3.0 MATERIALS AND METHODS

The present study entitled "***Bioremediation of methyl orange from aqueous solutions using Oedogonium subplagiostomum AP1***" was studied under the following phases:

PHASE I

3.1 Collection and identification of the alga

3.1.1 Collection and preparation of the alga

3.1.2 Identification of the alga

PHASE II

3.2 Batch biosorption experiments

3.2.1 Preliminary screening of dyes for decolourisation by alga

3.2.2 Preparation of the selected dye

3.2.3 Optimization parameters for dye removal by alga

3.2.4 Response Surface Methodology (RSM) studies for dye decolourisation

3.2.5 Desorption and recycling studies

3.2.6 Column studies for dye decolourisation

PHASE III

3.3 Sorption equilibrium and kinetic studies for dye removal

PHASE IV

3.4 Analytical methods for decolourisation studies

3.4.1 UV-Visible spectrophotometry

3.4.2 Fourier transform infrared spectroscopy (FT-IR)

3.4.3 Scanning electron microscopy (SEM) with Energy-Dispersive X-ray spectroscopy (EDX)

3.4.4 X-Ray Diffractometry (XRD)

PHASE V

3.5 Bioassay studies

3.5.1 Phytotoxicity

3.5.2 Zootoxicity

3.5.3 Microbial toxicity

3.5.4 Cytotoxicity and genotoxicity

PHASE VI

3.6 Reuse of dye desorbed algae and treated dye solution

3.6.1 Dye desorbed algae for compost production

3.6.2 Reuse of treated dye solution for dyeing fabrics

PHASE VII

3.7 Physicochemical characterisation of textile dyeing effluent using alga

PHASE VIII

3.8 In silico docking of azoreductase with methyl orange

PHASE I

3.1 COLLECTION AND IDENTIFICATION OF THE ALGA

3.1.1 Collection and preparation of the alga

The green alga was collected from free floating ripples in a fresh water pond near Perur, Coimbatore District, Tamil Nadu, India (Plate 1). The selected alga was not a rare species and there is no specific approval needed from any authority for its collection. Before use, the alga was washed with running tap water thrice and thereafter with double distilled water to remove the adhering larvae, sand and dirt. The collected alga was submitted for identification to Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

The washed biomass was shade dried at room temperature for 48hrs and sun dried for 10 days. The dried alga was pulverized to fine powder and stored in airtight container for further studies (Plate 2).

Plate 1 – Collection of alga from a pond in Coimbatore

Plate 2 - Powdered algal biomass

3.1.2 Identification of the alga

The genomic DNA from the alga was isolated using DNA extraction kit (Appendix 1). The nuclear encoded 5.8S rRNA was amplified by 35 cycles of PCR using the extracted genomic DNA as the template and two specific primers A1 [5^1-TCMGTAGGTGADCCWBCGS-3^1] and A2 [5^1-TCCTNCGYTKATKGVTADGH-3^1].

The PCR reaction for 5.8S rRNA gene was performed with a thermal program which consisted of pretreating at 94^{o}C for 5min. Initial denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 45 sec followed by 7 min at 72°C for post PCR reaction time or the last extension time. The PCR amplified DNA was subjected to gel electrophoresis using 1% agarose, stained with ethidium bromide for the visualization of bands. The 500bp DNA ladder was loaded to identify the amplified DNA. The PCR amplified DNA was purified using Qiaquick gel extraction kit (Appendix 2) and sequenced by dideoxy chain-termination method using the Big Dye Terminator version 3.1 kit and analysed using ABI 3500XL genetic analyser (Applied Biosystem, USA).

The 5.8S rRNA gene of the algal sequence was analysed at NCBI server (https://submit.ncbi.nlm.nih.gov/subs/genbank/) using BLASTN tool and the corresponding sequences of the homologous species were retrieved and used for phylogenetic analysis. The phylogenetic tree was constructed using Tree Builder Software and Weighbor (Weighted version of Neighbour Joining method) with alphabet size 4 and length size 1000 and bootstrap value of 100. The distance matrix was generated using Jukes - Cantor correction model (Ponnuswamy *et al*., 2013).

PHASE II

3.2 BATCH BIOSORPTION EXPERIMENTS

3.2.1 Preliminary screening of dyes for decolourisation by alga

A pilot study was conducted to screen the efficiency of the selected alga against different textile dyes namely Alizarin red S, Methyl orange, Aniline blue, Amido black 10B, Solochrome black T and Basic orange 21 (Analytical grade-Merck). Table 1 depicts the details of the dyes selected for the study.

Each dye was taken at a concentration of 100mg/L in an Erlenmeyer flask separately, inoculated with 100-700mg/L of biosorbent and incubated at room temperature (28±2^{o}C) for 5 days. At specific time interval, aliquots from each flask were removed aseptically and the decolourisation of dye was recorded spectrophotometrically at the given wavelength for the selected dyes (Phugare *et al*., 2011). The percentage removal was assessed using the equation,

$$\text{Decolourisation (\%)} = \frac{(A_i) - (A_t)}{(A_t)} \times 100 \quad \text{---------- (1)}$$

where A_i was the initial absorbance and A_t is the absorbance at incubation time t.

The dye which exhibited maximum decolourisation using alga was selected for further studies after preliminary screening.

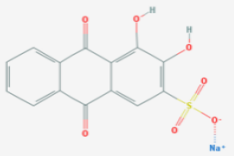
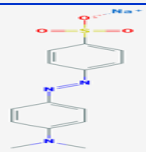
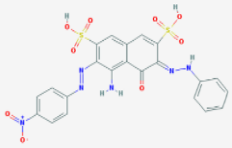
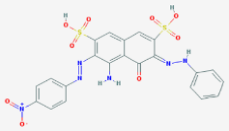
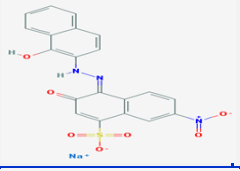
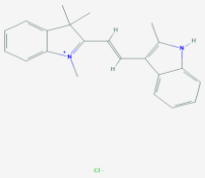
3.2.2 Preparation of the selected dye

Stock solutions of methyl orange were prepared at different concentrations ranging from 100-900mg/L. The wavelength maxima of the dye were measured spectrophotometrically by wavelength scan, against water as blank.

