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Laccase production and simultaneous decolorization of synthetic dyes in unique inexpensive medium by new isolates of white rot fungus

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ABSTRACT

Morphological and biochemical analysis of the newly isolated white rot fungal (WRF-1) strain has ability to secrete laccase in the economical medium consisted of synthetic dyes, groundnut shell (GNS) and cyanobacterial biomass (algal bloom) under submerged shaking condition at pH 5.0 and 30 °C ± 2 °C temperature. WRF-1 strain was found to decolorize synthetic dyes efficiently at pH 5.0 and 30 °C ± 2 °C temperature. The laccase activity of strain was purified to homogeneity by chromatography with yield up to 70%. The molecular mass of laccase was found to be 70 kDa by SDS-PAGE and isoelectric point was 4.8. Biotransformation of the dyes was followed spectrophotometrically and dyes were found to decolorize completely after 6 days of fermentation. LC-MS studies were used to decipher the degradation profile of synthetic dyes by WRF-1. Indigo carmine gets degraded to isatin sulfonic acid and 4-amino-3-methyl-benzenesulphonic acid whereas methyl orange degraded metabolites were identified as *p*-*N*,*N*′-dimethylamine phenyldiazine and *p*-hydroxybenzene sulfonic acid. Thus the study would give a road map for the production and application of laccase enzyme on a larger scale using low cost substrate.

1. Introduction

Laccases (*p*-diphenol: dioxygen oxidoreductases, EC 1.10.3.2) are multi-copper enzymes, which catalyzes the oxidation of variety of organic and inorganic substrates coupled to the reduction of molecular oxygen to water with the one–electron reaction mechanism. Laccases catalyze the oxidation of a broad range of substrates e.g., polyphenols, substituted phenols, diamines and also some inorganic compounds (Thurston, 1994). These enzymes are widely distributed in nature. Laccases or laccase like activity has been demonstrated in higher plants, in some insects and in a few bacteria. However the best known laccases are of fungal origin, especially those belonging to the class of white rot fungus. They are capable to delignify wood pulp, decolorize and degrade toxic environmental pollutants and synthetic dyes which are carcinogenic and hazardous to environment (Gianfreda et al., 1999; Bak et al., 2009). More recent approaches have focused on utilizing agro-wastes as a raw material for the production of microbial metabolites for food processing industries. This has gained increasing ground because agro-based raw materials such as coffee pulp and husk, sugar beet pulp, sugarcane bagasse and bran of pulses have the advantage of being used as a sole source of energy and C-pool and are environment friendly. This has particular relevance for agriculture-based countries like India wherein plenty of agro-waste is available as raw material. The component analysis of groundnut shell (GNS) reveals that it contains (wt% dry biomass) about 35.7% cellulose, 18.7% hemicellulose and 30.2% lignin (Raveendran et al., 1995), thus groundnut shell was choice substrate to study the ligninolytic enzyme activities in dependence with nitrogen source in submerged fermentation by white rot fungi. The diazotrophic cyanobacteria are cosmopolitan microbes contributing significantly to nitrogen economy of the biosphere. Many cyanobacteria are useful to mankind as food and feed for animals since they are not pathogenic and have high nutrient value (Dwi et al., 2001). Cyanobacteria are main constituents of water bloom in water resources such as lakes, ponds and dams. The occurrence of water bloom leads to deterioration of water quality because some cyanobacteria release toxic compounds and dead cells causes an objectionable odor. However, cyanobacteria are rich source of nitrogen, sugars, lipids and proteins, can be exploited for the production of useful compounds (Ueno et al., 1996).

Pollution of communal water bodies by waste dye stuff released from textile plants and dye houses represents a major environmental concern. Although presently a wide range of physical and chemical methods is available to decolorize dye-contaminated effluents, alternative processes based on biotechnological principles

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are attracting increasing interest (Kandelbauer et al., 2004). In the textile finishing industry, enzymatic degradation of indigo could have a potential both in stone-wash process and for the treatment of dyeing effluents. Several steps are involved in the manufacture of denim garments between dyeing and the final stone washing where excessive amounts of indigo are removed from the fabrics and discharged with the wastewater (Campos et al., 2001).

Azo dyes are synthetic organic compounds widely used in textile dyeing. This chemical class of dyes, which is characterized by the presence of at least one azo bond (−N═N−) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70%. They are designed to convey high photolytic stability and resistance towards major oxidizing agents. The release of azo dyes into the environment in effluent from textile dyeing plants has become a major concern in wastewater treatment, since they are highly recalcitrant to conventional wastewater treatment processes. The recalcitrance of azo dyes has been attributed to the presence of sulfonate groups and azo bonds (Couto, 2007). Azo dyes forming "forbidden aromatic amines" are toxic and potential carcinogens which possibly form toxic metabolites after hydroxylation or oxidation with cytochrome P450 inside mammalian cells (Tauber et al., 2008).

