3. **Materials and Methods**
Chapter 3
Materials and Methods

3.1. Chemicals and microbiological media

All chemicals used were of the highest purity and of analytical grade. ABTS (2, 2’-Azinobis, 3-ethylbenzothiazoline-6-sulfonic acid) was obtained from Sigma Chemicals Company (St. Louis, MO, USA). n-propanol, o-tolidine, hydroquinone, pyrogallol, guaiacol, catechol, DMP, o-danisidine, DAB, L-DOPA, MgCl$_2$, MnSO$_4$, CaCl$_2$, MnCl$_2$, ZnSO$_4$, and CuSO$_4$, sodium azide, L-cysteine, EDTA, NADH (Nicotinamide adenine dinucleotide reduced disodium salt), coomassie brilliant blue R-250 were obtained from Sisco Research Laboratories, India. DEAE-cellulose, 3,4-dimethoxy benzyl alcohol (veratryl alcohol), syringic acid, catechol, N,N’-dimethyl phenylenediamine, caffeic acid, Murashige and Skoog medium, clarigel were obtained from Hi-Media laboratory, India. Tartaric acid was obtained from BDH Chemicals (Mumbai, India). Protein markers were obtained from Bangalore Genei Pvt. Ltd. Ethyl acetate, hydrogen peroxide, dimethyl sulfoxide, glacial acetic acid and sodium chloride were obtained from Qualigens Fine Chemicals, Mumbai, India. Trichloroacetic acid was obtained from Thomas Baker Chemicals Limited, Mumbai, India. N,N’-methylene bisacrylamide-2X was obtained from Serva Feinbiochemica Heidelberg, New-York. TEMED (N,N,N,N’-Tetramethyl-ethylenediamine) was obtained from Fluka Chemicals, India. Methanol was obtained from sd fine- chemicals Ltd., Mumbai, India. BA, NAA, 2,4-D, HgCl$_2$, Bavistin were obtained from Hi-media, India.

The plant seeds viz. *Triticum aestivum* and *Phaseolous mungo* were obtained from local market, Kolhapur, India.

3.2. Textile dyestuff and textile industry effluent

Malachite Green, Methyl Orange and Methyl Red were obtained from Hi-media laboratory, India. The textile dyes viz. Red HE8B, Reactive Red 2 and Direct Red 5B, Red HE 7B, Golden Yellow HER, Patent blue, Scarlet GDR, Remazol Red and Brilliant Blue R were obtained from Manapasand textile industries, Ichalkaranji, India. Textile industry effluent was obtained from Mahesh textile processors, Ichalkaranji, India.

3.3. Selection of the plants

The plants that were screened for their dye decolorization potential were *Eucalyptus* species, Bamboo species, *Blumea malcomii* Hook. and *Typhonium flagelliforme* (Lodd.)
Blume plantlets were collected from the plants grown in the campus of Shivaji University, Kolhapur, India. *T. flagelliforme* plantlets widely growing in semi aquatic environment were collected from Sindhudurg district of Maharashtra, India, in the month of August and September. *Eucalyptus* and Bamboo plants were obtained from a local nursery in Kolhapur, India. Based on the decolorization potential exhibited by these plants, the plants having higher decolorization abilities were selected for further studies.

### 3.3.1. Decolorization experiments with wild plants

All decolorization experiments were performed in three sets. Initially, experiments were performed with wild plants of *Eucalyptus* species, Bamboo species, *B. malcolmii* and *T. flagelliforme*, to check the abilities of these plants to decolorize various dyes namely, Malachite Green, Direct Red 5B, Orange HE2R, Red HE8B and Reactive Red 2 and Brilliant Blue R. Experiments done with these plants were carried out in 250 mL Erlenmeyer flasks containing 100 mL of 20 mg L$^{-1}$ of dye solution in plain distilled water. Each flask contained three uprooted wild plants of *B. malcolmii*, each having dry weight of 0.614 g and one plant of each of the other three plants used. Dry weights of one plant of *Eucalyptus*, Bamboo and *T. flagelliforme* were 1.77 g, 1.89 g and 1.98 g respectively. The roots of all the plants were washed with running tap water to remove the adherent soil followed by treatment of the roots with 0.1% HgCl$_2$ (w/v) for 3 min after which the plants were thoroughly washed with distilled water and then immersed in dye solutions. Samples were withdrawn after regular time intervals, centrifuged to remove any particulate matter such as root hairs and absorbance was noted at the absorption maxima of the respective dyes. Percentage decolorization was calculated as mentioned in section 3.6. below. Since *B. malcolmii* and *T. flagelliforme* showed promising results, *in vitro* propagation of both these plants was carried out.