3.2.3 Optimization parameters for dye removal by alga

The decolourisation capability of alga was determined using the selected dye methyl orange. To a series of Erlenmeyer flask (1L) containing different concentrations of dye (100-900mg/L) varied amount of the algal biosorbent (100-700mg/L) was inoculated and incubated at different time intervals (1-10 days). Further, the experiments were executed to determine the effect of pH at different ranges (2-11) by adjusting with 1N HCl or 1N NaOH and by varying the incubation temperature (20°C-60°C), while keeping other conditions constant. The solution containing dye without alga served as control. All the optimization process parameters were conducted in triplicates.

Table 1 - Details of the dyes selected for the study

Name of the dye	Structural formula	Molecular formula	Molecular mass (g/mol)	Group	λ max	Appearance	C. I. No and CAS. No
Alizarin red S		$C_{14}H_7NaO_7S$	342.2559	Anionic	556	Orange yellow	58005: 130-22-3
Methyl orange		$C_{14}H_{14}N_3NaO_3S$	327.3340	Anionic	490	Less acidic (Red to Orange) and alkaline (Yellow)	13025: 574-58-0
Aniline blue		$C_{37}H_{27}N_3Na_2O_9S_3$	799.7995	Cationic	610	Blue colour	707: 28631-66-5
Amido black 10B		$C_{22}H_{16}N_6O_9S_2$	572.5272	Anionic	617	Dark Red to Black	20470: 1064-48-8
Solo chrome Black T		$C_{20}H_{12}N_3NaO_7S$	461.3799	Anionic	503	Dark blackish Brown powder	14645: 1787-61-7
Basic Orange 21		$C_{22}H_{23}ClN_2$	350.89	Cationic	490	Bright yellow orange	221-290-7: 3056-93-7

The samples were withdrawn for every 24h of incubation time and centrifuged at 10,000rpm for 10 minutes. The dye decolourisation was determined spectrophotometrically by monitoring the absorbance of the clarified samples so as to resolve the equilibrium time required for maximum dye decolourization. The percentage of dye removal (Eq.1) and the amount of dye biosorbed by the biosorbent (Denniz and Saygideger, 2010) was calculated as follows:

$$\text{Biosorption capacity } [q\ (mg/g)] = \frac{C_o - C_e}{M} \times V \quad \text{---------- (2)}$$

where, q represents the amount of dye biosorbed in mg/amount of the biosorbent in g, C_o and C_e are the initial and equilibrium concentrations of the dye (mg/L), V is the volume of the dye solution (L) and M is the mass of the biosorbent (g).

Under the above mentioned optimal conditions, the algal species was inoculated into methyl orange solution and the percent decolourisation and dye uptake was determined.

3.2.4 Response Surface Methodology (RSM) studies for dye decolourisation

Response Surface Methodology (RSM) is a statistical tool used to compute the functional relationship between the designed variables and the response obtained based on the experimental results. The objective of RSM approach is to optimize the process settings in an efficient use of the resources and to generate the best conditions for a system constituting different variables (Shdhukhan *et al*., 2009).

This method seems to be appropriate for fitting a quadratic surface and facilitate to optimize the effective parameters with a minimum number of trials, as well as to analyse the interaction between the parameters. In order to determine the existence of a relationship between the factors and the response variables, the data collected were analysed in a statistical manner using regression. A regression design is normally employed to model a response as a mathematical function (either known or empirical) of a few continuous factors and good model parameter estimates are desired.

Design of Experiment (DOE) is a well-accepted statistical technique able to design and optimize the experimental process that involves choosing the optimal

experimental design and estimate the effect of the several variables independently and also the interactions simultaneously.

The Box-Behnken Design (BBD) chosen for the present investigation was used to study the effect of variables towards their responses in optimization experiments. BBD is suitable for fitting a quadratic surface and aids to optimize the effective operational parameters as well as to analyse the interaction between the parameters. The RSM approach was applied to determine the operational levels of four factors namely dye and biosorbent concentrations, pH and contact time to identify the relationship between the response functions and process variables. The values used were based on the results of the preliminary experiments carried out to determine the decolourisation and dye uptake efficiency.

Based on BBD, the factors were analysed at two levels: -1 for low level, and +1 for high level. A total of 29 runs were performed to optimize the process parameters, and experiments were performed according to the experimental design matrix. In order to determine the existence of correlation between the factors and response variables, the data collected were analysed in statistical method using regression. For regression analysis a second-order polynomial equation was developed to estimate the response as a function to correlate the interaction of dependent and independent variables. In general, the response for the second-order polynomial is described as follows,

$$Y = ß_0 \Sigma ß_i X_i + \Sigma ß_i X_i ß_{ij} + \Sigma X_i X_j \text{ ---------- (3)}$$

where Y is the predicted response and $ß_0$, $ß_i$, and $ß_{ij}$ are constant regression coefficients of the model and X_i and X_j represents the independent variables.

Design-Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA) was used to evaluate the regression and graphical analyses with statistical significance. The 3D response surface plots were generated from the model, to visualize the relationship between the experimental variables and responses. From the response surface plots, the optimum values of the process variables were obtained. Lack-of-fit is a special diagnostic test for adequacy of a model that compares the pure error, based on the replicate measurements to the other lack-of-fit and model performance. F value is calculated as the ratio between the lack-of-fit

mean square and the pure error mean square. Pure error mean square is the statistic parameter used to determine whether the lack-of-fit is significant or not, at a significance level. F test was used to evaluate the significance of the model equation and model terms. The results were evaluated by applying the coefficient of determination (R^2), analysis of variance (ANOVA) and response plots (Selvankumar *et al*., 2017).

