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Original Research Article

Screening of Mycelia of Milky Mushroom (*Calocybe indica*) Strains for their Dyes Decolorization Potential

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A B S T R A C T

Milky mushroom is gaining popularity in the tropical parts of India and cultivated round the year while in subtropical parts it is preferred during summer seasons. The evaluation of five strains of *Calocybe indica* for the action of fungal mycelium to decolourize synthetic organic colorants was studied and compared in order to devise an easy decolorization and bioremediation strategy for the treatment of textile industry effluents. Solid state and broth culture studies were carried out in medium supplemented with 2.5, 5.0, 7.5 and 10 mg/100 mL of Amido-black, Congo red and Remazol Brilliant Blue R (RBBR) dyes to study the decolorization potential of Ci-1, Ci-3, Ci-6, Ci-7 and CBE 1515 strains of *C. indica.* The results showed that all the strains have a promising potential for dye decolorization but significant decolorization was shown by CBE 1515 for the dye Amido black.

Keywords

Calocybe indica, Dyes, Decolorization, Bioremediation

Introduction

Out of the total pollution load, about 8-20% has been reported to be contributed by incomplete exhaustion of the dye (Cristóvão *et al.,* 2009). Azo dyes constitute the biggest class of colors utilized economically. There are more than 8,000 synthetic items recorded in the shading list which are related with the coloring procedure, while more than 100,000 financially accessible dyes exist with more than 700,000 metric huge amounts of dyestuff created every year (Park *et al.,* 2004). The physical methods likeadsorption, flotation and chemical methods like fenton oxidation, chlorination, reduction, ozonization, ion exchange and incineration are mostly used to treat the effluents containing dyes (Cristóvão *et al.,* 2009). Though these techniques are compelling, they have a few disadvantages such as formation of hazardous by products, and intensive energy requirements. Therefore, the applications of microbial degradation as a better alternative are attracting much attention, and several microorganisms for degrading toxic compounds have been reported and their characteristics have been investigated (Park *et al.,* 2004).

Bioremediation involves the use of microorganisms to degrade, remove or detoxify contaminations of soil, water or sediments that otherwise threaten public health. Due to their ability to elaborate

various complex compounds many fungal strains are known to degrade a wide variety of complex compounds like xenobiotics, lignin and dyestuff. Moreover there are many reviews that have shown numerous fungal strains are accomplished in degrading different types of synthetic dyes for example azo, polymeric, heterocyclic, phthalocyanine dyes and triphenyl methane (Park *et al.*, 2004).

Among all fungal groups, the white rot fungi in particular release extracellular compounds like laccase, lignin peroxidase and manganese peroxidase, which are capable of breaking down lignin in lignocellulosic substrates. This method of ligninolytic degradation of many white rot fungi is directly involved in the degradation of varied recalcitrant compounds and dyes. The capacity of the white rot fungi to degrade color can be specifically corresponded with its capacity to degrade lignin, along with lignin the dye molecules get degraded. Use of white rot fungi is the one of idiomatic application in the technology of bioremediation to debase structurally distinct xenobiotic organo pollutants. (Tripathi *et al.*, 2007)

Materials and Methods

The qualitative screening (in solid medium) and quantitative screening (in aqueous medium) experiments were run in triplicates

Fungal culture and dye stuff

Calocybe indica (P and C) strains Ci-1, Ci-3, Ci-6 and Ci-7 were procured from germplasm collection bank of the Department of Microbiology, Punjab Agricultural University, Ludhiana. *Calocybe indica* (P and C) strain CBE 1515 was procured from the Department of Plant pathology, Tamil Nadu Agricultural University, Coimbatore. Three textile dyes which are mostly used in dye industry i.e., one Anthraquinone dye [Remazol Brilliant Blue- R (RBBR) dye] and two azo dyes [Amido black (AB) and Congo red (CR)] were used to study the dye degradation potential of *C. indica* strains at variable concentrations of dyes 30±2°C. The decolorization of dyes was recorded at the respective λ_{max} values.

Preparation of culture medium

To quantify the decolorization efficiency of *C. indica* strains potato dextrose agar (PDA) medium was used with the following composition: filtrate obtained from 250g peeled and boiled potatoes in 500-600 ml of water, dextrose 18g and agar-agar 20g. Dextrose and agar were dissolved in the extract and the volume was made to 1000 ml. The final pH was adjusted to 6.5 and medium was sterilized by autoclaving at 121°C for 20 minutes at 15 psi.

Preparation of stock and working concentrations of dyes

The stock solutions of 5000 ppm of Remazol Brilliant Blue- R dye, 5000 ppm of Amido black and 5000 ppm of Congo red were prepared using 0.25 micrometre sterile cellulose acetate syringe filter in distilled water and stored at 4°C in reagent bottles covered with black paper.