### 3.4. In vitro propagation of the selected plants

#### 3.4.1. Preparation of culture medium

The culture medium consisted of Murashige and Skoog’s (MS) basal medium (Murashige and Skoog, 1962) and was supplemented with plant hormones and sucrose (3%) as per the requirement of the experiment. The medium was solidified with 0.2% Clarigel. The
pH of the medium was adjusted to 5.8±0.05 before autoclaving. The medium was autoclaved at 15 psi and 120°C for 20 min.

### 3.4.2. In vitro propagation of Blumea malcolmii Hook.

#### 3.4.2.1. Sterilization and preparation of explants

The seeds of *B. malcolmii* were collected from the plants grown in the campus of Shivaji University, Kolhapur, India during the month of February. The seeds (Achenes) are small and brown. The collected seeds were washed thoroughly with running tap water and air dried on a filter paper after which they were pooled in an eppendorf tube and treated for 10 min with 0.2% bavistin (Carbendazim 50% WP BASF, India) containing few drops of detergent (Labolene). This was followed by 3-4 washes with sterile distilled water. Then the seeds were surface sterilized with 0.1% HgCl₂ (w/v) for 10 min. To eliminate traces of HgCl₂, the seeds were rinsed repeatedly with sterile distilled water under aseptic condition.

#### 3.4.2.2. Culturing and development of plantlets

These seeds were cultured and spread in Petri plates containing 30 mL of half strength (MS) basal medium. Cultures were maintained at 25±2°C with 16 h light and 8 h darkness. Half strength MS medium was found suitable for 80% of seed germination. After 3 weeks, the seeds started to germinate.

The germinated seedlings were aseptically excised and shoot tips were isolated. The individual shoot tips were cultured on MS+BA (2 mg L⁻¹) for shoot multiplication. In 6 weeks time, several shoots emerged from the cultured shoot tips, which again were excised and transferred to fresh medium of the same composition. Highly prolific shoot cultures were thus established. For plantlet regeneration, shoots were separated from shoot clusters and cultured on MS+NAA (1 mg L⁻¹) for the induction of roots. After 4-6 weeks, shoots started producing roots, which were long and profuse. These plantlets were then used for phytoremediation studies.
3.4.3. In vitro propagation of *Typhonium flagelliforme* (Lodd.) Blume

3.4.3.1. Sterilization and preparation of explants

The tubers were brought to the laboratory and were washed and cleaned thoroughly for 3 h under running tap water to remove the muddy soil. Afterwards, the tubers were dipped in liquid soap (labolene) for about 15 min and then treated with 0.2% bavistin, a fungicide (Carbendazim 50% WP BASF, India) for 20 min. This was followed by the addition of 2-3 drops of Tween 20 to all treatments. Subsequently, tubers were washed 3-4 times with sterile distilled water to remove the traces of detergents and bavistin. The explants were dipped for 3 min in 70% alcohol, followed by washing with sterile double distilled water and gentle shaking for 15 min in 0.1% (w/v) mercuric chloride (HgCl₂) solution in laminar air flow cabinet. The tubers were finally washed several times with sterile double distilled water to remove traces of HgCl₂.

3.4.3.2. Culturing the explants and development of plantlets

Surface sterilized explants were placed in test tubes (25x100 mm), containing 10 mL of medium and culture bottles (200 mL capacity), containing 40 mL of the autoclaved medium. All cultures were maintained at 25±2°C with 16 h light and 8 h darkness.

