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Research Article

BIODEGRADATION OF SYNTHETIC DYE BY ENDOPHYTIC FUNGAL ISOLATE IN *CALOTROPIS PROCERA* ROOT

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Abstract

In this study, based on colony morphology characteristics, a total of 19 fungal endophytes were isolated from root of *Calotropis Procera* a traditional Indian medicinal plant. All fungal isolates were screened for their dye degradation ability. The dyes used as test dyes were Rose Bengal (RB), azo dye Methyl Red (MR), Coomassie Brilliant Blue (CBB) and Methylene Blue (MB) and the concentration of each dye in the experiment was kept 100mg/L. Among the 19 fungal endophytic isolates (CPR1-CPR19), only one isolate CPR4 showed strong dye decolourization capability against all the four test dye. Dye decolourization ability by the isolate CPR4 was determined to be 97.4%, 87%, 65% and 45% for Rose Bengal (RB), Methyl Red (MR), Coomassie Brilliant Blue (CBB) and Methylene Blue (MB) respectively. Complete colour decolourization was observed with rose Bengal followed by Methyl Red. Glucose minimal medium was used for liquid and solid culture of fungal isolates. Fungal biomass production in the presence of four test dye was studied and compare with control culture of fungal endophytes. Effect of temperature, pH, stationary and agitation conditions on dye degradation was also studied.

Keywords: Synthetic dye; endophytic fungus; decolourization; Minimal medium.

Introduction

In recent past times, because of rapid growth of industrialization process, pollution attributable to dyes has been increased to the great extent. Globally, thousands of the dye stuffs are being synthesized every day. On an estimate about 10,000 commercially available dyes exist and more than 7×10^5 tons of dyestuffs are being produced commercially and annually. Considerable amount of different dyes and auxiliary chemicals are being used during dyeing process in textile dyeing industries and eventually discharges huge amount of strong colored wastewater containing a large amount of dye particulates/molecules (Yogesh *et al.*, 2012). The effluent is of significant environmental alarm since it drastically decreases oxygen concentration due to the presence of hydrosulfides and also creates hindrance in the passage of light through water body which is detrimental to water ecosystem, where great diversity of micro-flora and fauna exist. Waste water from dye utilizing industries contains an array of azo and other synthetic dyes in addition to its characteristically high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Mohamed *et al.*, 2014). Among these dyes, azo dyes are the most extensively used and these account for over 60% of the total number of dye structures known to be manufactured (Lamia *et al.*, 2011; Weeranuch *et al.*, 2014). These dye are being disposed in the environment in

the form of effluents during the process of synthesis and dyeing. Dyes are one of the major constituents of this type of environmental pollution. These are one of the largest, important and diffuse groups of synthetic dyes used regularly in textile dyeing and paper printing. Loss of reactive dyes through hydrolysis during industrial dyeing processes is up to 50% and up to 15% of it is contributed by azo dyes (Shekhar *et al.*, 2011). For the reason that many dyes discharged from the textile industry have been shown to be toxic, mutagenic and carcinogenic, release of synthetic dyes into the environment may give rise to the sever environmental pollution and threaten human health (Li *et al.*, 2014). The structural diversity of dyes arise from the use of diverse chromophoric groups (e.g. azo, anthraquinone, triarylmethane and phthalocyanine groups) and different application technologies (e.g. reactive, direct, disperse and vat dye (Ivana *et al.*, 2007). Physical or chemical methods including adsorption, precipitation, chemical degradation or photo degradation are usually challenging to completely degradation and decolourization of these dye. The physio-chemical remediation techniques also have financial and methodological disadvantages (Muhammad *et al.*, 2013). Conventional biological management methods such as activated sludge or biofilm system which are mainly predominant by bacteria population had very low dye mineralization efficiencies due to the narrow substrate range in various degrading bacteria (Q. yang *et al.*, 2009).

Biological decolourization of dyes via fungi, bacteria and algae peroxidases have also been reported (Cátia *et al.*, 2010). Microorganisms that inhabitate the diverse eco-physiological groups including white rot fungi have been reported to degrade and detoxify industrial dyes to a significant extent because they are remarkable producers of oxidoreductive enzymes (peroxidases, manganese peroxidases, lignin peroxidases and laccases) (S Saroj *et al.*, 2014, J. Axelsson *et al.*, 2006, Petr *et al.*, 2006, Perlatti *et al.*, 2014]. Till date only a little experimental works have been reported which explains the use of endophytic fungi degradation of synthetic dye.