3.2.5 Desorption and recycling studies

For desorption study, 400mg of the dye loaded biosorbent was inoculated into 1000ml Erlenmeyer flask pertaining the desorbing agents namely 0.1N HCl, 0.1N HNO_3 and 0.1N NaOH respectively. The flasks containing the mixture of dye loaded biosorbent (500mg/L) and the desorbing agents were agitated at 200rpm at 30^{o}C for five days at pH 6. At every 24h incubation period, the contents were filtered and the filtrate was analysed to determine the percentage desorption efficiency. It was determined by calculating the ratio between the amount of dye adsorbed on the biosorbent and the final dye concentration in the desorption medium (Akar *et al*., 2009), as shown in the following equation (4)

$$\text{Desorption Efficiency (\%)} = \frac{\text{Amount of dye desorbed}}{\text{Amount of dye adsorbed}} \times 100 \quad (4)$$

The recycling efficiency of the desorbed alga was evaluated by three consecutive cycles of adsorption-desorption. The biosorbent was inoculated into dye solution at optimized conditions and the percent decolourisation was recorded. The experiment was conducted in triplicates.

3.2.6 Column studies for dye decolourisation

A fixed bed column was set up in laboratory condition to assess the decolourisation efficiency of the alga on methyl orange. A borosilicate glass column (70cmX10cm) was chosen and clean glass wool was placed at the bottom of the column to retain the biosorbent and to prevent the disturbance of the bed by high flow rate. The glass column was packed with the biosorbent (1.2g/3L) and the bed was washed with distilled water for several times. The dye, methyl orange (1.5g/3L) was added into the column manually and the flow rate was varied from 10-120mL/hr.

At periodical interval, aliquot of the sample was collected and the percentage removal of dye was recorded.

PHASE III

3.3 SORPTION EQUILIBRIUM AND KINETIC STUDIES FOR DYE REMOVAL

Biosorption studies were carried out by assessing the isotherms, kinetics and thermodynamic process which provides a better understanding on the adsorption mechanism. The equilibrium isotherm is of primary importance for the design and optimization of the system for the removal of dyes by adsorption. Hence, the efficiency of the prepared adsorbent can be evaluated to develop suitable industrial adsorption system designs.

Isotherm models

Isotherms give an equilibrium relationship between the amounts of adsorbate adsorbed on the adsorbent surface and its concentration in the solution at a constant temperature. In the present study the experimental data have been subjected to two commonly used sorption isotherms, namely the Langmuir and Freundlich for the adsorption of dyes. The applicability of the Langmuir and Freundlich isotherm equations were compared by their correlation coefficient.

Kinetic models

The adsorption kinetics demonstrates the development of adsorption potentiality during time and its necessity for recognizing the categories of adsorption mechanism in the biosorption process. In order to comprehend the mechanism and dynamics which controls the biosorption process, adsorption kinetic models were evaluated to identify the potential adsorption process and to know the possible physicochemical interactions involved in adsorption phenomenon between the adsorbent surface and dye molecules. In the present study the adsorption of methyl orange on the alga was analysed using pseudo-first order (Lagergren, 1898) and pseudo-second order (Ho and McKay, 1999) kinetic models.

Thermodynamic studies

Thermodynamic parameters evaluate the nature of the adsorbate and its magnitude during adsorption process. It seems to be essential in which any chemical system tends to conquer a state of equilibrium, from the non-equilibrium state

(Milind *et al.,* 2010). The thermodynamic parameters such as Gibbs free-energy changes (ΔG^o), standard enthalpy changes (ΔH^o) and standard entropy changes (ΔS^o) were also studied for the better understanding of the adsorption mechanism.

PHASE IV

3.4 ANALYTICAL METHODS FOR DECOLOURISATION STUDIES

In order to expose the possible mechanism of dye decolourisation, various analytical techniques such as UV-Vis, FT-IR, SEM with EDX and XRD were used to identify the metabolites generated from methyl orange after algal treatment.

3.4.1 UV-Visible spectrophotometry

UV-Visible spectral analysis of the cell free sample, before and after dye decolourisation was performed using UV- spectrophotometer (Hitachi U2800, Tokyo, Japan). The changes in the absorption spectrum in the visible range of 200-800nm were recorded in comparison with the results from the control runs (Du *et al*., 2013).

3.4.2 Fourier transform infrared spectroscopy (FT-IR)

The FT-IR analysis of dye unloaded and loaded algae was carried out using Shimadzu 8400S FT-IR spectrophotometer (Japan) in the mid IR region of 400-4000cm^{-1} with 16 scan speed. The samples were prepared using spectroscopically pure KBr in the ratio of 5:95 and the pellets were fixed in the sample holder and analysed (Kalpana *et al*., 2012).

3.4.3 Scanning electron microscopy (SEM) with Energy-Dispersive X-ray spectroscopy (EDX)

The surface morphology of the dye unloaded and loaded algae were observed using scanning electron microscope. The dried algal samples were mounted on stubs coated with gold palladium (10nm) using a sputter coater and transferred to the sample chamber of SEM operated at 20 kV. The photomicrographs were recorded using SEM (model JSM 6100, Japan) at 5000 and 7500X magnification to confirm the surface adsorption of dye on the biosorbent. The existence of chemical components of the dye unloaded and loaded algae were determined using Energy-dispersive X-ray spectroscopy (Michalak *et al*., 2014).

3.4.4 X-Ray Diffractometry (XRD)

X-ray diffraction pattern of dry powdered samples of dye unloaded and loaded algal biomass was recorded in a Philips (PW-3710) diffractometer equipped with monochromatic CuKα radiation (λ=0.15406Å), with generator voltage of 40kV and tube current of 30mA. The samples were scanned at the rate of 3^{o}/min over the range of 10^{o}-100^{o} (2θ) with a step length of 0.058 (2θ) (Saraf and Vaidya, 2016).