Initially, tuber explants cultured on MS basal medium without growth hormones; produced dark green leaves from the tubers after two weeks. Afterwards, the shoots with tubers were divided into pieces of appropriate sizes and were cultured on MS medium supplemented with BA (2 mg L⁻¹) and 3% sucrose for further multiplication. After 4 weeks, 10 to 15 new shoots were formed and the regenerated shoots were sub cultured routinely every two weeks. This enabled to raise several fresh cultures leading to the enhancement of cultured biomass. The regenerated shoots were robust, healthy and dark green. The cluster of shoots was dissected aseptically and single shoots were carefully excised and transferred individually to fresh rooting medium. Rooting was observed on MS basal medium supplemented with NAA 1 mg L⁻¹. Initiation of 8-10 roots from the explants was observed after 2 weeks, which were short and thick. These *in vitro* grown plantlets were then used for further phytoremediation studies.
3.4.4. Development of callus and suspension cultures of B. malcolmii

*In vitro* plantlets developed in the laboratory as described earlier in section 3.4.2., were used for the development of callus cultures. The medium used for the development of callus cultures consisted of Murahige and Skoog’s medium supplemented with 2,4-D (5 mg L\(^{-1}\)), glutamine (100 mg L\(^{-1}\)), sucrose (3%), coconut milk (20%) and Clarigel. The medium was prepared and autoclaved as stated above and was poured into sterile Petri plates aseptically. Internodal segments of the shoots were excised and cut into small segments aseptically. These segments were then placed on the medium aseptically following which the plates were sealed with Parafilm. The plates were incubated in the dark. The medium was observed for the development of callus. The developed callus was further subcultured after every 3-4 weeks. 2 g of yellowish soft friable calli was excised and macerated with a sterile scalpel and suspended in 250 mL Erlynmeyer flasks containing 50 mL of liquid medium of the same composition as that used for the development of callus. The cultures were maintained with continuous shaking at 100 rpm which facilitated the dispersion of cells. 10 mL of the inoculums was further transferred into 250 mL Erlynmeyer flasks containing 40 mL of autoclaved medium. The suspension cultures were maintained thereafter by sub culturing them regularly after every 10 days and were used for phytoremediation studies.

3.4.5. Growth pattern of B. malcolmii cells

Growth pattern of B. malcolmii cells was examined by recording the fresh weight and dry weight of the cells at regular intervals. For these measurements, the cells were harvested by filtering the cell containing medium through a Whatmann filter paper. The initial packed cell volume of the culture was 15%.

3.5. Decolorization experiments with *in vitro* cultures

3.5.1.1. Decolorization experiments with *in vitro* plantlets

For tissue cultured plants, experiments were carried out under strictly aseptic conditions in 100 mL Erlenmeyer flasks with 50 mL of sterile MS basal medium containing 20 mg L\(^{-1}\) of the above mentioned dyes viz. Malachite Green, Red HE8B, Methyl Orange, Reactive Red 2 and Direct Red 5B that were used for screening. Tissue cultured plants having
same stage of growth, equal number of shoots and almost equivalent dry weights (average wet weight of the entire plant was found to be 8.039 g while the average dry weight was found to be 0.262 g and the shoot length was 10 cm) were used. Three plantlets were immersed in each flask. *T. flagelliforme* plantlets of the same growth stage, equal number of shoots and almost equivalent dry weights (average wet weight of the entire plant was 6.827 g and dry weight was 0.203 g while the shoot length was about 10 cm) were used for all the experiments to maintain uniformity. Initial screening for *T. flagelliforme* plantlets was carried out in 25x100 mm test tubes that contained 15 mL of autoclaved distilled water with the respective dyes (20 mg L\(^{-1}\)) viz., Direct Red 5B, Reactive Red 2, Methyl Orange, Red HE 8B, Brilliant Blue R, Malachite Green, Golden Yellow HER, Patent blue and Red HE 7B. One plantlet of *T. flagelliforme* was immersed in each of the dye solution containing tubes. Aliquots of the solution were aseptically removed at different time intervals, centrifuged at 5000 rpm for 10 min to separate any solid matter if present like root hairs and the absorbance of the clear solution was measured at the respective absorption maxima of the dyes screened.

Abiotic and biotic controls were kept for the experiments done with tissue cultured plants as well as wild plants. Abiotic controls were devoid of plants whereas biotic controls contained plants immersed in distilled water (in case of experiments with wild plants), tissue cultured plantlets immersed in autoclaved distilled water (in case of experiments with tissue cultured plantlets of *T. flagelliforme*) and in sterile MS basal medium (in case of experiments with tissue cultured plantlets of *B. malcolmii*).