Therefore an attempt was made to examine the possibility of utilizing cyanobacterial biomass (CBM) from water bloom as a source of nitrogen and essential growth factors to raw carbon source (groundnut shell) for production of laccase in the medium containing synthetic dyes by newly isolated strain of white rot fungus. Further studies were also made to analyze the degradation products using LC-MS.

2. Materials and methods

2.1. Morphological study of newly isolated fungal strain

Fungus was isolated from the bark of tree of University campus, Varanasi, India in the month of July–August from the decaying wood.

Tissue culture technique was employed for the isolation. The fungus growing on the bark of tree at BHU campus, Varanasi, India were removed and thoroughly washed under running tap water. These was then surface sterilized by treatment with 0.1% mercuric chloride for 30 s under aseptic condition. Then the fungus was washed with sterilized distilled water three-four times. These were then subjected to surface sterilization with 75% alcohol under aseptic conditions for 30 s and thoroughly washed with sterilized water. This fungus was then inoculated in a sterilized 10 g of groundnut shell (GNS) in petri plates, for an incubation period of one and half month (Revankar and Lele, 2006). The plates were moistened with sterile distilled water at regular interval of 4–5 days. In one of the plates white mycelium was developed which was further subcultured on PDA (potato dextrose agar) medium to get a pure culture.

Distinct and predominant fungal colonies were isolated from plates and characterized on their morphological and biochemical basis.

2.2. Cultivation conditions, enzyme production and dye decolorization study

Groundnut shell was collected from local market. Water bloom was collected from river Ganges, Varanasi, India in summer season. Cyanobacterial water bloom sample showed typical morphological characteristics of *Microcystis* sp. under a microscope. Cyanobacterial biomass was harvested by centrifugation (13,000×*g*, 10 min). Dry weight of cyanobacterial water bloom was determined by drying at 80 °C until a constant weight was obtained (Dwi et al., 2001). The spores from 4 days old slant dispersed into 3 ml of sterile water (10^6 spores/ml) was used as inoculum.

Enzyme production medium contained groundnut shell (4 g) and dry cyanobacterial biomass (1 g) in 100 ml acetate buffer of pH 5.0. The culture was incubated at 30 °C for 12 days in submerged shaking condition at 100 rpm. The culture was harvested on 12th day when maximum laccase titer was present, centrifuged and the supernatant was stored in sterile container in refrigerated condition (Mishra and Kumar, 2007).

Dye decolorization studies was carried out with four synthetic dyes (indigo carmine, methyl orange and malachite green) frequently used in textile industries. Production media was made with 0.02% (w/v) of dye comprised of GNS and dry cyanobacterial biomass with acetate buffer of pH 5.0 and incubated at 30 °C for 12 days of fermentation. The spectral studies of dye decolorization were carried out using the UV–Visible spectrophotometer.

Percentage decolorization was calculated according to the formula

$$D = 100(A_{ini} - A_{obs})/A_{ini}$$

where D is decolorization (in %), A_{ini} initial absorbance and A_{obs} observed absorbance.

2.3. Biochemical identification

2.3.1. Qualitative assay

(a) Plate assay method

On performing qualitative assay by using ABTS (2,2′–Azino-bis-[3-ethylbenzthiazoline-6-sulphonic acid]) as a substrate. The PDA plates were supplemented with 3.0 mM of ABTS and on this the white rot fungus was grown and evaluated for laccase production (Srinivasan et al., 1995; Prabu et al., 2006).

(b) Bavendam test

In the Bavendum test the substrate 10 mM guiacol (ortho-methoxy phenol) was used in potato dextrose broth and after that the three- four agar plugs having white rot fungus was added to it and examined for laccase positive strain (Soponsathien, 1998).

2.3.2. Quantitative assay

Laccase activity was determined by measuring the oxidation of 2, 2′–Azino-bis- (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at 420 nm ($\epsilon = 3.6 \times 10^4\ cm^{-1}\ M^{-1}$) (Niku-Paavola et al., 1998). The reaction mixture contained 1.0 ml ABTS (3.0 mM) and 0.8 ml sodium acetate buffer (0.1 M) of pH 5.0 and 0.2 ml aliquots of appropriately diluted enzyme extract at 60 °C temperature for 10 min.

One unit of laccase activity was defined as the amount of enzyme, which leads to the oxidation of 1 μM of ABTS per minute. The activities were expressed in U per milliliter of extracted fermented supernatant (U/ml).

2.4. Protein estimation

The protein concentration was estimated by using bovine serum albumin as standard (Lowry et al., 1951).

2.5. Determination of cell composition of CBM

Starch concentration of CBM was determined as total glucose concentration by dinitrosalicylic method after hydrolysis of the