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Biodegradation and detoxification of azo solvent dye by ethylene glycol tolerant ligninolytic ascomycete strain of *Pseudocochliobolus verruculosus* NFCCI 3818

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ABSTRACT

Ligninolytic microorganisms or enzymes mediated biodegradation of toxic pollutants was studied extensively; but hydrophobic waste management is a major environmental concern. For this reason, 80 fungal strains were isolated from various ligninocellulosic containing microhabitats and around 27.91% strains, demonstrated the ligninolytic activity. Screened isolates were further examined for organic solvent tolerance and the fungal strain LSF9 displayed better growth along with laccase (157 UL^{-1}), lignin peroxidase (LiP; 12 UL^{-1}) and manganese peroxidase (MnP; 8.6 UL^{-1}) activity in the presence of water miscible ethylene glycol. The strain LSF9 was further identified as *Pseudocochliobolus verruculosus* based on the morphotaxonomic and molecular approach. The *P. verruculosus* was able to degrade water insoluble Solvent yellow 2 and display 98% decolorization with maximum laccase activity (52.74 UL^{-1}). The degradation profile and metabolic fate of fungus treated dye was revealed by HPLC, FTIR and GC-MS analysis. The phytotoxicity study of fungus treated dye on *Triticum aestivum* (wheat) and *Vigna radiata* (mong bean) confirmed the nontoxic nature as compare to the phytotoxic Solvent yellow 2 dye. Thus, the present study suggests the potential of ethylene glycol tolerant *P. verruculosus* for environmental decontamination of solvent soluble azo dyes.

1. Introduction

Azo dyes are the most common synthetic colorants (~3000) preferred for coating, paper printing and textile dyeing because of their (i) wide variety of colour spectrum, (ii) better firmness profile, (iii) simple application, (iv) inexpensive synthesis, (v) ease in structural modification, and (vi) capacity to bind to most synthetic fibers (Bae and Freeman, 2007). The extensive application caused release of about 10–20% dye load into the environment (Wesenberg et al., 2003) which presents aesthetic objections (Banat et al., 1996), human health and ecological threats since azo dyes are, toxic, carcinogenic, mutagenic, and recalcitrant to biodegradation (Bae and Freeman, 2007; Celebi et al., 2012). Consequently, the azo dyes from effluents are not completely removed with conventional treatments in compliance with environmental legislation (Ali, 2010).

Amongst azo dyes, Solvent yellow 2 (SY2) is soluble in organic solvents and sparingly soluble in water. This property widened the application of SY2 to colour lacquers, polystyrene, varnishes, printing inks, stains, plastics, cosmetics, waxes, soaps, fats, oils and gasoline (Sabnis, 2010). As a result, release of organic solvent soluble SY2 raised serious environment concern (Pereira and Alves, 2012) since SY2 is documented as a potential mutagenic carcinogen, also implicated for genotoxicity, haematological effects, etc. (McCann and Ames, 1975). The solubility of SY2 in non-aqueous solvent system is major constrain for its biodegradation. The existing conventional chemical treatments using catalytic ozonation, photocatalysis, ultrasonic irradiation and electrochemical oxidation (Sharma et al., 2013), proved effective for water soluble azo dyes, but these techniques are impractical to remove azo solvent dyes as it generate a large amount of sludge, require more time and expensive (Robinson et al., 2001; Wang et al., 2008). In contrast, biological decolorization and degradation of azo dyes with bacteria, fungi, and algae have been of considerable interest due to their inexpensive and ecofriendly nature (Dafale et al., 2008; Jafari et al., 2014). Although, bacteria remove azo dyes from aqueous system

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by specific azo reductase activity that acts on (–N=N–) linkages; however, there is a high tendency to produce toxic and carcinogenic aromatic aryl amines as dead-end-products (Spadaro et al., 1992; Dawkar et al., 2009). Moreover, exposure of degradation products to oxygen may restore colour to the effluent. Alternatively, fungi have (i) strong resistance to dye toxicity (Pinedo-Rivilla et al., 2009), (ii) better capacity to mineralise diverse range of persistent priority organic pollutants including azo dyes in aqueous as well as non-aqueous media (Okazaki et al., 2002; Selvam et al., 2003) and (iii) ability to secret non specific and non-stereoselective oxidative enzymes (Wesenberg et al., 2003; Giardina et al., 2010) consisting of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Kuhad et al., 2004; Giardina et al., 2010). Hence, it is generally perceived to screen fungi for dye decolorization and biodegradation. The biodegradation of series of synthetic azo dyes have been reported by aerobic treatment (Kuhad et al., 2004; van der Zee and Villaverde, 2005) with white-rot basidiomycetes (Spadaro et al., 1992; Paszczynski and Crawford, 1995; Wesenberg et al., 2003; Pinedo-Rivilla et al., 2009). Besides, biotreatment of azo dyes with fast growing ascomycota has proved promising since the oxidative enzyme systems utilize natural redox mediators (Rodríguez-Couto, 2012; Adnan et al., 2015). The non-specific nature of fungal lignin degrading enzyme systems, such as LiP, MnP and laccase has already enabled to degrade a wide range of xenobiotics (Husain, 2006; Perlatti et al., 2012). However, each fungal strain has variable enzymatic profile and display different response. Hence, biotreatment with ligninolytic fungi are limited to water soluble azo dyes and a non aqueous system was suggested for decolorization of water insoluble solvent azo dyes (Sugimori et al., 1999; Okazaki et al., 2002). Several microbes secreting hydrolytic enzymes functional in organic solvents have been reported earlier (Rahman et al., 2005; Gupta and Khare et al., 2009; O'Reilly and Magner, 2011). Although, Spadaro et al. (1992) has demonstrated 29–46% and 4.5–23.1% degradation of Solvent yellow 2 and Solvent Yellow 14 dyes, respectively after 12 days by ligninolytic *Phanerochaete chrysosporium* in nitrogen limiting conditions; there have been few studies on decolorization and degradation of water insoluble dyes using organic solvent tolerant microbes secreting ligninolytic enzymes. Hence, newer biodegradation systems are required for removal of organic solvent soluble azo dyes. Also, Okazaki et al. (2002) employed microperoxidase (MP-11) for oxidative catalysis of azo solvent dyes such as solvent orange 7, solvent blue 11, solvent red 24, solvent green 3 in hydrophilic organic solvents viz. ethylene glycol and methanol as well as hydrophobic organic solvents viz. isooctane.