### 3.5.1.2. Decolorization experiments with suspension cultures of *B. malcolmii*

The dyes, Brilliant Blue R, Malachite Green, Scarlet GDR, Remazol Red, Golden Yellow HER, Red HE 7B, Red HE 8B and Reactive Red 2 were screened to determine the decolorization abilities of the suspension culture of *B. malcolmii*. 40 mL of the medium used for the growth of suspension cultures was added into different Erlenmeyer flasks and each of the eight dyes was added into the flasks separately at the concentration of 20 mg L\(^{-1}\). The dye containing medium was autoclaved and then each flask was inoculated with 10 mL of inoculum of the suspension culture which was grown for 10 days. The flasks were exposed to continuous shaking at 100 rpm at 25±2°C. Aliquots of the sample were withdrawn at regular intervals. The samples were filtered through Whatmann filter paper and the clear filtrate was used to determine the absorbance at the respective absorption maxima of the dyes used.
Abiotic controls constituted of the dye containing medium without the inoculum and biotic controls constituted of the medium inoculated with the suspension culture and devoid of the dye.

3.6. Calculation of percentage decolorization

Decolorization percentage for the respective dyes in all the experiments was calculated using the below mentioned formula:

\[
\text{Decolorization (\%) = } \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100
\]

3.7. Decolorization with increasing dye concentration and increasing amount of biomass

Based on the results obtained with screening experiments, Direct Red 5B was selected as the model dye for studies with \( B. \ malcolmii \) plantlets. To study the effect of dye concentration on the decolorization ability of the plant 10, 20, 40, 60, 80 and 100 mg L\(^{-1}\) were the selected concentrations with three plants in each 100 mL flask containing 50 mL of MS medium. Based on the results of the screening experiments, Brilliant Blue R (20 mg L\(^{-1}\)) was selected as the model dye for studies with the plant \( T. \ flagelliforme \) and all the further experiments involving decolorization studies were carried out in 250 mL Erlenmeyer flasks, containing 50 mL of autoclaved distilled water containing the dye. Three plantlets of \( T. \ flagelliforme \) were immersed in each of the flasks. For studying the effect of different concentrations of the dye Brilliant Blue R on decolorization, the concentrations selected were, 20, 40, 80, 160 and 320 mg L\(^{-1}\) with three plantlets immersed in each flask. For studying the effect of varying dye concentrations on the decolorization performance of suspension cultures of \( B. \ malcomii \) too the selected dye concentrations were 20, 40, 80, 160 and 320 mg L\(^{-1}\) which were added into flasks containing the medium used for the development and maintenance of suspension cultures which has been described above and 10 mL of 10 day old inoculum was added to each of the flasks. The flasks were exposed to continuous shaking at 100 rpm and 25±2°C. The absorbance was noted after every 12 h in case of suspension cultures. To study the effect of increasing number of \( B. \ malcolmii \) and \( T. \ flagelliforme \) plantlets on the decolorization of the dyes Direct Red 5B and Brilliant Blue R 1, 2, 3, 4 and 5 plants were used respectively in different flasks containing 20 mg L\(^{-1}\) of the
dyes, respectively. To study the effect of increasing biomass on the removal of the dye Brilliant Blue R, 5, 10, 15 and 20 ml of 10 day old inoculum of the suspension culture of *B. malcolmii* was added into flasks containing 40 mg L$^{-1}$ of the dye BBR. The flasks were exposed to continuous shaking at 25±2°C and the absorbance was noted after every 6 h.