Qualitative and quantitative screening procedure

Radial growth studies in solid medium

The potato dextrose agar medium plates supplemented with different working concentrations of 0.5% (w/v) of RBBR, Congo red and Amido black were inoculated with 5 mm diameter mycelial bit, which

was10 days old fungal cultures of Ci-1, Ci-3, Ci-6, Ci-7 and CBE-1515 strains individually per plate under aseptic conditions. The inoculated plates were incubated at 30°C in the dark for period of 10 days. The plates were observed on daily basis for radial growth of fungal mycelium and change or appearance of any color on the agar plates. The intensity of color disappearance or appearance was compared in each concentration of supplemented compounds with that of uninoculated plates as well as among the five strains of *C. indica.* The positive controls were run by inoculating the unsupplemented agar plates of respective media with *C. indica* cultures individually.

The mycelia growth and zone of dye clearance were recorded by recording the colony diameter (in cm) at 2 days interval up to 10^{th} day.

Decolorization studies in aqueous medium

The pre sterilized 50 ml PDB flasks were supplemented with different working concentrations of 0.5% (w/v) of RBBR, Congo red and Amido black then inoculated with 5 mm diameter mycelial bit, which was 10 day old fungal cultures of Ci-1, Ci-3, Ci-6, Ci-7 and CBE-1515 strains individually per flask under aseptic conditions. The flasks were then incubated at 30± 2°C under dark conditions at 100 rpm for 10 days. The dye decolorization was recorded at 2 day interval by recording absorbance 610nm for both RBBR and Amido black while Congo red was recorded at 565nm. Dye decolorization efficiency of different dyes was calculated by using following formula:

$$\% \text{ decolorization} = \frac{(\text{Initial Absorbance} - \text{Final Absorbance})}{\text{Initial absorbance}} \times 100$$

Results and Discussion

Decolorization studies in agar medium

Though all the five strains were able to degrade the synthetic dyes; but on the basis of color intensity and time required for positive results in the form of appearance or disappearance of zone, the strains Ci-3, Ci-7 and CBE 1515 were found to be having higher potential to degrade the synthetic dyes whereas the strains Ci-1 and Ci-6 were shown less potential to degrade the dyes. The brownish red color zones were formed in Amido black supplemented plates even at higher supplementation rates indicating the oxidative polymerization of Amido black by the laccase produced extracellularly into the medium. In RBBR supplemented plates production of colorless halo rings was observed in lower concentrated plates (25 ppm and 50 ppm) while there was small or no zone production was observed in two higher concentrated plates (100 ppm and 200 ppm).The supplementation rate of Congo red in growth medium adversely affected the mycelial extension rates and observed no color zone formation.

Decolorization studies in aqueous medium

Amido black supplemented liquid medium

The trend for potential dye decolorizing strain has been varied at different incubation period. On 8^{th} day of incubation maximum percent decolorization was observed by the strain CBE 1515 in PDB flasks supplemented with 100 ppm (72.12%) of Amido black, which is on par with 200 ppm (71.67%) of dye supplementation. Following the strain CBE 1515, strains Ci-6 and Ci-7 had shown maximum percent (68%) removal of dye at 100 ppm of dye

supplementation. The concentration of dye has also shown adverse effect on decolorization potential of fungal strains. In general, maximum decolorization was observed at 100ppm which was at par with 50ppm concentration followed by 25pmm and minimum decolorization was observed as we proceed to higher concentration like 200ppm. Color change of dye from bluish black to reddish brown in Amido black supplemented broth was observed, which could be due to the oxidation of dye molecules by laccase enzyme produced by growing mycelium. The intensity of colour change is directly proportional to the concentration of synthetic dye.

RBBR supplemented liquid medium

Maximum of 72% of RBBR (50 ppm) has been observed to be decolored in PDB flasks inoculated with the strain Ci-3 on 8th day of incubation, followed by the strain Ci-7 that could remove dye to an extent of 62% and minimum potential for RBBR decolorization was observed in flasks inoculated with the strain Ci-1. The decolorization percent was varied as the concentration of RBBR was increased from 25 ppm to 200 ppm. In general, maximum mean per cent of dye decolorization was observed at 50ppm concentrated flasks (60.35%) followed by 100ppm (56.62%) and 200ppm (58.50) which was at par. The minimum decolorization was observed at lower concentration i.e., 25ppm (49.31%)

Congo Red supplemented liquid medium

Highest percent removal (68.92%) of Congo red was achieved by the strain Ci-3 in PDB flasks supplemented with lower (25 ppm) concentration of dye, followed by the strain CBE 1515 (58%). Three strains Ci-1, Ci-6 and Ci-7 were found to be less potential for Congo red decolorization. It was observed that the decolorization potential was indirectly proportional to the concentration of synthetic dye. As the concentration was increased the percent decolorization was decreased. The trend for different concentrations of Congo red on percent decolorization was observed as follows 25 ppm (52.72%) > 50 ppm (51.12%) > 100 ppm (46.63%) > 200 ppm (39.29%) on 8th day of incubation.