This study aims to investigate the potential of isolated endophytic fungi from root of traditional Indian medicinal plant, *C. procera*, to tested for their dye degradation /decolourization ability against four test dye; Rose Bengal (RB), azo dye Methyl Red (MR), Coomassie Brilliant Blue (CBB) and Methylene Blue (MB)

Material and Methods

Plant Material

Plant samples were collected from the surrounding areas of Agricultural fields of Banaras Hindu University (BHU). Identity of the plant was confirmed on the basis of external morphology characteristic features. A complete mature and healthy plant was rooted out from soil surface. The samples were collected in sterile polythene bags and brought to the laboratory in an icebox. Samples were preserved at 4 °C in a refrigerator and processed for isolation of endophytic fungi immediately.

Isolation of Endophytic Fungi

Dye degrading endophytic fungus used in this study was isolated from the healthy root of plant; *Calotropis procera*. To eliminate epiphytic microorganisms, Root and stem samples of *C. procera* were washed thoroughly in running tap water for 15 min followed by four times washing with double distilled water to decrease the concentration of microorganisms from the sample surface. The surface treatment and the effectiveness of surface sterilization were checked following to the method of Schulz et al (Suredra *et al.*, 2012). Surface microorganisms of root were removed by dipping in 70% (v/v) ethanol for 2–3 min and in aqueous solution of sodium hypochlorite (4% v/v) for 3–4 min followed by washing with 70% (v/v) ethanol for 30s. The tissues were then rinsed three times with sterile double distilled water and allowed to surface dry in sterile conditions. Complete sterilization was confirmed by inoculating a very small volume of last washing water was inoculated into petri dishes containing peptone 5 (g/l), malt extract 5 (g/l), sodium chlorite 1.0 (g/l) and agar 2.5%. The pH of the medium was adjusted to 5.5 (Schulz *et al.*, 2009). After surface sterilization, Roots were cut aseptically into small pieces of 0.5 cm length and transferred to sterilized petri dishes containing (g/l): yeast extract 3.0, malt extract

3.0 peptone 5.0, dextrose 10 and agar 2.5%. The pH of medium was adjusted to 5.6. To check bacterial growth inoculation medium was supplemented with streptomycin (150 µg/ml) and chloramphenicol (150 µg/ml) (Schulz *et al.*, 1993). The petri dishes were incubated at 28°C and the plates were observed each day. Hyphal tips of developing fungal colonies were subcultured separately. Fungal isolates were distinguished based on its colony morphology.

Dye Decolourization Experiments

To test decolourization of dye, the isolated endophytes were grown onto glucose minimal (GM) agar plates and initially screened for their ability to decolorize Rose Bengal, Coomassie Brilliant Blue, methylene blue and one azo dyes (Methyl red). The Glucose minimal agar medium contained: glucose; 10(g/l); ZnSO₄ ·7H₂O, 0.01(g/l); MgSO₄ ·7H₂O, 0.5(g/l); K₂HPO₄ (g/l); CuSO₄ ·5H₂O, 0.05(g/l); FeSO₄ ·7H₂O, 0.01(g/l); KCl, 0.5(g/l); NaNO₃, 3(g/l); and agar, 20(g/l) (A. Amirita et al 2012). The pH of the agar medium was adjusted to 5.5 and autoclaved at 121 °C for 15 minute, and apical part of 6-day old fungal mycelium was used as inoculant. Agar plug (6mm diameter) containing fungal mycelia were placed onto medium containing petri plates. All inoculated Petri plates were kept in an incubator at 28.5 °C. The four dyes were added separately into GM agar plates at concentration of 100 mg/liter. A little amount (30 µg/1000 ml) of streptomycin was also added in the medium to avoid any bacterial contamination. Another set of four petri plates containing same medium composition with dyes (100mg/l) were also prepared and these petri plates were not inoculated and used as control to compare the visual disappearance of colour from the inoculated Petri plates. Plates were on a regular basis monitored and observed for visual disappearance of colour for a period of 20 days. On the basis of observation, most efficient dye decolorizing isolate was selected for further dye decolourization in liquid GM medium. Dyes were added to the 50mL GM liquid medium in 250mL Erlenmeyer flask to a final concentration of 100mg /l and each flask was inoculated with a piece of 5 mm² agar plugs from a 6-day old fungal culture and incubated under static condition at 28±2 °C. Flask with the respective dye and no fungal inoculums was used as control. All cultures were incubated for a period of 20 days, and sampled at every 5-day interval. Each culture condition was repeated three times to avoid error in data calculation.