### 3.8. Decolorization of mixture of dyes and textile industry effluent

#### 3.8.1. Analysis of color removal in case of mixture of dyes and textile effluents

True color values were determined in case of textile effluents and mixture of different textile dyes by using the American Dye Manufacturers’ Institute (ADMI 3WL) tristimulus filter method. The original textile effluent was filtered using filter paper. The effluent used for the analysis of ADMI values was 1:5 diluted. A synthetic mixture of textile dyes was prepared by adding six different textile dyes viz. Red HE 8B, Red HE 7B, Brilliant Blue R, Reactive Red 2, Direct Red 5B and Malachite Green to distilled water to attain a concentration of 20 mg L$^{-1}$, similar to that used for individual textile dyes. For experiments performed with *B. malcolmii* plantlets, the synthetic dye mixture was prepared in MS medium to attain a concentration of 20 mg L$^{-1}$. The solution was autoclaved and then was used. For decolorization experiments, 3 plantlets of *T. flagelliforme* and *B. malcolmii* were separately suspended in 250 mL Erlenmeyer flasks containing 50 mL of the solution containing the dye mixture and 50 mL of the dye effluent. For the analysis of color removal by suspension cultures of *B. malcolmii*, the dye mixture was prepared in the medium used for suspension cultures and 10 mL of inoculum was added into 40 mL of the medium containing the dye mixture (30 mg L$^{-1}$). 5 mL of the filtered effluent was added to 35 mL of the medium and after autoclaving these flasks, 10 mL of inoculum was added. ADMI values of control samples of the effluent and mixture of dyes as well as test samples, which were withdrawn after 10 days of exposure of the plantlets and 1 day of exposure of the suspension cultures to the industrial effluent and mixture of dyes respectively were calculated. The ADMI removal ratio was calculated (APHA, 1998) as follows:

\[
\text{ADMI removal ratio (\%)} = \frac{\text{Initial ADMI (oh) } - \text{Observed ADMI (t)}}{\text{Initial ADMI (oh)}} \times 100
\]

Where, ADMI (oh) is the initial ADMI value while ADMI (t) is the value obtained after a reaction time of 10 days for the plantlets and 1 day for suspension cultures.
3.8.2. Characterization of the textile effluent and mixture of dyes

BOD (Biological Oxygen Demand) and COD (Chemical Oxygen demand) of the industrial effluent and the mixture of dyes used were determined for characterization. The COD value was determined by using following procedure. The interference of chloride was removed by using mercuric sulfate. Then, the mixture of diluted sample (before and after treatment), $K_2Cr_2O_7$ and silver sulphate which was used as a catalyst, was refluxed in a reflux flask equipped with condenser on a COD Digestion Apparatus (Spectralab, 2015D, India) for 1.5 h. The refluxed sample was titrated against ferrous ammonium sulfate (FAS) as titrant. Distilled water was used as a blank and similar condition was used for the blank. Finally, COD was calculated by following formula:

$$\text{COD (mg/L)} = \frac{(A-B) \times N \times 8 \times 1000}{\text{Sample Size of sample}}$$

Where, $A = \text{mL of FAS that was used for the blank}$, $B = \text{mL of FAS that was used for the effluent sample}$, $N = \text{the normality of FAS}$, and $8 = \text{the mill equivalent weight of oxygen}$. The sample size used was 20 mL (Ghodake et al. 2009). BOD of the sample was determined by measuring the dissolved oxygen levels of the control samples (uninoculated effluent and solution of mixture of dyes respectively) and test samples (effluent and the solution of mixture of dyes to which $B. malcolmii$ and $T. flagelliforme$ plantlets were exposed for 10 days while suspension cultures of $B. malcolmii$ were exposed for 1 day) before and after incubation for 3 days. Winkler’s Iodometric Method was used for this estimation. (APHA, 1995, Ghodake et al., 2009) and further BOD was calculated as follows.

$$\text{BOD, mg L}^{-1} = (D_0-D_3) \times \text{dilution factor}.$$ 

Where, $D_0 = \text{Initial DO in the sample}$ 
$D_3 = \text{DO after 3 days}.$

3.9. Preparation of cell free extracts

Roots of the control plants (those suspended in plain distilled water) and test plants (those exposed to dye solutions) were used for the preparation of cell free extracts. The roots were initially detached from the shoot system, weighed and then cut into fine pieces after which they were suspended in ice cold 50 mM potassium phosphate buffer (pH 7.4) and ground finely in a mortar and pestle. The tissue was then homogenized in a glass homogenizer with intermittent cooling and then the extract was centrifuged at 8000 rpm for
20 min at 4°C. The supernatant thus obtained after centrifugation was used as intracellular enzyme source. After removal of plants the remaining dye solution after centrifugation was used as a source of extracellular enzymes. In case of *Blumea* suspension cultures, the cells were separated from the medium by filtration through a Whatmann filter paper. The filtrate was used as the source of extracellular enzymes while similar procedure as that mentioned above was used for the extraction of intracellular enzymes.

### 3.10. Enzyme assays

Activities of lignin peroxidase, laccase and tyrosinase were assayed spectrophotometrically at the room temperature where reference blanks contained all components except the enzyme (0.2 mL). The volume of reaction mixture was adjusted by the respective buffer. One unit of enzyme activity was measured as change in the absorbance per min.

