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Research article

Wastewater cleanup using *Phlebia acerina* fungi: An insight into mycoremediation

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ABSTRACT

The scarcity of available drinking water has led the researchers to develop novel and cost-effective ways of bioremediation process for wastewater treatment. Bioremediation is a cost-effective and environmentally sound method for the removal of toxic compounds. Such approach is not only a chemical-less effort but also an energy savior. In the present work *Phlebia acerina,* a white rot wood rotting fungi have been used to degrade the toxic wastewater pollutants. Congo Red (CR) and Eriochrome Black T (EBT) have been selected as model pollutants to test the wastewater cleaning ability of the fungus. The Lignin modifying enzyme (LME) and Cellulolytic enzyme assays (CMC) potential of *Phlebia acerina* helped in understanding the dye degradation mechanism. Under the optimum conditions, the fungi was able to degrade as high as 92.4% CR while the EBT was degraded to a maximum of 50%. *Phlebia acerina* was found to show first-order kinetics of dyes degradation. Further, the seed germination and antimicrobial assay of treated and untreated water were carried out in order to establish the formation of non-toxic end product after degradation.

1. Introduction

Basidiomycetes or white rot fungi are common inhabitants of forest litter and fallen trees (Balachandra Dass, 1990). They are capable to depolymerize and degrade plant components including the most recalcitrant-lignin. The three major classes of extracellular enzymes manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase are believed to be important in the degradation by fungi (Bohacz, 2017; Ramos et al., 2016; Rytioja et al., 2014). A new group of ligninolytic scheme containing peroxidase is combining structural and functional properties of the LiP and MnPs, are the versatile peroxidases (VPs). In addition, an enzyme involved in hydrogen peroxidase production such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) are considered to belong to the ligninolytic system (Roncal et al., 2012). Some white rot fungi produce all three types of ligninolytic enzymes, but some have a tendency to secrete only one or two. The enzymes involved in the ligninolytic system are effective against a broad spectrum of aromatic compounds which are the part of dye (Han et al., 2014). This ability of the fungi have attracted the scientific attention on the use of white rot fungi in the dye degradation (Balachandra Dass, 1990; Selvakumar et al., 2013; Yang et al., 2016). The enzymes have high biotechnological interest as demonstrated by several studies, reporting their role for green processes such as wood pulp delignification, dye decolourization in the textile industry, ethanol production, wine processing, treatment of polycyclic aromatic hydrocarbon, bioremediation and for the realization of biosensors. The increasing demand for these enzymes from fungi have intensified the search for microorganisms having a high level of enzyme activities and for improved degradation of dyes as well as other xenobiotics.

Keeping the extraordinary properties of lingo-cellulolytic fungi, the two model dyes Congo Red (CR) and Eriochrome Black T (EBT) were selected for the experimentation. CR is a sodium salt of 3,3′-([1,1′-biphenyl]-4,4′-diyl)bis(4-aminonaphthalene-1-sulfonic acid) and red in colour. It has a very strong affinity towards cellulose fibres, therefore,

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used to dye the fibres in cotton textile, paper and wood pulp industries. It has been found that CR is carcinogenic and mutagenic to many organisms (Debnath et al., 2014). Another model dye, EBT is a sodium salt of the 1-Naphthalenesulfonic acid, 3-hydroxy-4-[(1-hydroxy-2-naphthyl)azo]-7-nitro. It is a complexometric indicator used in complexometric titrations such as water hardness estimation. EBT is known to have many toxicological effects to organisms such as irritation to eyes, damage to skin and respiratory tract. The instantaneous effects of EBT intake are nausea, vomiting and gastrointestinal irritations (Kaur and Singhal, 2015).

The researchers are more attracted towards advanced oxidation processes (AOP) based on free radical attack, as they generate no hazardous sludge (Chakma et al., 2015; Hassaan et al., 2016; Safdar et al., 2015). However, the sonochemical break down of the dyes is also trending due to its faster processing and lesser chemical consumption (May-Lozano et al., 2017; Wu et al., 2013). The major disadvantage of these quick methods is the requirement of external energy source such as UV radiation, adsorbent or degrading particles (Ding et al., 2016; Fida et al., 2017; Lučić Škorić et al., 2016) ultrasonic waves (Im et al., 2013) and catalysts (Bhatti et al., 2012; Gupta et al., 2017). To date, different low-cost biosorbents, such as alginate (Elwakeel et al., 2017a,b,c), bio-debris (Elwakeel et al., 2017a,b,c) and chitosan (Elwakeel et al., 2017a,b,c) etc. have been used to clearify wastewater. But the problem of sludge generation is the major hurdle in this field. Also, the problem of their restoration after the completion of the degradation process because their release in the environment may cause severe toxicity to the environment (Zhang et al., 2016). Other biological agents such as bacteria are also very effective in the degradation of harmful dyes. For example Sudha et al., 2018 used *Enterobacter aerogenes* for the complete degradation of azo dyes DB71 and DG 28. Many other studies by Elfarash et al., (2017) and Martorell et al., 2017 used micro-organisms to remove harmful contaminants from the environment. These methods have the major drawback as the bio-agents used in them are mostly pathogenic in nature. Their use in massive remediation can cause several ecological issues. Thus the choice of non-pathogenic bio-agent is highly emphasized in bioremediation.

