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Materials and Methods

2. 1. Chemicals, microorganisms culture conditions

2. 1. 1. Chemicals

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3'3'-diaminobenzidine tetrahydrochloride (DAB), HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) and riboflavin were purchased from Sigma-Aldrich. USA. Tartaric acid, catechol, n-propanol, ethylene diamine tetraacetic acid (EDTA), calcium carbonate ($CaCO_3$), *o*-Toludine (3, 3-Dimethyl benzedine), hydroquinone, L-DOPA (Dihydroxyphenylalanine), Diethyl aminoethyl (DEAE) cellulose, NADH (Nicotinamide adenine dinucleotide reduced disodium salt), glucose 6-phosphate dehydrogenase and D-glucose 6-phosphate disodium salt were purchased from Sisco Research Laboratories, India. Bacteriological peptone, yeast extract, beef extract and agar-agar were obtained from Hi-media Laboratories Pvt. Ltd., Mumbai, India. Ethyl acetate, H_2O_2 and NaCl were obtained from Qualigens Fine Chemicals, Mumbai, India. N, N'-Methylene bisacrylamide-2X was obtained from Serva Feinbiochemica Heidelberg, New-York. The TEMED (N, N, N', N'-Tetramethyl ethylenediamine) was obtained from Fluka Chemicals, India. Molecular weight markers were obtained from Bangalore GeNei, India. All chemicals used were of the highest purity available and of the analytical grade.

2. 1. 2. Dyes

Malachite green, Amido black 10B and Methyl red were purchased from Hi-media, India. Brown 3REL, Brilliant blue G, Navy blue, Yellow brown, Remazol red, Scarlet RR and Orange HE 2R were kind gift from Manpasand textile industry, Ichalkaranji, India.

2. 1. 3. Plant seeds

The seeds of *Sorghum bicolor* and *Triticum aestivum* were obtained from local market, Kolhapur.

2. 1. 4. Microorganism and culture conditions

Galactomyces geotrichum MTCC 1360 (GGO) was obtained from Microbial Type Culture Collection, India. Pure culture was maintained on malt yeast agar slants at 4°C. The composition of malt yeast medium was (g l^{-1}): Malt extract 3, yeast extract 3, peptone 5, and glucose 10. *Bacillus* sp. VUS were procured from contaminated soil and maintained on nutrient agar slants at 4°C. The yeast extract medium used for decolorization studies was composed of (g l^{-1}): NaCl 5 and yeast extract 5. Malt yeast medium was used for decolorization studies by GGO.

2. 2. Development of the consortium-GB

Consortium-GB was prepared by aseptically transferring the biomass of *Galactomyces geotrichum* MTCC 1360 harvested by centrifugation at 6000 rpm for 20 min (1cm pieces of mycelium cut from agar slants were used to inoculate 100 ml malt yeast medium in 250 ml Erlenmeyer flasks and grown at 30°C for 24 h) to the flask containing 24 h grown *Bacillus* sp. VUS strain (5 ml suspension was inoculated at 0.1 optical density at 620 nm to 250 ml Erlenmeyer flasks containing 100 ml of yeast extract medium and grown at 30°C). During preparation of consortium cell density of *Bacillus* sp. VUS was (24 h) 0.42 optical density at 620 nm and wet weight of *G. geotrichum* MTCC 1360 (24 h) was 2 g.

2. 3. Decolorization experiments

Decolorization using *G. geotrichum* MTCC 1360 was done by growing the organism for 24 h at 30°C in 250 ml Erlenmeyer flasks containing 100 ml malt yeast medium. After 24 h, different industrial dyes (Malachite green, Orange HE 2R and Amido black 10B at 50 mg l^{-1}, and Methyl red and Scarlet RR, at 100 mg l^{-1} concentration) were added in each flask and incubated at 30°C for 150 rpm on orbital shaking incubator. Consortium-GB was used for different dyes like Brown 3REL, Brilliant blue G, Navy blue, Yellow brown and Remazol red at 50 mg l^{-1} concentration, individually and incubated at static

condition, at 50°C (pH 7). The detail decolorization study of Brown 3REL and Brilliant blue G with consortium-GB was done at 50°C with pH 7 (shaking condition) and pH 9 (static condition) respectively. Methyl red (distilled water) and Scarlet RR (malt yeast medium) decolorization study was carried out using *G. geotrichum* MTCC 1360 at 50°C for 150 rpm on orbital shaking incubator at pH 3 and 12 respectively. An aliquot (3 ml) of the culture media was withdrawn at different time intervals, centrifuged (4000 g for 10 min) and supernatant was separated. Methanol (2 ml) (for Brilliant blue G acetone was used) was added to the cell pellet to extract bound dye followed by centrifugation (4000 g for 10 min). Decolorization of various dyes was monitored by measuring the absorbance of culture supernatant mixed with methanol extract at their respective λ_{max} (Table 1).

