 2014). All the endophytic fungi isolated from *Simarouba glauca* were grown in a qualitative agar media containing 4 mM guaiacol and screened for ligninase enzyme production (Kawai et al., 1988).

Fungal DNA was extracted using the method followed earlier (Sathish et al., 2014). Molecular identification was made by amplifying rDNA Internal Transcribed Spacer (ITS) regions: ITS1 (forward primer) and ITS4 (reverse primer) (Dai et al., 2010) and sent for sequencing at Chromous Biotech Pvt. Ltd, Bangalore. Blast searches of ITS sequences were made in National Center for Biotechnology Information (NCBI) database for the closest match and sequence was submitted to GenBank to get an accession number.

2.2. Electron beam irradiation of endophytic fungus

Freshly grown endophytic fungal disc was placed on the center of a 95 mm polypropylene plate containing PDA media and grown aseptically for 3–4 days at 30 °C until the fungus grows about 30 mm in diameter and whole plate was exposed to electron beam radiation (EBR) keeping fungal colony facing the radiation source. Dosimetry of the EBR on fungal growth was measured using dose ranging from 0 to 15 kGy at Microtron center, Mangalore University, Karnataka and dose above 4 kGy was found lethal for the fungal growth using this method. Independent fungal plates were exposed to different doses (0.2, 0.4, 0.8, 1.2 and 2.0 kGy) of EBR, below the lethal dose. Similarly one fungal plate was processed without radiation along with the irradiated plates and considered as 0.0 kGy or non-irradiated fungus.

2.3. Laccase enzyme production and assay in post irradiated endophytic fungi

Five days old EB irradiated (0.2, 0.4, 0.8, 1.2 and 2.0 kGy) and non-irradiated (0.0 kGy) endophytic fungal cultures from PDA were transferred to 100 ml modified Kirk's media (Composition: glucose, 10 g; peptone, 5 g; yeast extract, 1 g; ammonium tartrate, 2 g; KH_2PO_4,1 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCl, 0.5 g; and trace elements, 1 ml per liter in pH 5) (trace elements composition per liter is $CuSO_4{\cdot}5H_2O$, 0.01 g; $FeSO_4{\cdot}7H_2O$, 0.05 g; $MnSO_4{\cdot}7H_2O$, 0.01 g; $ZnSO_4{\cdot}7H_2O$, 0.07 g; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.01 g per liter) and grown at 30 °C (Tien and Kirk, 1983). Aliquots of cultures were drawn at regular intervals, centrifuged at 8000 rpm for 20 min and supernatant was used for the laccase enzyme assay.

Laccase enzyme production in all the endophytic fungal samples which are irradiated with different doses of EBR were assayed along with the non-irradiated endophytic fungus using above method.

The irradiated fungus with EBR dose 0.2 kGy was taken for all future studies along with the control (non-irradiated). The fungal culture supernatant was considered as the cell-free crude enzyme (4.32 ± 0.34 Uml^{-1} for non-irradiated and 11.15 ± 0.25 Uml^{-1} for 0.2 kGy EBR irradiated) and used for all studies unless mentioned elsewhere.

2.3.1. Laccase activity

The laccase activity was determined spectrophotometrically by monitoring oxidation of 2, 2-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) at 420 nm (Oses et al., 2006; Tien and Kirk, 1983). The reaction mixture (200 µl) containing 20 µl of 5 mM ABTS, 80 µl of 0.1 M acetate buffer (pH 5) and 100 µl of crude enzyme was incubated at 30 °C for 2 min and absorbance was measured. One unit of the laccase enzyme activity was defined as the amount of enzyme that transforms 1 µM of the substrate in 1 min. Laccase enzyme activity was analyzed for every 24 h for 16 days, in order to find out peak time of highest enzyme production.

2.3.2. Effect of temperature and pH on laccase activity

The effect of different temperature (20, 25, 30, 37, 40, 45 and 50 °C) at pH 5 and effect of different pH (4, 5, 6, 7, 8 and 9) at temperature 30 °C on laccase activity was studied. The experiment was carried out using crude laccase enzyme and incubated at respective conditions for 15 min before the assay. All the above experiments were performed in triplicates (Sadhasivam et al., 2008).

2.3.3. Laccase activity amidst metal ions

The effect of different concentrations (0, 0.5, 1, 5 and 10 mM) of individual metal ions on laccase activity was studied for partially purified laccase enzyme (40 U each). Twelve types of metal ions were used from the following salts LiCl, $ZnSO_4$, $HgCl_2$, $MnCl_2$, Pb $(C_2H_3O_2)_2$, $CoCl_2$, $CuSO_4$, $NiCl_2$, $FeSO_4$, K_2CrO_4, Ca $(NO_3)_2$ and KCl. The cell-free crude extract was precipitated with pre-chilled acetone 1:2 ratio (v/v) and the precipitate was dissolved in 0.1 M acetate buffer (pH 5) and used as partially purified laccase enzyme (Murugesan et al., 2009). Similarly, the effect of mixture of metal ions (Pb^{+2}, Cr^{+2} and Hg^{+2}; 2 mM each) on laccase enzyme activity was measured. All the experiments were performed in triplicates.

2.4. Decolorization of RBBR dye

The cell free crude enzyme was taken and 50 mg l^{-1} of RBBR was added to check the rate of RBBR dye decolorization by the EB irradiated fungus. The percent decolorization was measured spectrophotometrically (Thermo Scientific Multiskan GO) by reading optical density (OD) at 595 nm. The readings were taken at regular time intervals and represented as percentage of dye degradation using the following formulae (Palmieri et al., 2005; Jasińska et al., 2012).

$$\% \text{ decolorization} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

2.4.1. Optimization of physicochemical parameters for enzymatic dye decolorization

Decolorization of the RBBR by cell-free crude enzyme from EB irradiated fungus was studied for the effect of different temperature (20, 25, 30, 37, 40, 45 and 50°C) at pH 5, effect of different pH (4, 5, 6, 7, 8 and 9) at temperature 30°C and effect of different dye concentration (50, 100, 150, 200 and 250 mg l^{-1}) at pH 5 and temperature 30°C. Effect of temperature and pH on decolorization was studied at a constant dye concentration of 50 mg l^{-1} (Saratale et al., 2009a, 2009b).

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