Periodically 3ml of the culture broth was sampled in an Eppendorf tube and centrifuged at 10,000 rpm for 5min to obtain the supernatant. Fungal biomass was determined by drying the fungal mycelium to a constant weight at 70 °C. The dye disappearance of each supernatant was determined spectrophotometrically by monitoring the absorbance at the wavelength maximum for each dye (549nm for Rose Bengal, 520 nm for Methyl red, 470 nm for Coomassie Brilliant Blue and 664 nm for Methylene blue). The

decolorizing rate of each dye by cell-free culture was determined by the difference in the absorbance at the maximum wavelength between the initial and sampling values at each sampling time.

Percentage of decolourization was calculated according to the following formula

$$\text{Percentage of Decolourization (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c is the absorbance at the maximum absorption wavelength of dye in the control flask at time, t , and A_s is the absorbance at the maximum absorption wavelength of dye in the sample flask at time, t

Molecular identification of endophytic fungus CPR 05

Molecular identification of endophytic fungus CPR-5 was conducted on the basis of 18S ribosome RNA sequence using polymerase chain reaction (PCR) cloning technology. Genomic DNA was isolated from fungal mycelia using PrepMAN Ultra Sample Preparation Reagent kit according to the manufacture's recommendation (Applied Biosystem). A pair of ITS primer IT1 (5'GTAGTCATATGCTTGTCTC 3') [Qiagen] and IT4 (5'CTTCCGTC AATTCCTTTAAG 3') [Qiagen] were used to amplify the highly specific and conserved sequence for endophytic fungi targeting the gene encoding for 18S rRNA (Wu *et al.*, 2002). PCR was carried out in a programmable thermal controller (Biorad). In PCR reaction mixture of a total volume of 2.5 μ l, it contained 10 μ l template DNA, 14.5 μ l PCR Master Mix (PCR buffer, 4 mM MgCl₂, 0.4 mM of each dNTP, 0.05U/ μ l Taq polymerase (Fermentas), 1 μ l of each primer (IS1 and IS4) and double distilled water to make up the volume. The amplification was performed for 34 cycles having preset programming of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C. After the final cycle has been completed, the amplification was extended for 10 at 72°C. The amplified DNA fragment (approximately 400 bp) was purified and was sequenced by Genetic Analyzer. The analysis and comparison of the sequence were performed with nucleotide Basic Local Alignment Search Tool (BLAST) of GenBank (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree was produced using BLAST pair wise alignments.

Results and Discussions

We have isolated total number of nineteen endophytic fungi from the root of *Calotropis Procera* and these fungi were named according to the source from which they were isolated and followed by a number (CPR- *Calotropis procera* root). All endophytic fungi (CPR1-CPR19) were individually tested for their potential to degrade all four test dye. Dye decolourization activity of the isolated endophytic fungi was ascertained by using an agar plate method which showed decolourization and production of clear halo. Among all the nineteen isolates, CPR1, CPR2 CPR5, CPR6, CPR7 CPR11 CPR13 and CPR18, showed dye degrading

ability for at least one test dye. CPR 3, CPR 4, CPR 8, CPR 9 and CPR 10 and CPR19 did not show any decolourization activity for at least any test dye. CPR R5 demonstrated prominent dye degrading ability, among all of the nineteen fungal isolate tested, for all four test dye (RB, MR, CBB and MB). Coomassie Brilliant Blue, Methylene Blue were partially degraded by endophytic isolate CPR 5 and results are listed in Table 1. Both RB and MR in the agar medium appeared to undergo decolourization within 3 to 4 days after fungal inoculation and were completely decolorized within a period of 6 days

Table 1: Decolourization of Rose Bengal, Methyl red, Coomassie Brilliant Blue, and Methylene Blue on agar plate by the 19 isolated endophytic fungi in *Calotropis Procera* after 20 days of cultivation

Fungal Isolates	Dye tested for Decolourization			
	RB	MR	CBB	MB
CPR1	-	+	-	-
CPR2	+	-	-	-
CPR3	-	-	-	-
CPR4	-	-	-	-
CPR5	+++	++	+	++
CPR6	+	-	-	-
CPR7	-	+	-	-
CPR8	-	-	-	-
CPR9	-	-	-	-
CPR10	-	-	-	-
CPR11	+		-	+
CPR12	-		-	-
CPR13	-	+	-	-
CPR14	-	-	-	-
CPR15		-		-
CPR16	-	-	+	-
CPR17	-	-	-	-
CPR18	-	-		+
CPR19	-	-	-	-