Peroxidase activity was assayed by the procedure of Shanmugam et al., (1999). It was determined by monitoring the formation of propanaldehyde at 310 nm in a reaction mixture (2.5 mL) containing 100 mM *n*-propanol, 250 mM tartaric acid and 10 mM H$_2$O$_2$. Enzyme activity was expressed as units of enzyme min$^{-1}$ mg of protein$^{-1}$.

Laccase activity was determined by monitoring the formation of oxidized ABTS at 420 nm in a reaction mixture (2.0 mL) containing 10% ABTS in the 100 mM acetate buffer (pH 4.8) (Hatvani and Mecs, 2001). Enzyme activity was expressed as units of enzyme/min/mg of protein.

Tyrosinase activity was assayed using 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 the 100 mM potassium phosphate buffer (pH 6.8). Enzyme activity was expressed as units of enzyme min$^{-1}$ mg of protein$^{-1}$.

NADH-DCIP reductase activity was assayed by the procedure of Salokhe and Govindwar (1999). DCIP reduction was monitored at 595 nm and calculated using an extinction coefficient of 19 mM$^{-1}$ cm$^{-1}$. Reaction mixture (5.0 mL) prepared was containing 50 mM substrate DCIP in 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. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 µM of DCIP min$^{-1}$. 
Riboflavin reductase NAD(P)H: Flavin oxidoreductase was monitoring the decrease in absorbance at 340 nm. Cell free extract 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 mol L^(-1) cm^(-1) (Russ et al, 2000). One unit of enzyme activity was defined as µg of riboflavin reduced min^(-1) mg protein^(-1).

Azoreductase activity was assayed by modifying earlier method (Kalyani et al, 2008) 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) and 20 µM NADH. Enzyme activity was calculated by using molar extinction coefficient of 42.8 mM cm^(-1) of Methyl red (Jadhav et al, 2008). One unit of enzyme activity was defined as µM of MR reduced min^(-1) mg protein^(-1).

**Scanning electron microscopy of biosurfactant treated cells**

Permeabilized cells and control cells were fixed in 2% (w/v) glutaraldehyde for 4 h at 4°C, washed with saline solution, and dehydrated for 5 min in increasing ethanol concentrations (30, 50, 70, and 90% v/v) and for 30 minutes in absolute ethanol. The samples were air dried then coated with gold in argon atmosphere to an approximate thickness of 50 nm with the help of sputtering. The Scanning electron microscopy (SEM) observations were carried out using a scanning device JEOL JSM-6360.

**3.11. Phytotoxicity studies**

The ethyl acetate extracted products of degradation of Direct Red 5B by *B. malcolmii* and Brilliant Blue R by *T. flagelliforme* plantlets and suspension cultures of *B. malcolmii* were dried and dissolved in 20 mL of water to make the final concentration of 1000 ppm. *Phaseolus mungo* and *Triticum aestivum* seeds were used for phytotoxicity studies and the experiments were carried out at room temperature by placing 10 seeds in separate 10 mL solutions containing the dye, products of metabolism of the dye and water. The samples were watered every day. Germination (%), the length of plumule (shoot) and radical (root) was recorded after 8 days.
3.12. Mediator studies with cell suspension cultures

With a view of enhancing the decolorization potential of suspension cultures of *B. malcolmii* for the dye Brilliant Blue R, different known redox mediators were added into the medium. The suspension culture medium was autoclaved with the dye Brilliant Blue R (40 mg L\(^{-1}\)) and sterilized solutions of the different mediators (2 mM) were aseptically added into each of the respective flasks followed by inoculating them with 10 day old suspension cultures. The flasks were exposed to continuous shaking at 25°C. Decolorization percentage was calculated as described in section 3.6. The mediators used for these studies were ABTS (0.4 µM), HOBT, acetosyringone, syringic acid, vanillin, hydroquinone, DMP and pyrogallol.