PHASE V

3.5 BIOASSAY STUDIES

In spite of the effectiveness of the biological treatments, microbes can transform dyes into compounds more toxic than the original. Hence there is a need to assess the toxicity of degraded metabolites after biological treatment for toxicological assays such as phytotoxicity, zootoxicity, microbial toxicity and cytogenotoxicity. These eco-toxicological assays play a vital role in the safe cleanup of environmental measures.

3.5.1 Phytotoxicity

Phytotoxicity test was assessed to evaluate the toxicity of the degraded metabolites on the flora and to explore its reuse potential in the field of irrigation.

3.5.1.1 Selection of the experimental plant

Tagetes erecta (Marigold/ Cottage Red) is a perennial herbaceous plant belonging to the family Asteracea was selected for this study. It is usually cultivated for ornamental purposes and it does not require special conditions for successful cultivation which makes it very popular among the gardeners worldwide. It requires adequate sunlight and well-drained soil for successful growth. Dyes extracted from marigold flowers are used in textile, food and beverage industries and also as a nutritional supplement in cosmetic industry. It also possesses anticancer, antimicrobial and anti-insecticidal properties.

3.5.1.2 Experimental design

The experiment was conducted in field under three treatments for duration of 60 days. For field level cultivation, nine square pits were made with 30X30X30cm (length, width, depth) and each treatment was replicated thrice (Plate 3).

The temperature of the selected site was 20-30^{o}C with no rainfall. The treatments used for the study were T_1 - Tap water (Control), T_2 - Untreated dye solution and T_3 - Treated dye solution.

Plate 3 - Experimental land for plant growth studies

The seeds of marigold were collected from Tamil Nadu Agricultural University, Coimbatore District, Tamil Nadu, India. Healthy seeds were selected and surface sterilized with 0.1% $HgCl_2$ solution for 2 min and washed with tap water to remove the surface contaminants. In each pit, 20 healthy seeds were sown and irrigated with tap water (T_1), untreated (T_2) and treated dye (T_3) solutions respectively.

3.5.1.3 Biometric observations

The plants were uprooted on 7th and 60th day after sowing and washed in running water to remove the soil particles adhered on the roots and dried with filter paper to remove the water contents. On the 7th day, the biometric parameters namely germination percentage, seedling length, vigour index and phytotoxicity index were calculated.

Germination percentage is an estimate of the viability of seed population, which was calculated by the following equation

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seed sown}} \times 100 \quad \text{---------- (5)}$$

The seedling length (shoot and root length) of the germinated seeds were measured from each treatment. The shoot and root length was measured from the base of the primary leaf and root to the base of the hypocotyl separately. The mean shoot and root length were expressed in centimetre.

The vigour index was calculated adopting the method of Abdul Baki and Anderson (1973).

Vigour index = Germination percentage X (Root length + Shoot length) ---- (6)

Phytotoxicity index (PI) was calculated based on the germination and root elongation of marigold seeds exposed to different treatments

$$PI = 1 - \frac{R_{LT}}{R_{LC}} \quad \text{---------- (7)}$$

where R_{LT} is the root length in the untreated and treated dye solutions and R_{LC} is the root length in the control (Mekki *et al*., 2007).

Similarly, on 60th day height of the plant, number of flowers, size and weight of the flowers were recorded. The cost economy of the marigold flowers produced per year after irrigation with treated dye solution was calculated.

Statistical analysis

The data obtained was statistically analysed by one way analysis of variance ($P < 0.05$) using statistical software Sigma stat 3.1.

3.5.1.4 Characterisation of soil samples

The physicochemical characteristics of the soil exposed to tap water, untreated and treated dye solutions were analysed. The soil was collected from a depth of 15cm with a wooden spade, dried, crushed and sieved. The dried soil samples were analyzed for the physicochemical parameters namely pH, electrical conductivity, total nitrogen, total phosphorus, total potassium, sodium, calcium, iron, copper, zinc, nickel and manganese by adopting the standard

procedures as depicted in Table 2. The data obtained were statistically analysed by mean ± SD.

Table 2 - Physicochemical characterisation of the experimental soil

Parameters	Method of analysis	References	Appendix No.
pH	pH meter	Jackson (1973)	3
EC (mmhos/cm)	Conductivity bridge		4
Macronutrients (kg/ha)			
Total nitrogen	Microkjeldhal method	Subbaih and Asija (1956)	5
Total phosphorus	Flame photometry	Olsen *et al*. (1954)	6
Total potassium and sodium			7
Calcium	Titrimetry	Subbaih and Asija (1956)	8
Micronutrients (mg/kg)			
Iron	Atomic absorption spectrophotometry	Lindsay and Norvell (1978)	9
Copper			
Zinc			
Manganese			
Nickel		APHA (1998)	10

3.5.2 Zootoxicity

Toxic impact of untreated and treated dye solutions on the fingerlings of *Labeo rohita* were determined by behavioural, haematological, biochemical and histopathological changes.

3.5.2.1 Selection of experimental fish

The Indian major carp *Labeo rohita* (Plate 4) popularly known as Rohu is selected as the experimental animal for the present study due to its rapid growth, high sustainability, tolerance at high stocking density and survival ability in oxygen depleted water. It is a bottom feeder which mainly consume algae and aquatic plant The taxonomic classification of the selected fish is given below

Scientific Classification	
Phylum	Chordata
Subphylum	Vertebrata
Division	Gnathostomata
Superclass	Pisces
Class	Osteichthyes
Subclass	Actinopterygii
Superorder	Teleostei
Order	Cypriniformes
Family	Cyprinidae
Genus	*Labeo*
Species	*rohita* (rohu)

Plate 4 - Labeo rohita - Experimental fish

3.5.2.2 Procurement and acclimatization of experimental fish

The freshwater fingerlings of *Labeo rohita* (length 7.2±0.4cm and weight 8.3±1g) was procured and transported to laboratory from National fish seed farm, Department of Fisheries, Bhavani Sagar, Erode, Tamil Nadu, India in clean aerated polythene bags. The collected fishes were safely brought to the laboratory and acclimatized under laboratory conditions for 20 days in fish tank disinfected with potassium permanganate solution to prevent from fungal infection (Plate 5).