RB=Rose Bengal, MR=Methyl Red, CBB=Coomassie Brilliant Blue, MB=Methylene Blue (-): no dye decolourization; (+): partial or weak dye decolourization; (++) : complete dye decolourization; +++: Very rapid and complete dye decolourization

Methylene Blue and Coomassie Brilliant Blue were also partially decolorized by isolate CPR5 with the production of halo, and no observable dye was absorbed by the fungal mycelium. Ngieng *et al.*, 2013, also demonstrated dye decolourization in *Melastoma malabathricum* (Senduduk). To further test dye decolourization ability of the fungal isolate CPR5 in liquid medium, isolate was cultured in glucose minimal liquid medium based on the

ability to decolorize all the dyes on the agar plate. The experimental results obtained in liquid medium showed that isolate CPR4 was proficiently decolorized all of the four different dyes tested to a different extent (Fig. 1).

Molecular analysis of fungus provided a powerful technique for assessing fungal identity. The use of molecular tools aided in rapid and clear identification of fungi to the genus

and strain level. In this study, fungal molecular identification of the most active isolate CPR-05, based on 18S ribosome RNA sequence analysis showed that isolate CPR-05 was identified as *Aspergillus niger* species. The percentage of identity was found to be 96%. Phylogenetic tree analysis indicated that 18S ribosome RNA sequence of CPR-05 strain was closely related to *Aspergillus niger* (Fig. 2).



Fig. 1: Dye decolorization by isolate CPR4 on agar plate medium containing (1) Rose Bengal, (2) Methylene blue, (3) Methyl Red, (4) Coomassie Brilliant Blue. In each set of figure, control petri plate is at right side

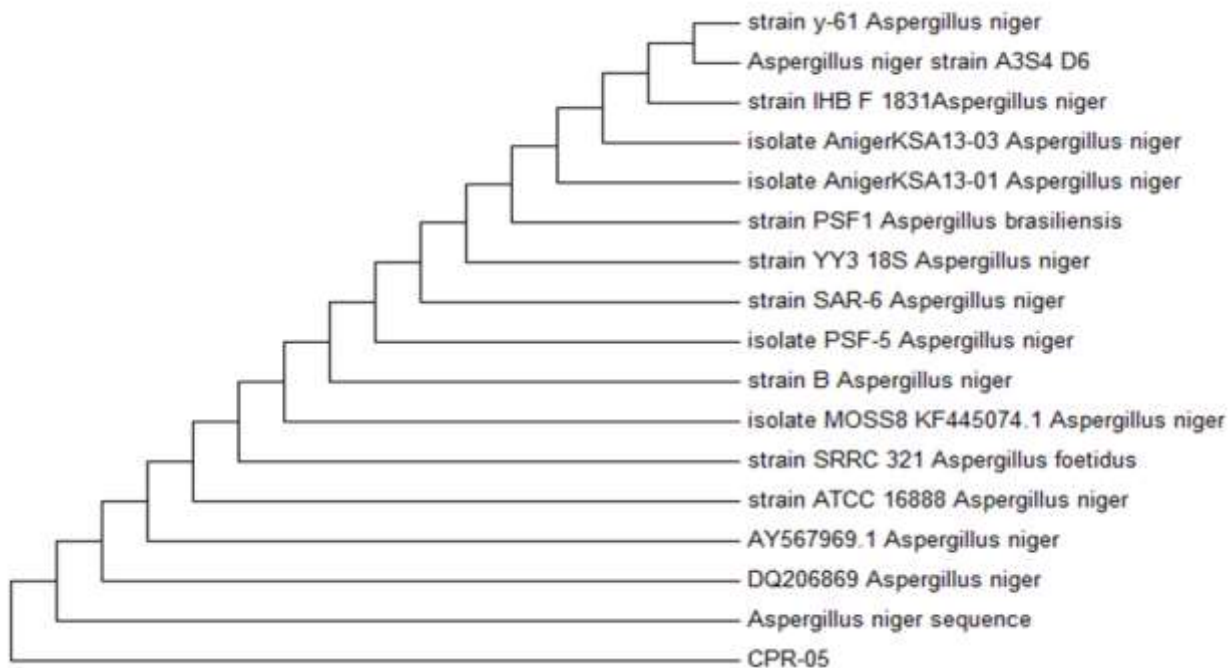


Fig. 2: Phylogenetic tree based on 18S rRNA gene sequence, showing the relationship between fungal endophytic isolate from *C. procera* (isolate CP- 05) and reference strains.

