MATERIALS AND METHODS
3.1 Materials

3.1.1 Algae as biosorbents

Mature thalli of *Caulerpa taxifolia*, *Chaetomorpha media*, *Enteromorpha intestinalis* and *Ulva lactuca* were collected from the coastal regions of Sindhudurga District along the west coast of Maharashtra, during the growing season from August to February and brought to the laboratory in sealed polythene bags.

The sites of collection of seaweeds were:

- Malvan -16.40°N, 73.28° E.
- Kunakeshwar - 16.40° N, 73.19° E.

Four seaweeds were selected in the present study for removing dyes from aqueous solution. A brief description of selected seaweed species is given below.

3.1.1.1 *Caulerpa taxifolia* (Vahl) C. Agarth

- Division: Chlorophyta
- Class: Bryopsidophyceae
- Order: Bryopsidales
- Family: Caulerpaceae
- Genus: *Caulerpa*
- Species: *taxifolia* (Vahl) C. Agarth

It is commonly known as ‘killer alga and aquarium Caulerpa’. It is highly invasive, colonizing in huge areas. It has flattened feather-like branches (fronds) with 5-65 cm length. Leaf-like pinnules are oppositely attached to midrib, flattened, slightly curved upwards and tapered at both base and tip. Branches extend upward from horizontal stolons. Stolons are 2 to 3 m long and 1-2 mm in diameter, attached to underwater surfaces such as rocks, mud or sand via root-like rhizoids. Natural habitat is tropical and temperate coastal lagoons to ocean waters; usually in water up to 50 m deep. It contains a chemical ‘Caulerpicin’ which is noxious to fishes and some predators (Lemee et al., 1997. Plate.I).

3.1.1.2 *Chaetomorpha media* ( C. Agarth ) Kutz .

- Division: Chlorophyta
- Class: Ulvophyceae
- Order: Cladophorales
Family - Cladophoraceae
Genus - Chaetomarpha
Species - media (C. Agarth) Kutz

It is also known as 'green hair algae'. It occurs in marine intertidal zone. It has dark green, filamentous clumps attached to hard, rocky substratum with rhizoids. Filaments are 4 to 10 cm. in length, unbranched and dense. Tip of the filaments appears colourless because of escape of zoospores (Sahoo, 2010. Plate.I).

3.1.1.3 Enteromorpha intestinalis (L.) Knee
Division: - Chlorophyta
Class - Ulvophyceae
Order - Ulvales
Family - Ulvaceae
Genus - Enteromorpha
Species - intestinalis (L.) Knee

It is commonly known as ‘gutweed’. It occurs in upper littoral zone on rocky bottom. Thallus is pale green to dark green at maturity, one cell thick, wrinkled and convoluted, intestine like, grows up to 20 to 40 cm, often branching is from base. Young plant is attached to the substratum by basal rhizoidal cell or circular disc. Mature thallus becomes free floating. At low tide it tends to bend and float on the surface (Sahoo, 2010 . Plate.I).

3.1.1.4 Ulva lactuca (L)
Division - Chlorophyta
Class - Ulvophyceae
Order - Ulvales
Family - Ulvaceae
Genus - Ulva
Species - lactuca (Linnaeus)

It is also called as 'sea lettuce'. It is common on rocks in littoral or sublittoral zones in marine habitat. It is a thin, flat green alga growing from a discoid hold fast. Margin is somewhat ruffled and often torn. Thallus may vary from 18 to 30
PLATE I

Chælæra taxifælia (Vahl) (C.Agæth)  Chaætomæra medæa (C.Agæth) Kætz

Enteræoræpha intestinalis (L.) Kæe  Uæa lactææa (L.)

Biosorbent Green Sea Weeds
Habitat of Biosorbent Green Seaweeds
cm in length and consists of two layers of cells arranged irregularly in cross section. Chloroplast is cup shaped with 1 to 3 pyrenoids (Sahoo, 2010. Plate.I).

Habitat of the above algae is shown in Plate II.

3.1.2 Preparation of biomass (adsorbent):

Algal material was first washed with filtered sea water, and then with fresh water for several times to remove sand particles, dirt and epiphytes. After drying in shade at room temperature, it was ground to a powder and then passed through sieves having different mesh size to obtain fine (0.1 to 0.84 mm), medium (0.84 to 2 mm) and coarse (above 2 mm) powder. This powdered material was stored in separate airtight containers in a cool and dry place at room temperature for further use.

3.1.3 Procurement of dyes (adsorbate):

In the present study Methylene Blue and Malachite Green were selected as the model adsorbates. They were purchased from Merck Specialties Pvt. Ltd.; Mumbai. A brief description of these dyes is given below.

3.1.3.1 Methylene Blue (M.B.): It is a cationic thiazine dye, dark green in colour that turns to deep blue when dissolved in water or alcohol.

M.B. is widely used for printing, dyeing of wool, tanning of leather, and as a coating for paper (Jiang et al., 2009). It is also used as a sensitizer in photo oxidation of organic pollutants (Aksu and Karabayir, 2008). Besides it is widely used in biology, chemistry and medicine as a biological stain, bacterial stain, and indicator for pasteurized milk and as an oxidizing agent (American Chemistry Council, 2010). It is employed for the treatment of methaemoglobinemia (Brent and Curry, 2005).