The percent decolorization (Saratale et al., 2006) and average decolorization rate (Khehra et al., 2005) was measured at different time interval. All decolorization experiments were performed in three sets. Abiotic controls (without microorganisms) were always included. The percentage decolorization was calculated according to the following formulation:

$$\text{Percentage of decolorization} = \frac{A_b - A_a}{A_b} \times 100$$

A_b is the absorbance at the maximum absorption wavelength of dye before decolorization and A_a the absorbance at same wavelength after decolorization.

The average decolorization rate was calculated as follows

$$\text{Average decolorization rate} = \frac{C \times \%D \times 1000}{100 \times t}$$

Where; C = Initial concentration of dye (mg L^{-1}), %D = Dye decolorization (%) after time t.

2. 4. Effect of physicochemical parameters on decolorization

2. 4. 1. Effect of temperature

To study the effect of temperature on decolorization, the consortium and *G. geotrichum* MTCC 1360 were pre grown for 24 h at 30°C and incubated at different temperatures (5, 30 and 50°C) after addition of dye. Flasks were incubated at respective temperatures for 30 min before addition of dye.

2. 4. 2. Effect of pH

Study of effect of pH on decolorization performance of consortium-GB and *G. geotrichum* MTCC 1360 was done by adjusting the pH of decolorization medium (pH adjusted after preparation of consortium-GB) at various pH values (3, 5, 7, 9 and 12 pH). The pH of the decolorization medium was adjusted by using 0.2 M NaOH and HCl.

2. 4. 3. Effect of initial dye concentration

Effect of initial dye concentration on decolorization of Brown 3REL (consortium-GB), Methyl red and Scarlet RR (*G. geotrichum* MTCC 1360) was studied by the addition of different dye concentrations (100, 200 and 300 mg l^{-1}). Brilliant blue G (consortium-GB) was studied at concentration of 100 and 200 mg l^{-1}, to determine the effect of initial dye concentration.

2. 4. 4. Effect of additional carbon and nitrogen sources

Different carbon sources like starch, sucrose, malt extract and glucose (1%) were used. Peptone, beef extract, urea and casein (1%) were used as nitrogen sources for consortium-GB.

2. 4. 5. Effect of initial biomass concentration

Effect of initial biomass concentration on decolorization was studied with *G. geotrichum* MTCC 1360 by the addition of different concentrations (1, 2, 3 and 4 g, wet wt) of biomass. The culture was pre grown for 24 h at 30°C and then the biomass was transferred (centrifuged at 13,000 g for 30 min) to

distilled water for Methyl red decolorization, while for Scarlet RR biomass was transferred to sterile malt yeast medium containing dye.

2. 4. 6. Effect of glucose and molasses

Effect of glucose on decolorization of Methyl red by *G. geotrichum* MTCC 1360 was studied at various concentrations (10, 30 and 60 g l^{-1}). Effect of molasses at concentration (1, 2, 3 g l^{-1}) on decolorization performance of *G. geotrichum* MTCC 1360 for Methyl red was studied at 30°C (pH 3). Effect of glucose and molasses at same concentration was studied for Scarlet RR in synthetic medium. The culture was pre grown for 24 h at 30°C and then the biomass was transferred (centrifuged at 13,000 g for 30 min) to distilled water for Methyl red decolorization, while for Scarlet RR biomass was transferred to synthetic medium.

2.4.7. Effect of $CaCO_3$ and EDTA on dye decolorization of Scarlet RR by G. geotrichum MTCC *1360*

Different concentrations (10-100 mg l^{-1}) of calcium carbonate ($CaCO_3$) were added in malt medium during the growth of *G. geotrichum* MTCC 1360. Inhibitor ethylenediamine tetraacetic acid (EDTA) was added to the medium after 24 h growth of *G. geotrichum* along with dye and its effect on decolorization performance was studied at various concentrations (0.01-0.05 mM) at 50°C (pH 12).