During the acclimatization period, the fishes were fed daily with rice bran and groundnut oil cake (2:1) which had no detectable amount of dye. The water in the acclimatization tank was renewed daily to remove the excess amount of feed and excretory materials. Fishes showing abnormal characteristic behaviour was removed from the tank. At the end of the acclimatization period healthy fingerlings were separated and subjected to experimental study.

3.5.2.3 Experimental design

The mortality of the selected fish *Labeo rohita* was studied at 7^{th} day by introducing 15 fishes into the tubs filled with 20L of tap water which served as control (T_1), untreated dye (T_2) and treated dye (T_3) solutions separately (Plate 6).

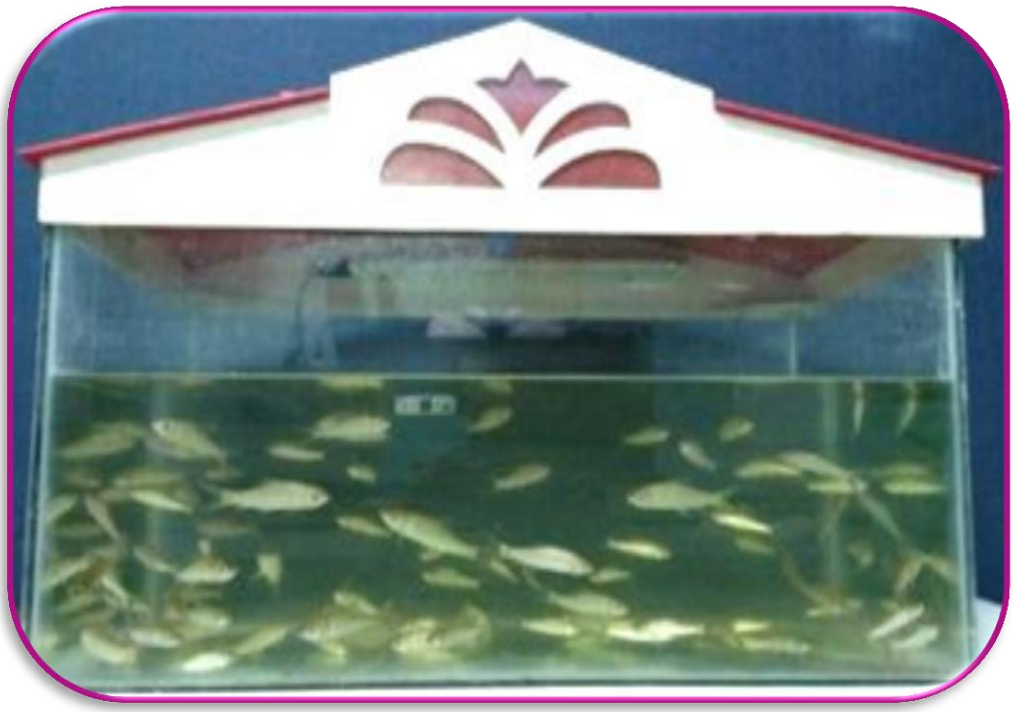


Plate 5 - Acclimatization of the experimental fish

Plate 6 - Experimental setup for fish bioassay studies

T_1 - Tap water (Control)

T_2 - Untreated dye solution

T_3 -Treated dye solution

For each treatment three replicates were maintained and the treatment water samples were renewed daily. Dead fishes were removed immediately to prevent the contamination and to maintain desired concentration of oxygen. The mortality of the fishes were recorded daily and the cumulative percentage of fish mortality (Amte and Mhaskar, 2013) were noted on 7^{th} day using the formula given below

$$\text{Mortality (\%)} = \frac{\text{Number of fishes dead}}{\text{Total number of fishes}} \times 100 \quad (8)$$

The fishes from each treatment were subjected to haematological, biochemical, enzymological and histological studies.

3.5.2.4 Haematological, biochemical and enzymological analysis

The blood was collected from the experimental fishes by cardiac puncture using heparinised syringes and transferred into heparin coated vials. The whole blood was used for haematological studies and the remaining was centrifuged at 10,000rpm for 20 min to separate the plasma and used for biochemical assays.

After drawing blood from fishes, they were washed with distilled water and blotted dry with absorbent paper. The liver was isolated from the control and experimental fishes. 100mg of each tissue was weighed and homogenized with 2.5ml of 0.25M sucrose solution in ice cold condition. The homogenates were centrifuged at 6000rpm for 20 min and the clear supernatant fluid was taken for enzyme assay (Hogeboom *et al*., 1948).

The collected whole blood, plasma and the supernatant obtained from the liver samples were subjected haematological, biochemical and enzymological analyses. The parameters analysed and the methodology adopted was given in Table 3. The data obtained were statistically analysed by mean±SD. The data obtained was statistically analysed by one way analysis of variance ($P < 0.05$) using statistical software Sigma stat 3.1.

3.5.2.5 Histological examination

For histological investigation, the fishes exposed to tap water, untreated and treated dye solutions were dissected to collect the tissues of gills, liver and kidney. The dissected tissues were immediately fixed in 10% formalin and washed in distilled

water, dehydrated in graded ethanol series (30%, 50%, 70%, 85% and 100%), infiltrated with xylene and embedded in paraffin wax at 56-60^{o}C. The tissues embedded in paraffin wax were sectioned using a rotator microtome (5µm) and treated with xylene to remove paraffin and subsequently washed in 90%, 70%, 50% and 30% alcohol (Bancroft and Cook, 1994).