3.13. Extraction of the products obtained after decolorization

In case of test plants, after being immersed in dye solutions, the plants were removed and the remaining dye solution was centrifuged to remove any solid materials if present. In case of degradation studies with suspension cultures, the cells from the dye decolorized flasks were removed by filtering the sample through Whatmann filter paper. The supernatant and filtrate were then mixed with an equal volume of ethyl acetate and the extracts were then evaporated in vacuum over anhydrous Na\(_2\)SO\(_4\) and dried. The crystals obtained were dissolved in a small volume of HPLC grade methanol and were further used for analytical studies. Similarly for control samples, viz. medium in which *B. malcolmii* plantlets had been immersed, autoclaved distilled water in which *T. flagelliforme* plantlets were immersed and medium inoculated with suspension culture were used for extraction of products in order to compare the results with the original dye and the extracts of the test solutions.


3.14.1. UV-Visible spectroscopy

Decolorization of all the individual dyes and the synthetic mixture of dyes in culture supernatants was monitored qualitatively using UV-visible spectroscopic analysis (Hitachi U-2800; Hitachi, Tokyo, Japan).
3.14.2. High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out (Waters model no. 2690; Waters Corp., Milford, MA) on C18 column (symmetry, 4.6x250 mm) by using HPLC grade methanol as mobile phase (75:25) with flow rate of 1 mL min\(^{-1}\) for 10 min and UV detector at 270 nm.

3.14.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analyses of the original dyes, the degraded products and the control samples were 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 holders for analyses.


Rotary vacuum concentrated sample was dissolved in methanol and GC-MS analysis of metabolites was carried out using a QP 5000 mass spectrophotometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm × 30 mm; XTI-5). The initial column temperature was 40°C for 4 min, then increased linearly at 10°C min\(^{-1}\) to 270°C and held at 4 min. The temperature of injection port was 275°C and GC-MS interface was maintained at 300°C. The helium was used as carrier gas with flow rate 1 mL min\(^{-1}\) and 30 min run time. The compounds were identified on the basis of mass spectra and using the NIST library stored in the computer software of the GC-MS (version 1.10 beta Shimadzu).

3.15. Purification and characterization of extracellular laccase

3.15.1. Growth of suspension cultures of \textit{B. malcolmii} and preparation of sample

Ten day old cultures of \textit{B. malcolmii} were used for purification studies. The cell cultures were filtered with a Whatmann filter paper under cold conditions (4°C). The filtrate was used as a crude source of extracellular laccase.
3.15.2. Purification techniques

The enzyme was dialyzed in a dialysis bag against sodium phosphate buffer (pH 7.4) for 4 h at 4°C to remove media components.

3.15.2.1. DEAE cellulose anion exchange chromatography

The purification was carried out manually at cold condition. The procedure for DEAE-cellulose anion exchange chromatography as follows. Activated DEAE-cellulose was packed in the column and pre-equilibrated with 0.2 M carbonate bicarbonate buffer (pH 8.0). The following conditions were maintained during purification of enzyme using DEAE-cellulose anion exchange chromatography (Table 3.2).

Table 3.2. Detailed conditions for purification of enzyme using DEAE-anion exchange chromatography.

<table>
<thead>
<tr>
<th>Required conditions</th>
<th>Maintained conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working temperature</td>
<td>5-10°C</td>
</tr>
<tr>
<td>Column type</td>
<td>Cylindrical glass column</td>
</tr>
<tr>
<td>Column height</td>
<td>14 cm³</td>
</tr>
<tr>
<td>Column width</td>
<td>1 cm³</td>
</tr>
<tr>
<td>Column flow rate</td>
<td>0.88 mL min⁻¹</td>
</tr>
<tr>
<td>Fraction volume</td>
<td>3 mL</td>
</tr>
<tr>
<td>Total fractions collected</td>
<td>75</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.2 M Potassium phosphate buffer (pH 8.0)</td>
</tr>
<tr>
<td>Eluent</td>
<td>NaCl (0 to 0.5 M)</td>
</tr>
<tr>
<td>Elution</td>
<td>Continuous gradient elution</td>
</tr>
</tbody>
</table>

3.15.3. Characterizations of purified enzyme

3.15.3.1. Polyacrylamide gel electrophoresis

Proteins in the enzyme preparation were analyzed by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing PAGE using
vertical slab gel electrophoresis unit (Table 3.3). Activity staining was carried out using non-denaturing PAGE. L-DOPA was used as a substrate for the activity staining of phenol oxidase (Eisenman et al., 2007). The molecular mass of purified enzyme was determined by SDS-PAGE using high molecular mass-standards such as phosphorylase b (98 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) lactoglobulin (18.4 kDa) and aprotinin (6.5 kDa). Protein bands were visualized using silver staining method.