2. 5. Preparation of cell free extract

The consortium-GB was prepared as mentioned in 2.3 and individual organisms were grown in their respective medium for 24 h at 30°C and centrifuged at 9000 g for 30 min. This biomass of consortium and individual organisms were separately suspended in 0.1 mM sodium phosphate buffer (pH 7.4) and used for sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining temperature below 4°C and giving

12 strokes each of 30 s with 1 min interval. These extracts were then used as enzyme source.

2. 6. Enzyme assays

Lignin peroxidase (LiP) activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM n-propanol, 250 mM tartaric acid, 10 mM H_2O_2 and 0.2 ml of crude enzyme (Shanmugam et al., 1999). Laccase activity was determined in a reaction mixture of 2 ml containing 0.2 ml of crude enzyme, 5 mM 3'3'-diaminobenzidine tetrahydrate (DAB) in 0.1 M acetate buffer (pH 4.8) and measured increase in optical density at 410 nm (Flurkey et al., 1995). Tyrosinase activity was assayed by the procedure of Zhang and Flurkey, (1997). It was determined by monitoring the formation of catechol quinone at 495 nm in a reaction mixture (2.0 ml) containing 0.01% catechol in 100 mM potassium phosphate buffer (pH 6.8) and 0.2 ml of crude enzyme. All enzyme assays were carried out with reference to blanks containing all components except the enzyme. All enzyme assays were run in triplicate and average rates were calculated. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg of $protein^{-1}$.

NADH–dichlorophenol indophenol (NADH-DCIP) reductase activity was determined using a procedure reported earlier by Salokhe and Govindwar, (1999). DCIP reduction was monitored at 595 nm and calculated using an extinction coefficient 19 mM^{-1} cm^{-1}. The reaction mixture (5.0 ml) prepared was containing 50 mM substrate (DCIP) in the 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml enzyme. From this, 2.0 ml reaction mixture was assayed spectrophotometrically by the addition of 50 mM NADH.

Riboflavin reductase NAD (P)H:Flavin oxidoreductase was measured spectrophotometrically by monitoring the decrease in absorbance at 340 nm. Cell free extract (0.1 ml) was added to a solution (final volume, 1 ml) containing 100 µM of Tris-HCl (pH 7.5), 25 µM of NADPH and 0.003 U l^{-1} of

riboflavin. Reaction rates were calculated by using a molar extinction coefficient of 6.3 mM $l^{-1}cm^{-1}$ (Russ et al., 2000).

Azoreductase activity was determined by monitoring the decrease in the Methyl red concentration at 440 nm in a reaction mixture of 2.2 ml, containing 152 µM Methyl red, 50 mM sodium phosphate buffer (pH 5.5), 20 µM NADH and 0.2 ml of crude enzyme. One unit of enzyme activity was defined as µg of Methyl red reduced min^{-1} mg $protein^{-1}$.

2. 7. Extraction and analysis of the metabolites

2. 7. 1. Extraction of the metabolites for analysis

Culture broth was centrifuged at 13,000 g for 30 min after 100% decolorization of studied dye. Equal volume of ethyl acetate was used to extract metabolites from clear supernatant. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. Control dye sample was prepared by the addition of dye in respective media and extracted with ethyl acetate and used as a control. Since it is extracted from the medium and the higher concentration of dye, media component does not interfere in HPLC and FTIR. If any, the effect will be automatically subtracted from the results of samples.

2. 7. 2. UV-Vis spectrophotometric analysis

UV-Vis spectrophotometric analysis was carried out at initial time (zero time) when dye was added to the medium and after complete degradation of dye. Scans of clear supernatant broth were taken in the visible region of 400-800 nm.