Table 3 - Haematological, biochemical and enzymological parameters analysed in the experimental fishes

Parameters analysed	Methods of analysis	References	Appendix No.
Haematological parameters			
RBC (10^6 cells/mm^3)	Diluting fluid method - Haemocytometer	Rusia and Sood (1992)	11
PCV (%)	Microhematocrit reader	Nelson and Morris (1989)	12
Hb (g/dl)	Spectrophotometry – Cyanmethemoglobin method	Drabkin (1946)	13
WBC (10^3 cells/mm^3)	Trucks fluid method - Haemocytometer	Rusia and Sood (1992)	14
Erythrocyte indices			
MCV (fl)	Calculation	Blaxhall and Daisly (1973)	15
MCH (pg)			
MCHC (g/dl)			
Biochemical parameters			
Plasma Glucose (mg/100ml)	Spectrophotometery	Trinder (1969)	16
Plasma Protein (µg/ml)		Doumes *et al.* (1971)	17
Enzymological parameters			
AST (IU/L)	Spectrophotometery	Reitman and Franckel (1957)	18
ALT (IU/L)			19

Finally paraffin free sections were washed with distilled water, stained with haematoxylin for 10min and washed in running tap water. Finally the tissues were stained in eosin for 10min, examined under microscope and photographed. The changes in the tissues of the experimental fishes were compared with the control fishes.

3.5.3 Microbial toxicity

The microbial toxicity was performed to examine the toxic effect of the degraded products or metabolites of methyl orange in relation to microbial flora. The bacterial isolates selected for the present study includes *Klebsiella pneumoniae, Streptococcus epidermis, Vibrio cholerae, Pseudomonas aeruginosa, Shigella* species*, Bacillus cereus, Proteus vulgaris, Staphylococcus aureus, Escherichia coli, Salmonella enteritis, Enterococcus faecalis, Yersinia enterocolitica* and *Acinetobacter* species*.* The fungal isolates are *Aspergillus flavus, Aspergillus niger, Acremonium* species*, Rhizopus* species and *Trichoderma viride.*

Agar well diffusion was carried out to assess the microbial toxicity against the selected bacterial and fungal isolates (Bauer *et al.,* 1966). Three wells were bored on sterile Muller-Hinton agar (bacteria) and Rose Bengal Chloramphenicol agar (fungi) plates using a sterile cork borer separately. Each plate was swabbed with 100µl of the selected bacterial and fungal cultures separately. To each well in the respective medium, tap water, untreated dye solution and degraded metabolites of dye were added separately and the plates were incubated at 37^{o}C for 24hrs (bacteria) and at room temperature for 5 days (fungi) to measure the zone of inhibition. The size of inhibition zone (mm) surrounding the well represented the index of toxicity against the pollutants. The data obtained were statistically analysed by mean±SD (Saratale *et al*., 2010).

3.5.4 Cytotoxicity and genotoxicity

3.5.4.1 Selection of *Allium cepa*

Allium cepa is one of the most extensively used experimental plant in cytogenotoxicity studies because it is sensitive, has rapid growth, suitable chromosomes and exhibit good correlation with mammalian genetic composition (Evseeva *et al.,* 2003). In the present study, the toxicity potential of the untreated dye

and treated dye solutions were investigated by assessing their cytotoxic and genotoxic effects on *Allium cepa* (Datta *et al.,* 2018).

3.5.4.2 Cytotoxic and genotoxic evaluation of untreated and treated dye solution on *Allium cepa*

The purple variety of *Allium cepa* (2n=16) of equal size (2-2.5cm width and 2-4 g weight) were purchased from the local market. The onion bulbs were air dried for 5 days and the outer dry brown scales were removed. The poorly preserved bulbs that have started with shooting green leaves and mouldy were discarded. The dried roots were carefully removed with a sharp blade and placed in distilled water to protect the primordial cells from drying up (Rank, 2003).

Five onion bulbs were grown in glass beakers containing 200ml of tap water (T_1) which served as control, untreated dye (T_2) and treated dye solutions (T_3) separately at room temperature for 5 days. The test solutions were replaced for every 24h and the experiments were carried out in triplicates. At the end of 5^{th} day the germinated bulbs were removed and the root length was measured using a calibrated ruler. The results of mean root length were statistically expressed as mean±SD. The data obtained was statistically analysed by one way analysis of variance ($P < 0.05$) using statistical software Sigma stat 3.1. The morphological changes in the root tip exposed to different treatments were also observed. The root growth of *Allium cepa* in different treatments were observed and calculated as follows:

$$\text{Root growth (\%)} = \frac{\text{Mean root length of treatments}}{\text{Mean root length of control}} \times 100 \quad \text{---------- (9)}$$

The root meristems of 2cm long were cut from each treatment separately, washed in distilled water and fixed in Carnoy's fluid [acetic acid : ethanol (1:3)] for 5min. The fixed root tips were hydrolysed in 1N HCl at 60^oC for 5min to soften the tissue. The root tips were macerated and stained with acetoorecin for 15min, covered with coverslip and squashed. A total number of 1000 cells from five scorable slides per treatment were observed under light microscope to view the different mitotic stages and chromosomal aberrations. The mitotic index (MI), Mitotic depression/inhibition (MD) and frequency of chromosomal aberrations were

calculated using the following formula described by Fiskesjo (1993), Fiskesjo (1985) and Baskare *et al*. (2000) respectively.

$$\text{Mitotic index (MI)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells scored}} \times 100 \quad (10)$$

$$\text{Mitotic depression / inhibition (MD)} = \frac{(\text{MI in control group} - \text{MI in treatment groups})}{\text{MI in control group}} \times 100 \quad (11)$$

$$\text{Chromosomal aberration (\%)} = \frac{\text{Number of chromosomal aberration}}{\text{Total number of cells}} \times 100 \quad (12)$$

PHASE VI

3.6 REUSE OF DYE DESORBED ALGAE AND TREATED DYE SOLUTION

3.6.1 Dye desorbed algae for compost production

Due to the disposal problem, the desorbed algae can be used as an organic waste for algal composting.