**Table 3.3.** Composition of polyacrylamide gel and buffer used for nondenaturing and SDS-PAGE electrophoresis.

<table>
<thead>
<tr>
<th>30% Acrylamide stock (mL)</th>
<th>4x Separating buffer (mL)</th>
<th>Distilled water (mL)</th>
<th>10% ammonium persulfate (mL)</th>
<th>TEMED (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.01</td>
<td>7.5</td>
<td>11.4</td>
<td>0.2</td>
<td>0.05</td>
</tr>
</tbody>
</table>

4x separating buffer = 0.5 M Tris-HCl buffer (pH 8.3).
4x stacking buffer = 1.5 M Tris-HCl buffer (pH 6.8).
1x running buffer = 0.125 M Tris-HCl buffer (pH 8.3).
SDS-PAGE was run by using 0.4 ml of 10% SDS.

**3.15.3.2. Optimum conditions for catalytic activity of purified laccase**

The optimum pH and temperature for enzyme was calculated by observing the oxidation of substrate at different pH viz. 2, 3, 4, 5, 6, 7, 8, 9 and 10 and different temperatures viz. 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C. pH 3.0, 4.0, 5.0 and 6.0 were maintained by using sodium citrate and sodium acetate buffers while pH 7.0, 8.0 and 9.0 were maintained by using potassium phosphate buffer and pH 9.0 and 10 were maintained by using sodium carbonate-sodium bicarbonate buffer.

**3.15.3.3. Substrate specificity of the purified laccase**

Substrate specificity of the purified enzyme was studied by using several phenolic and nonphenolic compounds, such as 3,4-dimethoxy benzyl alcohol, guaiacol, 2,6-dimethoxy
phenol, L-DOPA, o-tolidine, hydroquinone, catechol, ABTS, caffeic acid, pyrogallol, p-cresol, syringic acid, o-danisidine and syringaldazine.

3.15.3.4. Kinetic constants of purified enzyme

Kinetics of purified enzyme was studied by observing the oxidation of increased concentration of ABTS at optimum condition. Michaelis constant (K_m) and maximum rates (V_max) were determined by using ABTS in the range of concentrations 0.125-1.25 µM pH 4.8 and 30°C. The reaction was followed in a spectrophotometer (Hitachi U-2800) and data were plotted according to Lineweaver-Burk.

3.15.3.5. Spectral characteristics of purified laccase

Sixty µg of laccase in potassium phosphate buffer (pH 8.0) was subjected to wavelength scan (200 to 800 nm) on UV-visible spectrophotometer (Hitachi UV 2800).

3.15.3.6. Effect of metal salts, inhibitors and stabilizers on the activity of purified laccase

We have studied the effect of different metal salts (1 mM; MgSO_4, CaCl_2, MnCl_2, MnSO_4, ZnSO_4, CoCl_2, and CuSO_4) and inhibitors viz. sodium azide, EDTA and L-cysteine (5 mM) on the activity of purified enzyme.

The enzyme activity was determined by using ABTS as substrate. Heat inactivated enzyme was used as a control. All the experiments were run in triplicates and average value was calculated.

3.15.3.7. Decolorization of the textile dyes using purified laccase

Four structurally different dyes viz. Brilliant Blue R, Malachite Green, Reactive Red 2 and Direct Red 5B were studied for their decolorization by using purified laccase.

The reaction mixture for the degradation of textile dyes using purified laccase contained the respective dyes (100 mg L^{-1}), 0.2 M sodium acetate buffer (pH 4.8) and 0.5 mL enzyme. To study the decolorization of the respective dyes in the presence of a redox mediator (ABTS), the reaction mixture was prepared as above and it contained 0.2 mL of ABTS as an additional component. The reaction mixture was incubated at 30°C at static as
well as shaking conditions. For all the dyes studied, two different controls were maintained as follows:

1. The first control contained the buffer and the respective dye (100 mg L\(^{-1}\)).
2. The second control contained the buffer, the respective dye and 0.2 mL of ABTS.

   The volume of all the samples was maintained by appropriately adjusting the volume of the buffer used.

3.15. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Readings were considered significant when \(P<0.05\).