The brownish red colour zones were produced by all five strains of *C. indica* on Amido black supplemented plates, which was the strong indication of production of extracellular lignin degrading enzymes. Laccase positive reactions can be easily identified by the color reaction of laccase with synthetic dye and guaicol. The oxidative polymerization of synthetic dye and guaicol are catalyzed by the enzyme laccase results in the formation of reddish brown zones in the medium (Kiiskinen *et al.,* 2004). The brownish red color zones formed in present study indicates the oxidative polymerization of Amido black by the laccase produced extracellularly into the medium. The present study results were in similarity with aqueous culture studies done by Kaur and co-workers (2016) using *P. florida* and *C. indica* for decolourizing Amido black and Sudan black.

It was observed that both the cultures resulted in significant decolorization of Amido black 10 B which having greater solubility PDB as compared to Sudan black. It was also found that the increased dye concentration resulted in decreased fungal decolorization efficiency. Amido black 10 B was decolorized up to 80 percent using both *P. florida* and *C. indica*. The decolorization at different dye concentration did not vary significantly; however maximum decolorization was observed at 150mg/l concentration (Poornima *et al.,* 2014) (Fig. 1 and 2; Table 1)

Fig.1 Diameter of fungal colonies (cm) of *C. indica* strains on agar plates supplemented with different concentrations of three chemically different dyes

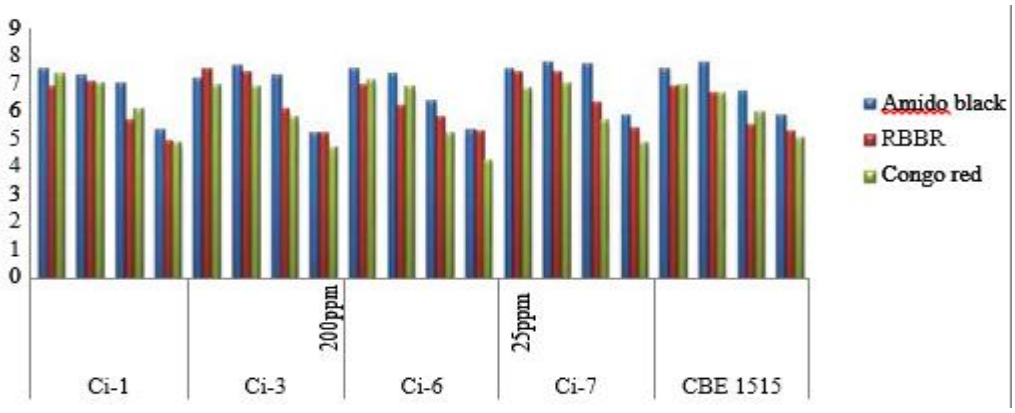
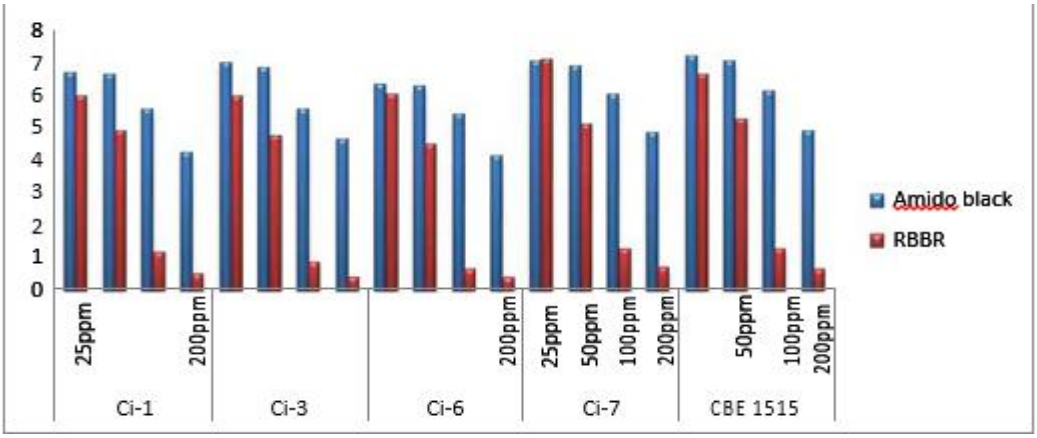