3.6.1.1 Selection of earth worm

Earthworm selection is very important factor because only few species are able to survive and adjust to a particular type of environment. The exotic earthworm namely *Eudrilus eugeniae* is commonly used for breaking down all the organic waste. *Eudrilus eugeniae* is a tropical earthworm commonly called African night crawler belongs to the family Eudrilidae. It is large in size, grows rapidly, fast breeder and capable of decomposting large quantity of organic materials into usable vermicompost.

3.6.1.2 Preparation of algal compost bed

The pit made for algal composting is about 2x3x3m in size. The pit for experimental setup was prepared by filling a layer of garden soil upto 10cm. The organic waste materials for composting (dye desorbed algal powder + garden waste + cow dung - 2:1:2) were added to the pit. The materials were allowed to decompose for 20 days and the moisture content was maintained by watering regularly. The heat produced due to decomposition of the materials was reduced by

sprinkling water over the bed. After 20th day, the predigested compost was subjected to composting using the selected earthworm species.

3.6.1.3 Inoculation of the worms into the vermibed

The composting bed was inoculated with 100 earthworms and covered with coconut husk to protect from birds and insects. The vermibed was left for 60 days without disturbing the worms and water was sprinkled daily to maintain the moisture content and body temperature of the worms. The appearance of black granular crumbly powder on the top of vermibed indicates the harvesting stage of the compost (Plate 7).

Plate 7 - Compost pit

3.6.1.4 Collection of algal compost

The algal compost was harvested on 60th day and sun dried to remove the gases released. The dried algal compost was powdered with a mortar and pestle and analysed for the physicochemical characteristics namely pH and electrical conductivity (Appendix 3 and 4), moisture (Appendix 20), organic carbon (Appendix 21) and macronutrients namely nitrogen, potassium and phosphorus (Appendix 5, 6 and 7). The cost economy for the algal compost produced per year was calculated.

3.6.2 Reuse of treated methyl orange solution for dyeing

In order to save water and reduce pollution, recycling of waste water is need of the hour. Hence an attempt has been made to utilize the treated dye solution for dyeing the fabrics.

Experimental setup for dyeing

The fabric selected for the present study is cotton which is exclusively used as a house hold fabric by the people in all levels due to its comfortness and good absorbency. Desizing of the selected fabric was carried out as a first pre-treatment process for dyeing. Desizing is the process of removal of starch from the fabric in order to make the fabric absorbent. The selected fabric (5g) was made wet with tap water and soaked in a bath containing 0.8% soap solution. The mixture was heated to boil with continuous stirring for 1hr at 90^{o}C and the fabric was rinsed thoroughly in running water and dried in shade. The scouring process which is the second pre-treatment process was done to remove the natural impurities such as grease, wax and fats present in the fabric. Scouring is carried out by boiling the fabric with 3% NaOH at a material ratio of 1:20 for 1hr. The scoured fabric is rinsed and dried in shade. Followed by scouring, bleaching of the fabric is done in a bath containing 2L of tap water, 3g of sodium bicarbonate and 2g of hydrogen peroxide. The bath containing the mixture of chemicals along with the fabric was boiled for 1hr. The bleached fabric was rinsed well and dried in shade.

Dyeing was carried out using treated dye solution and tap water. The following protocol was followed for dyeing

Dye	: 500mg/L
Material: Liquor ratio	: 1:30
Time	: 1hr
Temperature	: 90^{o}C

The dyed fabric were subjected to soaping by immersing in 2g/L detergent solution and stirred continuously for 30min at room temperature. The fabric was rinsed in tap water, dried in shade and subjected to evaluation.

Fabric evaluation

The fabrics dyed with tap water (control) and treated dye solution (test sample) was subjected to test various evaluation parameters namely fabric weight, fabric thickness, fabric strength, fabric elongation, fabric stiffness and colour fastness.

Fabric weight (g)

Fabric weight of control and test samples was determined using GSM cutter. It is a device with four blades to cut the fabric in a circular specimen of 100cm^2. The samples were cut, weighed accurately and the values were expressed in grams/square meter of the fabric.

Fabric thickness (mm)

Fabric thickness gauge was used to measure the thickness of the dyed fabrics. The gauge has an anvil and pressure foot through which the pressure was given to the foot to make the gauge dial zero. The samples were placed between the cleaned pressure foot and anvil without any pressure and the reading shown by the dial was noted. The thickness was determined at 5 different places of the sample which were 2 inch away from the selvedge.

Fabric strength (kg) and elongation (inch)

The strength of the fabric is a measure of its resistance to tensile load or stress in either warp or weft directions. Elongation is the increase in length of a fabric during tension test which is expressed in length (inch). The control and test fabrics were tested for tensile strength using Eureka cloth tensile strength tester. Each sample was cut by 12 inch X 2 inch both in warp and weft directions which were two inches apart from selvedge. The samples were placed between the upper and lower clamp of the tester and the dial reading was set to zero by adjusting the pendulum over the quadrant scale. The elongation pointer was checked for its position in zero. Before starting the machine, the pendulum lock was released and machine was switched to run. When the fabric started to break, the machine was switched off and dial reading (kg) was noted. The elongation of the fabrics was noted from the scale in inches.

Fabric stiffness

Fabric stiffness was tested using stiffness tester. The control fabrics were cut to the size of 15cm X 2.5cm using the template. The sample was placed on the platform with the template at top of it, so that the leading edges coincide. Both were slowly pushed forward until the leading edges of the sample and the template projected beyond the edge of the platform. The sliding of the samples was stopped when it cut both the index lines. The bending length of the sample was read from the scale opposite to datum line engraved on the side of the platform and the readings were recorded. The mean values of the bending length in warp and weft wise direction was calculated for both the samples.