Table 2. Fungal biomass production in presence of dye and glucose minimal liquid medium for period of 20 days

Fungal biomass produced (mg/mL)						
Dyes	0 Day	4 Days	8 Days	12 Days	16 Days	20 Days
RB	0.10	0.28	0.81	0.71	0.83	0.81
MR	0.10	0.38	0.89	0.75	0.72	0.71
MB	0.10	0.22	0.76	0.48	0.38	0.38
CBB	0.10	0.18	0.52	0.42	0.32	0.31
Control (no dye)	0.10	0.67	4.68	8.55	13.87	12.85

Table 3: Decolonization activity of aspergillus sp. under stationary and agitated conditions

S.N.	Days	% of dye decolourization							
		Stationary Conditions				Agitated conditions			
		Test Dyes				Test dyes			
		RB	MR	CBB	MB	RB	MR	CBB	MB
1	0	-	-	-	-	-	-	-	-
2	2	1.05	2.01	0.86	2.12	1.85	2.71	0.86	2.92
3	4	9.15	7.47	4.12	5.11	11.15	8.87	4.89	6.41
4	6	34.46	35.31	9.47	10.54	34.96	35.91	10.31	11.84
5	8	58.67	51.89	17.67	14.99	58.87	52.59	17.69	15.10
6	10	78.54	71.09	23.80	24.98	80.54	72.02	24.10	26.28
7	12	86.76	80.12	29.54	37.08	86.00	81.42	29.98	37.08
8	14	89.34	82.09	33.91	54.43	91.34	82.79	33.99	54.86
9	16	94.82	83.99	44.64	67.31	95.32	85.94	45.44	68.63
10	18	89.41	82.67	42.12	59.45	89.41	83.67	42.12	60.15
11	20	73.09	79.62	37.73	54.21	81.09	79.97	37.89	55.13
12	22	64.94	79.02	35.09	51.43	78.94	79.02	36.19	54.85
13	24	61.52	69.87	32.12	49.73	63.52	68.87	33.73	54.13

Among the four different dyes which were used for decolourization teste, Rose Bengal observed to be to be decolorized the fastest and to the greatest extent by isolate CPR4, up to 97.4% decolourization in 4 days. The azo dye, Methyl red was decolorized up to 87% in same time period. Decolourization of MB and CBB was somewhat slow with decolourization efficiency reaching to 65% after 8 days for MB. Decolourization efficiency of 45%, which is very less in comparison to other three dye explained earlier was found to be for CBB. The four different dye groups tested, rose Bengal and methylene blue were decolorized to a greater extent as compared to two

other group dyes (MB and CBB). Decolourization of the mono azo dye Methyl red was also greater as compared to the decolourization of both Methylene blue and Coomassie Brilliant Blue. On the other hand when CPR5 was used to decolorize all the four test dye in glucose minimal liquid medium relative dye decolourization ability of fungal isolate was surprisingly different from that observed in agar medium. To compare the biomass production, the isolate CPR4 was inoculated in 4 flasks containing 50 ml glucose liquid minimal medium and separately four dyes were added in each flask at the concentration of 100 mg/l. Initially each flask was inoculated with about 10 mg

mycelia. The biomass production was calculated after every 5 days interval. When first dry biomass was weight, the biomass produced by the isolate in liquid medium containing Rose Bengal dye was lower(0.28mg/ml) than that of the biomass produced (0.30) in the medium containing methyl red. Biomass produced in remaining two liquid medium (with dye MB and CBB) was comparatively low , it was calculated to be 0.22 mg/ml and 0.18 mg/ml for MB and CBB respectively indicating that CBB produces more hindrance in fungal metabolic activity and as a result

was decolorized with slowest rate in comparison to other three test dye. When dye decolorization activity was performed under stationary and agitated condition, decolorization efficiency of aspergillus species showed a noticeable difference in between these two condition and decolorization efficiency was found higher in agitated condition (Table 3).