Fig.2 Diameter of colored zone formed on synthetic dye supplemented agar plates



Working concentrations of various stock solutions

S.No	Compound	Stock concentration	Working concentration (ppm)	Volume of stock added to 200 ml of medium
1	RBBR	0.5% (w/v)	25,50,10 0,200	1ml, 2ml, 4ml, 8ml
2	Amido black	0.5% (w/v)	25,50,10 0.200	1ml, 2ml, 4ml, 8ml
	Congo Red	0.5% (w/v)	25,50,10 0,200	1ml, 2ml, 4ml, 8ml

Table.1 Per cent decolorization of three different synthetic dyes by *C. indica* strains in Aqueous Medium

Strain	Concentration (ppm)	% decolorized		
		Amido black	Congo Red	RBBR
Ci-1	25	56.67	51.57	38.03
	50	59.81	50.43	57.00
	100	62.14	48.24	55.75
	200	38.63	38.81	56.39
	Mean	54.16	52.26	51.79
Ci-3	25	60.46	68.92	56.99
	50	57.70	64.32	72.05
	100	65.37	58.90	68.67
	200	50.79	34.43	58.69
	Mean	57.74	56.64	63.17
Ci-6	25	55.74	32.96	49.61
	50	58.07	37.63	61.33
	100	68.92	37.83	58.69
	200	44.41	31.69	58.92
	Mean	56.78	35.02	57.13
Ci-7	25	55.77	42.11	56.58
	50	61.32	36.45	53.32
	100	68.99	35.63	62.51
	200	64.15	36.43	57.63
	Mean	61.21	37.65	56.99
CBE 1515	25	58.92	58.05	45.36
	50	67.07	56.77	58.07
	100	72.12	52.55	43.25
	200	71.67	55.13	60.91
	Mean	63.76	55.62	51.89
CD (P≤5%)	Strain(A)	2.69	1.87	2.29
	Concentration (B)	2.41	1.67	2.05
	A*B	5.39	3.74	4.59

Average of triplicates; Incubation temperature: 30°C; Agitation: 100 rpm

During the primary screening process RBBR dye showed consequent results on radial growth, decolorization zone and potency index values of four fungi *P. chrysosporium, P. sanguineus, P. sajor-caju* and *P. radiate*. At optimal growth temperatures of each fungus, *P. chrysosporium* decolorized about 95.0% of the RBBR plate after 6 days of incubation, followed by *P. sanguineus* (32%), *P. sajor-caju* (28.9%) and *P. radiate* (19.2%). When incubated at 35°C

P. sajor-caju showed 70.5% and 92.2% reduction in halo and colony areas, respectively on the 6th day of incubation. However *P. radiate* did not show any decolorization of RBBR or growth. The halo to colony ratio (potency index) of RBBR decolorization at respective fungal optimal growth temperatures was in order of *P. radiate, P. sajor-caju, P. sanguineus* and *P. chrysosporium* on the 2nd day of incubation. In contrast, the halo to colony ratio of RBBR decolorization at 35°C was in the order of *P. sajor-caju, P. chrysosporium, P. sanguineus* and *P. radiata* (Teck *et al.*, 2011).

The results are in accordance with the studies made by Palmieria and his associate's decolourisation estimation by removal of blue color during fungal growth. The disappearance of blue color in RBBR supplemented medium proceeds through an initial development of pinkish blue and later colorless.

The complete decolorization of the blue color was achieved at the 8th day of fungal growth only in the presence of veratryl alcohol using either MYP or PDY broths. *P. ostreatus* have greater efficiency (>90%) in decolourizing RBBR, it can able to decolourize the dye within 3–6 days at low (5µM) and high (50µM) dye concentration (Palmieria *et al.*, 2005)

Nithya *et al.*, compared plain and nanoparticle culture and found that the plain culture could degrade only 67% of the dye as compared to nanoparticle, the complex nature of Congo red might be the reason for minimal decolorization efficiency of plain culture. A slower rate of decolorization was attributed to higher molecular weight, structural complexicity of the dyes (Hu and Wu 2001). Congo red and Crystal Violet were transformed by 47% and 65% respectively, but were never completely degraded even after extended incubation times *Trametes* sp. SQ01 laccase, as the purified enzyme. In contrast, Congo Red, Crystal Violet, Cresol Red and CBB G250 were completely degraded by *Trametes* sp. SQ01 itself in liquid culture, provided that enough time was allowed (Yang *et al.*, 2009). The absence of zone of inhibition on agar plates indicated that the fungal degraded dye metabolites are nontoxic to beneficial micro-flora (Seema and Quazi 2013).

The edible, protein rich and non sporulating mushroom (*C. indica*) mycelia can help to improve the quality of effluents released by textile and dyestuff industries. Use of these fungi for decolorization of textile effluents can be an effective method of bioremediation.

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