2. 7.3. High performance liquid chromatography (HPLC)

The biodegraded product was characterized by HPLC analysis which was carried out (Waters model no. 2690) on C_{18} column (symmetry, 4.6 × 250 mm) with methanol as mobile phase at flow rate of 0.75 ml min^{-1} and the detector at 440 nm for Brown 3REL, 605 nm for Brilliant blue G and 510 nm

for Scarlet RR along with UV detector (238 nm). For study of Methyl red decolorization by *G. geotrichum* MTCC 1360 mobile phase used was acetonitrile: water (1:1) with flow rate of 1 ml min^{-1} and detector at 440 nm and UV detector at 238 nm. Samples were analysed on HPLC at λ max of dye and λ max of product in UV-range using dual detector.

2. 7. 4. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was done in the mid IR region of 400-4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out.

2. 7. 5. Gas Chromatography-Mass Spectroscopy (GC-MS)

The GC-MS analysis of metabolites were carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280°C with oven conditions as: 80°C kept constant for 2 min increased up to 200°C with 10 min^{-1} raised up to 280°C with 20°C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the National Institute of Standards and Technology NIST library.

2. 8. Phytotoxicity

We have assessed the toxicity of the untreated and treated samples in the concentration range 1000-4000 ppm for Methyl red. Tests were carried out according to the International Standard Organization (1994) on two kinds of seeds commonly used in the Indian agriculture: *Sorghum bicolor* and *Triticum aestivum* The phytotoxicity study was carried out at room temperature (28±2°C). The study parameters were percent inhibition of germination, height of roots and shoot. Germination (%) and length of shoot and root was recorded after 7 days.

In our preliminary experiments to set the methodology, we did experiment by taking ethyl acetate extract of controlled medium. No significant change in the growth parameters was observed in ethyl extract of the medium when compared to seeds grown in distilled water. Control dye sample was prepared by the addition of dye in respective media and extracted with ethyl acetate and used as a dye control.

2. 9. Partial purification of laccase (EC 1.10.3.2, p-diphenol:oxygen-oxidoreductase)

2. 9. 1. Cultivation of Galactomyces geotrichum MTCC 1360 *and sample preparation*

G. geotrichum MTCC 1360 was grown in malt yeast medium at 30°C for 24 h and biomass was collected by filtration through Whatman filter paper no 1. This mycelium was suspended in 0.1 mM sodium phosphate buffer (pH 7.4) and gently homogenized and disrupted by sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining temperature below 4°C and giving 9 strokes each of 1 min with 1 min interval. This extract was then centrifuged at 13,000 g for 30 min at 4°C and resulting supernatant was used as crude source of laccase.

2. 9. 2. Protein content and enzyme activity

The protein concentration of fractions collected during column chromatography was monitored by absorbance at 280 nm. Lowry method was used for accurate estimation of protein concentrations (Lowry et al, 1951). Laccase activity was determined as mentioned in 2.6. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg $protein^{-1}$.

2. 9. 3. Ion exchange chromatography

The crude enzyme was applied on the activated DEAE cellulose column (15 × 120 mm) pre-equilibrated with 0.1 mM sodium phosphate buffer (pH 7.4). The column was washed with the same buffer with two column volume,

and the enzyme was eluted with 0 to 1.0 M NaCl linear gradient at the flow rate 0.4 ml min^{-1}. The elution was carried out by using automatic linear gradient system (BIO-RAD).

All fractions were tested for laccase activity. The fractions containing laccase activity were pooled and dialyzed overnight against 0.1 mM sodium phosphate buffer (pH 7.4). Dialyzed enzyme was lyophilysed (Operon model) and used as partially purified laccase for the study of optimization of physicochemical parameters and for the dye decolorization.

2. 9. 4. Polyacrylamide gel electrophoresis analysis (PAGE)

Protein with laccase activity was evaluated with native PAGE (10% separating and 4% stacking gel), run on a slab gel unit (Genetech Laboratories). Composition of 10% separating gel was 13.3 ml stock acrylamide solution (30% acrylamide, 0.8% bisacrylamide), 8 ml Tris-HCl (pH 8.8) and 18.1 ml distilled water. Composition of 4% stacking gel was 1.35 ml stock acrylamide solution, 0.1 ml Tris-HCl (pH 6.8) and 7.5 ml distilled water. Both, separating gel and stacking gel was prepared (after degassing) by the addition of ammonium persulfate (5%) and 20 µl N, N, N', N' Tetramethyl-ethylenediamine (TEMED).