Colour fastness tests

Dyeing not only imparts attractiveness on the fibre but it also retains colour on it. Colour fastness measures the resistance of the fabric to change in colour when they are exposed to various agents like sun light, pressing and crocking. The grey scale employed for colour fastness test is 1-5 grades. Scale 1 denotes poor fastness and 5 represents excellent colour fastness of the fabric (Smith, 2006). In this study, three colour fastness tests namely fastness to sun light, wet and dry crocking, wet and dry pressing were carried out.

Fastness to sun light

Colour fastness is an important property to determine the resistance of a material to change its colour characteristics when the fabric is exposed to sunlight or an artificial light source. To test the fastness to light, the control and test fabrics of 16cm X 5cm were cut and divided into 8 equal parts and covered with black chart. The fabric pieces were exposed to sunlight day wise for 7 days and a control was maintained for comparison which was done using grey scale and the fabrics were rated.

Wet and dry crocking

Crocking is the rubbing fastness test which represents the transfer of colourant from the surface of the coloured fabric to an adjacent area of same fabric or to another surface by rubbing action. Sasmira crock meter was used to determine the fastness to crocking. Each of the dyed fabric was cut in the measurement of

25 X 20 cm and mounted on flat base. The desized white cotton fabric was mounted in a ring on the rubbing finger. Each sample was given 10 rubs based on standardisation. The colour transfer from the wet dyed sample to the white material was used for wet crocking. The procedure adopted was same as that of dry crocking. The colour transfer from the dyed sample to the white material was assessed using grey scale.

Wet and dry pressing

Two sets of control and test samples were cut at a measurement of 10 X 10 cm and were covered at either side with 5 X 5cm of desized white fabric. The prepared fabric were pressed for 10 seconds to assess its colour fastness to dry pressing while the others were covered with wet white cloth and pressed for 10 seconds to assess its colour fastness to wet pressing. The colour change in the dyed fabrics was graded using grey scale.

PHASE VII

3.7 PHYSICOCHEMICAL CHARACTERISATION OF TEXTILE DYEING FFLUENT USING ALGA

Recycling and reclamation of textile dyeing effluent is a promising alternative to conserve or reuse the effluent as a supplement to reduce or eliminate environmental pollution. The textile dyeing waste water was collected from a local textile dyeing factory, Tirupur, Tamil Nadu, India. Dried alga (400mg) was inoculated into 1L of textile dyeing effluent and allowed to grow for 5 days at room temperature.

The physical (colour, odour, pH, electrical conductivity, turbidity, total dissolved solids, total suspended solids and total solids) and chemical (alkalinity, total hardness, biological oxygen demand, chemical oxygen demand, chlorides, sulphates and nitrates) parameters were analysed in the textile dyeing effluent treated with the selected alga and the decolourisation percentage was also recorded. The data obtained were statistically analysed by mean±SD. The physicochemical parameters analysed, the method of analyses, appendices and their respective references were depicted in Table 4.

Table 4 - Physicochemical characteristics of textile dyeing effluent

Characteristics	Method of analysis	References	Appendix No.
Physical characteristics			
Colour	Visual	-	-
Odour	Smell	-	-
pH	pH meter	APHA, 1998	3
Electrical conductivity (mmhos/cm)	Conductivity meter	APHA, 1998	4
Turbidity	Visual	-	-
Total Dissolved Solids (mg/L)	Filtration	APHA, 1998	22
Total Suspended Solids (mg/L)	Filtration	APHA, 1998	23
Total Solids (mg/L)	Calculation	APHA, 1998	24
Chemical characteristics			
Alkalinity (mg/L)	Titrimetry	APHA, 1998	25
Total hardness (mg/L)	Titrimetry	APHA, 1998	26
Biological Oxygen Demand (mg/L)	Titrimetry	APHA, 1989	27
Chemical Oxygen Demand (mg/L)	Titrimetry	APHA, 1998	28
Chloride (mg/L)	Titrimetry	Vogel, 1964	29
Sulphate (mg/L)	Turbidimetry	APHA, 1998	30
Nitrate (mg/L)	Nitrate electrode method	APHA, 1998	31

PHASE VIII

3.8 *IN SILICO* DOCKING OF AZOREDUCTASE WITH METHYL ORANGE

Molecular interaction with substrate and binding sites can be studied using docking simulation which provides a holistic approach for the development of effective bioremediation. In this *in silico* study, azoreductase from the selected alga has been taken as an ideal model to understand the interaction with methyl orange.

3.8.1 Preparation of the ligand

The 3D structure of the ligand methyl orange was downloaded from the PubChem database (PubChem ID- 23673835). A single 3D structure with low energy and correct chirality was generated using LigPrep (SchrÖdinger software suite).

3.8.2 Preparation of target protein

The 3D structure of the target protein azoreductase was retrieved from the PDB database (PDB ID - 2V9C). Accurate target protein structure is necessary for successful molecular docking studies. The tool protein preparation wizard (SchrÖdinger software suite) was used to correct the problems in the target protein such as missing hydrogen atom, incomplete side chains and loops, ambiguous protonation states and flipped residues.

3.8.3 Molecular docking using Glide

Glide 5.9 uses a hierarchical series of filters to pursuit for potential locations of the ligand in the active site region of the receptor. The receptor grid was generated at the receptor site bound by a ligand and/or by selecting the active site residue of the protein. The prepared ligand (methyl orange) was then docked to the selected protein (azoreductase) using Glide 5.9 (SchrÖdinger software suite).

Conformational flexibility handled in Glide 5.9 is an extensive conformational search amplified by an empirical display that rapidly removes unsuitable conformations. The docking was performed using Standard Precision mode (SP) and the docked protein and ligand (complex structures) were viewed with Glide posse viewer.

The results obtained and the observations recorded were discussed in the subsequent chapter.