Table 4 Temperature effect on Decolourization activity of aspergillus sp. after a period of 16 days

S.N.	Temp.(°C)	% of dye decolourization							
		Stationary Conditions				Agitated conditions			
		Test Dyes				Test dyes			
		RB	MR	CBB	MB	RB	MR	CBB	MB
1	15	2.58	2.32	0.32	1.32	4.78	6.32	3.32	2.36
2	17	13.68	9.75	7.75	9.71	14.58	11.55	8.95	10.81
3	19	51.93	11.87	9.17	10.37	51.93	11.87	9.87	12.39
4	21	67.52	24.59	23.73	25.39	67.52	24.59	23.99	25.39
5	23	73.21	31.99	29.98	26.65	73.21	32.59	24.54	26.65
6	25	77.95	47.64	37.37	31.33	78.15	47.64	37.37	31.33
7	27	81.45	58.43	49.43	39.67	83.85	59.63	49.69	39.87
8	29	94.21	84.63	44.63	64.57	95.15	84.98	44.87	65.37
9	31	85.13	64.61	41.61	63.61	85.13	67.69	41.71	64.66
11	33	82.14	61.73	41.55	60.73	84.14	64.83	41.59	63.23

Table 5: Effect of pH on Decolourization activity of aspergillus sp. after a period of 16 days

S.N.	pH	% of dye decolourization							
		Stationary Conditions				Agitated conditions			
		Test Dyes				Test dyes			
		RB	MR	CBB	MB	RB	MR	CBB	MB
1	3.0	22.89	23.87	9.32	16.92	23.12	24.11	8.32	17.43
2	3.5	46.83	38.67	16.56	26.75	46.98	41.86	18.45	28.83
3	4.0	66.92	49.54	24.87	34.62	70.33	54.67	24.53	34.99
4	4.5	75.48	58.71	36.65	46.23	76.24	61.66	37.52	47.24
5	5.0	86.59	65.93	42.89	57.12	89.31	68.12	42.98	59.22
6	5.5	91.09	84.77	46.32	65.26	94.76	85.89	46.29	69.23
7	6.0	89.89	79.27	42.67	64.67	91.21	77.62	43.12	59.28
8	6.5	81.98	76.22	43.71	63.65	81.29	77.93	44.22	48.34
9	7.0	67.34	59.04	38.12	54.81	68.23	68.21	38.99	55.67

This may be due to enhanced supply of oxygen, which is a key oxidizing molecule required for almost many biological oxidation reduction reactions occurring inside fungi, resulting in rapid and enhanced degradation of dye. In another experiment (Table 4), when dye decolorization activity of fungus was optimized taking temperature as a variable factor, fungus showed maximum dye decolorization efficiency at its physiological temperature range (27-29 °C). Below and above of this temperature decolorization efficiency was observed to be low. One of the possible explanation for this could be that all biological reaction, especially enzymatic reaction occur at a narrow range of temperature.

In the last experiment (Table 5) when decolorization efficiency of fungus was tested under different pH, Fungus showed maximum efficiency at pH 5.5

Although efficiency of fungus at pH 6.0 was also noticeable as compared to pH 5. From these experimental results it shows that dyes belonging to chemically different groups are not decolorized to the same extent and the structural differences in the dye molecule strongly affect the decolorization process. Thus among the nineteen fungal isolate tested, isolate CPR4 may be a proficient source for decolorization of dye used in textile and paper industry.

Conclusions

In this study preliminary screening of endophytic fungal isolate revealed their potential to degrade Rose Bengal, Methyl Red (MR), Coomassie Brilliant Blue (CBB) and Methylene Blue (MB). In current days textile and paper industry has been explored extensively and a huge quantity of water containing a large amount of used synthetic dye is discharged in running water bodies. These dyes adversely affect the ecosystem of these water bodies. The dyes may be toxic to aquatic plant and animals, stops light from entering into deep of water level as a result cause hindrance in photosynthetic reaction of aquatic plants. Similarly, many of dye have been reported to reduces oxygen solubility in water making unavailability of dissolved oxygen required for all metabolic activity of aquatic animals, as a result death of large number of organisms may occur. Therefore, before release of these industrially used dyes, they could first be treated with fungal isolate to make them nontoxic or to be utilized by fungus. Therefore the endophytic fungal isolate might play a very important role and could be a resourceful basis for degrading and removal of most of the discharged synthetic dye from paper and textile industry. Still there is more research work remain, using advanced analytical tools, to be explored, pathway of metabolism of these synthetic dye by endophytic fungus should be detailed, molecular mass and structural information of degraded product must be determined to know their chemical properties. Endophytic fungi could

play a most important role in bioremediation of various dyes.

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