Surpassing the above issues, mycoremediation is considered as a better option for wastewater treatment as no external energy is required except for the food for fungal growth. For basidiomycetes, the food is dead and decaying plant debris which is very cheap in terms of cost and also environmentally non-toxic. Moreover, the management of the water post-treatment is not required as the fungi are an integral part of the environment. Therefore, the mycoremediation of the toxic dyes is considered more beneficial than other conventional and latest advanced methods due to its environmentally friendly nature. The present piece of work aims to isolate a new species of basidiomycetes fungi collected from District Kinnaur, Himachal Pradesh, India producing ligninolytic enzymes. During the course of study, a two-step screening was used in order to facilitate isolation of potential species of fungi. The potential of the isolate- *Phlebia acerina* in fungal degradation of xenobiotics was ascertained keeping two different azo dyes as a model (CR and EBT). *Phlebia acerina* fungi used in our experimentation of dye degradation have no records of its toxicity, thus the method is non-toxic, cheap and eco-friendly by nature.

2. Material and methods

2.1. Study area and organism

Wood sample were collected from a forest area in District Kinnaur, Himachal Pradesh, India. The sampling area located at latitude between 31° 05′50′′ and 32° 05′15′′north latitude and longitude between 77° 00′ 45′′and 79° 00′ 35′′ east longitude (Fig. S1). Permissions for the collection of fungal species were not required as the location is a scenic spot. Also, the collection procedure did not involve any endangered, vulnerable or protected species and the fungus was common in this location. Decaying twigs, dead wood and plant barks were collected in sealable plastic bags and taken to the laboratory for investigation. The fungi were photographed (Fig. S2 a) and the sample was aseptically transferred to Petri plates containing malt extract agar media and the plates were incubated at 30 °C for 5–7 days. Once the fungal colonies were formed in agar plate they were sub-cultured into other plates to obtain pure culture for future studies.

2.2. Chemicals employed

Malt Extract Agar (MEA), $C_4H_{12}N_2O_6$, yeast extract, KH_2PO_4, $CaC1_2.2H_2O$, $MgSO_4.7H_2O$ carboxymethylcellulose (CMC), Endoglucanase, NaCl and KOH, $CuSO_4.5H_2O$, $Fe_2(SO_4)_3$, $MnSO_4.H_2O$. Azure B, agar, glucose, Mueller Hinton (MH) agar, Congo Red (CR) and Eriochrome Black T (EBT) were purchased from Sigma Aldrich. All the chemicals were used as obtained, without any further purification.

2.3. Instruments used

Microscopy of the mycelium was carried out using Nikon Eclipse E200 light microscope with an attached camera. Field emission scanning electron microscopy (FESEM) of the pure and dye adsorbed mycelium was carried out using Hitachi SU8010 electron microscope. Fourier transform infrared spectroscopy (FTIR) was used to determine the interactions between fungal biomass and test dyes. The spectra were measured within the range of 400–4000 cm^{-1} using Perkin-Elmer (RX1) spectrophotometer. UV–vis. the spectroscopic analysis of dye removal experiments was performed using LABINDIA 3000 + spectrophotometer.

2.4. Identification of isolates

The morphological identification of the isolates was attempted by microscopic observations (Fig. S2 b-d). The obtained specimens were mounted in 4% KOH and CR (for staining) and studied under a microscope. The molecular analysis was conducted by Bioserve Biotechnologies Pvt. Ltd. India. DNA was isolated from the fungal culture, amplified by PCR using ITS 1 and ITS 4 primers. The 400–900 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI database.

2.4.1. Screening and isolation of lignocellulolytic fungi

2.4.1.1. Cellulolytic enzyme assays. Cellulolysis basal medium (CBM) was prepared using 5 g of $C_4H_{12}N_2O_6$ and 0.1 g yeast extract with 1 g of KH_2PO_4, 0.001 g $CaC1_2.2H_2O$ and 0.5 g $MgSO_4.7H_2O$ in 1L distilled water and stored as 10X sterilized stock (Dashtban et al., 2010). The cellulolytic activity of enzymes was detected by staining of CMC. Endoglucanase 1.8% w/v agar was added to the CBM medium supplemented with 1% w/v low viscosity CMC. The medium was autoclaved, dispensed into Petri dishes, allowed to solidify and inoculated with discs of the test fungi and incubated at 30 °C. After 5 days, the plates were covered with 2% aqueous CR (for staining) and allowed to stand for 15 min. The stain then washed from the agar surface with distilled water and the plates then flooded with 1M NaCl to de-stain for 15 min. The NaCl solution was thoroughly washed. CMC degradation around the colonies appeared as a yellow opaque area