Sample buffer composition in ml^{-1} distilled water was 0.5 ml, Tris-HCl buffer (pH 6.8), 0.5 g sucrose, 0.1 ml bromophenol blue (0.5%, w/v). Electrode buffer composition was (g l^{-1}): Tris 6, glycine 14.4 (pH 8.2-8.4). The gels were stained with Coomassie brilliant blue R-250 (0.1%, w/v) in methanol/acetic acid/water (4:1:5, v/v/v) for 1 h at room temperature followed by destaining using methanol/acetic acid/water (4:1:5, v/v/v). Zymogram analysis for laccase activity was performed in 1 mM L-dopa in 0.1 M acetate buffer (pH 4) and incubated at 37°C overnight. The gel was then washed for 1 h with the same buffer.

2. 9. 5. Characterization of partially purified laccase

2. 9. 5. 1. Determination of molecular weight

The molecular mass of the purified laccase was determined by calculating the relative mobility of standard protein markers (Genie, India) run alongside (205,000 Da, Myosin rabbit muscle; 0.5 mg ml^{-1}, 97,400 Da, Phosphorylase b; 0.5 mg ml^{-1}, 66,000 Da, Bovine serum albumin; 0.5 mg ml^{-1}, 43,000 Da, Ovalbumin; 0.75 mg ml^{-1}, 29,000 Da, Carbonic anhydrase; 0.5 mg ml^{-1}, 20,100 Da , Soyabean trypsin inhibitor; 2.0 mg ml^{-1}, 14,300 Da, Lysozyme; 0.75 mg ml^{-1}) on PAGE.

2. 9. 5. 2. Thermal stability and pH dependence

The effect of temperature on stability of partially purified laccase was determined by incubating the laccase for pre-determined time periods (10 min) at various temperatures (30, 40, 50, 60, 70 and 80°C) in 0.1 M sodium-acetate buffer (pH 4.0) and then determining the residual laccase activity with the DAB as substrate. The pH dependence of the partially purified laccase was determined spectrophotometrically with DAB as a substrate in the pH range 2.0-8.0 (0.1 M of KCl-HCl buffer; pH 2.0, glycine-HCl buffer; pH 3.0, Sodium-acetate buffer; pH 4.0-5.0 and Sodium-phosphate buffer; pH 5.0-8.0).

2. 9. 5. 3. Substrate specificity and inhibition studies

The substrate specificity of purified laccase was determined spectrophotometrically in acetate buffer (0.1 M, pH 4). The oxidation of substrates by partially purified laccase was determined spectrophotometrically at the specific wavelength of each substrate and at final concentration of substrates as shown in table 2. The laccase concentration used for the oxidation of each substrate was the same. The effect of potential inhibitors on laccase activity was determined using DAB (5.0 mM) as a substrate in sodium-acetate buffer (0.1 M, pH 4) in the presence of an inhibitor like sodium azide and L-cysteine at various concentrations (1, 5, 10 and 20 mM). The partially purified

laccase was incubated with inhibitors for 10 min and the enzyme activity was assayed. Control samples were maintained without the laccase inhibitors.

Table 2. Substrates used to determine the activity of partially purified laccase.

Substrate	Concentration (mM)	Absorbance (nm)
Hydroquinone	5	250
3-3'-DAB	5	410
O-Tolidine	2	366
ABTS	0.66	420
L-dopa	5	475

2. 9. 5. 4. Effect of metal ions

The metal ions used at different concentrations (1, 5, 10 and 100 mM) were $MgCl_2$, $CaCl_2$, $HgCl_2$ and $CuSO_4$. The partially purified laccase was incubated with metal ions for 10 min and the enzyme activity was assayed. Control samples were maintained without the metal ions.

2. 10. Textile effluent deolorization by consortium-GB

The textile effluent decolorization studies were carried out at various temperatures (30, 40 and 50°C) by inoculating consortium-GB and individual organisms along with respective medium, separately in 100 ml effluent. The time required for decolorization of effluent by consortium and individual organisms was noted down. The decolorization of textile effluent was confirmed by scanning textile effluent along with decolorized effluent by UV-Vis spectrophotometer from visible range of 400-800 nm. % decolorization was calculated using OD at 510 nm (λ max of the effluent).

2. 11. Statistical Analysis

The data were analyzed by One-way analysis of variance (ANOVA) using Tukey-kramer multiple comparisons test.