Therefore, solvent stable/ tolerant ligninolytic fungal strains with their enzymes are useful to decolorize and biodegrade solvent dyes in organic media. Towards this, the most preferred approach is to isolate solvent tolerant fungal strains from ecohabitats so that ligninolytic oxidative enzymes could catalyze oxidative cleavage of solvent dyes in organic solvent media. In lieu of the above facts, the foremost goal was to search for solvent tolerant ligninolytic fungi. The present study describes screening and identification of ligninolytic enzymes secreting solvent tolerant fungal isolate from lignocellulosic eco-habitats for its wider applications like decolorization and degradation of azo solvent dye (SY2) in non-aqueous conditions. This study also demonstrated the biodegradation pathway of SY2 by the efficient ligninolytic fungal isolate.

2. Materials and methods

2.1. Chemicals and reagents

2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), guaiacol, veratryl alcohol, 2, 6 dimethoxyphenol (DMP), 1-hydroxybenzotriazole (HBT), Lignin peroxidase, laccase from *Trametes versicolor* were procured from Sigma-Aldrich (St. Louis, USA). Solvent yellow 2 dye was the generous gift from Mukund Polychem Ltd., Jalgaon (India). Other chemicals and media used were of analytical grade and obtained from Hi-Media, Mumbai.

2.2. Enrichment and isolation of fungal strains

Samples (100 g) were collected from different solvent contaminated soil, decaying wood, soil-water sediments of nearby local industry in Jalgaon, Maharashtra and Gujarat for isolation of fungi as per Liu et al. (2009). The samples (lignin containing soil, sediment, rotten and decaying wood) were sieved (2 mm mesh size), homogenized and 5 g of each sample was added into 250 ml Erlenmeyer flasks containing 20 ml sterilized saline. Each flask was agitated on rotary shaker for 30 min to ensure complete distribution, then spread onto PDA and incubated at 28 °C for a week. The fungal isolates were periodically subcultured and maintained routinely on the same medium at 4 °C.

2.3. Screening of fungi for ligninolytic activity

The fungal isolates were examined for secretion of ligninolytic enzymes viz. ligninase, phenoloxidase, laccase and peroxidase. Initially, the fungal strains were grown on PDA and incubated at room temperature for a week. After incubation, fungal mycelium plug (~5 mm^2) of each strain was cut from the colony margin for inoculation onto PDA containing 0.5% (w/v) tannic acid and guaiacol (4 mM) for detection of phenoloxidase (Lopez et al., 2006) and laccase (D'Souza et al., 2006), respectively. The peroxidase activity was examined by drop screen method by adding few drops of equal parts of pyrogallol (1%) and H_2O_2 (0.4%) reagent to the 5 d old fungal culture grown on PDA for yellow-brown colouration (Lopez et al., 2006). Ligninase of each fungal isolate was determined as per Sundman and Nase (1971) using minimum mineral medium supplemented with kraft lignin. After 7 days of incubation, each plates was flooded with reagent containing $FeCl_3$ and $K_3[Fe(CN)_6]$ for green colour to media and clear zone around growth of fungal strains.

Similarly, fungal strains were also pre-grown aerobically in 250 ml Erlenmeyer flask containing 100 ml PDB at 28 °C with shaking at 120 rpm for a week. Cell-free supernatant (100 µl) of each isolate was examined for ligninolytic activity in 96 well microtiter plate containing 100 µl of 2, 6 DMP (5 mM) for laccase and equal parts of 1% pyrogallol and 0.4% H_2O_2 for lignin peroxidase. The reaction mixture was incubated at room temperature for 10 min to detect orange and yellow coloration, respectively. Lignin peroxidase and laccase (*Trametes versicolor*) were used as positive control.

2.4. Enzyme assay

LiP activity was assayed as per Tien and Kirk (1988) using veratryl alcohol and monitored at A_{310} for the formation of veratraldehyde (ε_{310}=9300 M^{-1} cm^{-1}) in sodium tartrate buffer (pH 2.5). MnP activity was estimated by the oxidation of 2, 6-DMP and 1 mM $MnSO_4$ at A_{469} in 50 mM sodium malonate (pH 4.5) (Moreira et al., 2001). Laccase activity was carried out using 2, 6-DMP (ε_{469}=27500 M^{-1} cm^{-1}) in 100 mM acetate buffer (pH 4.5) (Chakroun et al., 2010). One unit of enzyme activity (U) was defined as the amount of enzyme that oxidized 1 µmol of substrate into product per min under experimental conditions.

2.5. Screening of solvent tolerant fungal strains

The fungal strains that exhibited ligninolytic activity were grown on PDA amended with different solvents (organic solvents with dibutyl

Each fungal strain was inoculated with two mycelia agar plugs (~5 mm^2) in 500 ml glass bottle containing 50 ml PDB and overlaid with 5–25% (v/v) of organic solvents. Each bottle was sealed with