Detrimental Effects: Although M.B. is not considered as a very toxic dye, it can reveal very harmful effects on living things. M.B. can cause eye burns which may result in permanent injury to the eyes of human and animals. It also causes cyanosis, convulsion, tachycardia, dyspepsia and skin irritation. After inhalation, symptoms such as difficulties in breathing, vomiting, diarrhea and nausea can occur in human (Jiang et al., 2008; Rafatullah et al., 2010).

3.1.3.2 Malachite Green (M.G.): It is a triaryl methane dye. It is dark green in colour, crystalline and prepared by condensing one part of benzaldehyde with two parts of dimethyl aniline in presence of concentrated sulfuric acid or zinc chloride
It has been widely used for dyeing leather, wool, jute and silk (Jiang et al., 2008).

**Detrimental effects:** M.G. discharged into water bodies even at a low concentration affects the aquatic life and causes detrimental effects on liver, gill, kidney, intestine and gonad of aquatic and terrestrial animals. It also causes serious hazards in human like irritation to the gastrointestinal track upon ingestion. Contact of M.G. with skin causes irritation, redness and pain (Daneshvar et al., 2007). Both clinical and experimental observations have reported that M.G. is a multiorgan toxin. It decreases food intake, growth and fertility rates; causes damage to liver, spleen, kidney and heart; inflicts lesions on skin, eye, lungs, bones and produces teratogenic effects. It is highly cytotoxic to mammalian cells. Incidences of tumor in lungs, breast and ovary in rat are also reported. It also acts as a respiratory poison. Decrease in RBC count (dyscrasia), Hb (anemia) and HTC (%), increase in WBC count (leucocytosis) and delay in blood coagulation are reported (Gupta et al., 1997; Srivastava et al., 2004; Yonar and Yonar, 2010).

Technical information of Methylene Blue and Malachite Green is given below

**Table 3. Properties of Methylene Blue and Malachite Green**

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Methylene Blue</th>
<th>Malachite Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other Names</td>
<td>Swiss Blue</td>
<td>Victoria Green B</td>
</tr>
<tr>
<td>Colour Index Name</td>
<td>Basic Blue 9,</td>
<td>Basic Green 4</td>
</tr>
<tr>
<td>Colour Index Number</td>
<td>52015</td>
<td>42000</td>
</tr>
<tr>
<td>Class</td>
<td>Thiazin</td>
<td>Triarylmethane</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>[7-(dimethylamino)</td>
<td>N-(4-[(4- (dimethyl amino)</td>
</tr>
<tr>
<td></td>
<td>Phenothiazin-3-ylidene]</td>
<td>Phenyl] (Phenyl) Methyldiene</td>
</tr>
<tr>
<td></td>
<td>dimethylanazanium chloride</td>
<td>Cyclohexa-2,5-dien-1-ylidene) -N-Methyl methanaminium chloride.</td>
</tr>
<tr>
<td>Ionisation</td>
<td>Basic</td>
<td>Basic</td>
</tr>
<tr>
<td>Empirical Formula</td>
<td>C_{16}H_{18}NSCl</td>
<td>C_{23}H_{25}ClN_{2}</td>
</tr>
<tr>
<td>Chemical Formula</td>
<td></td>
<td>[\text{Chem Spider, 2009; Stain File, 2005} ]</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>319.85 g/mole</td>
<td>364.91 g/mole</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} )</td>
<td>664 nm</td>
<td>617-619 nm</td>
</tr>
<tr>
<td>Colour</td>
<td>Blue</td>
<td>Green</td>
</tr>
</tbody>
</table>
3.1.4 Preparation of dye solution:

Stock solution of dye was prepared by dissolving accurately weighed dye powder in double distilled water at a concentration of 1 g/L and left overnight to make the dye powder fully dissolved. The bottle was covered with an aluminum foil in order to prevent decolourization caused by light and stored in dark at room temperature.

Dye solutions of desired concentration were prepared by diluting the stock solution using distilled water and used for the batch experiments.

3.2 Methods:

3.2.1 Batch adsorption experiment:

Batch mode adsorption studies for individual dyes were carried out to investigate the effect of different parameters on its removal by seaweed biomass.

Biosorption experiment was set up in a 250 ml Erlenmeyer flask containing weighed adsorbent and 50 ml dye solution. The flask was sealed with aluminum foil and kept on a rotary shaker at room temperature (27±2°C). The dye solution was agitated at a constant speed (rpm) on a rotary shaker. After desired time intervals the adsorbate was removed from the adsorbent by centrifuging the content of flask. Absorbance of dye solution was recorded before and after the batch experiment on a UV-VIS spectrophotometer (Systronics, 2205) at the corresponding X max. The amount of dye adsorbed onto the adsorbent was calculated using the difference between the dye concentration in solution before and after the biosorption process. The initial and final concentrations of dye were calculated from the standard graph by interpolation technique.

3.3 Optimization of physico-chemical factors for biosorption:

Kinetic parameters related to the biosorption process viz. pH, agitation time, adsorbate concentration, adsorbent dosage and agitation speed were varied for each dye and its uptake was recorded.

3.3.1 Effect of pH:

In order to determine the effect of pH on the adsorption of dye, the experiment was set up at different pH values ranging from 1 to 12 and keeping all other parameter constant. The pH of dye solution was adjusted to the desired value by adding either 0.1N HCl or 0.1N NaOH. The experiment was carried out using 50 ml
of dye solution at 100 mg/L concentration with 100 mg adsorbent dose and agitated at equilibrium time. The equilibrium time varied with the dye and adsorbent under consideration. Optimum pH giving maximum dye removal was determined from this study.

3.3.2 Effect of agitation time:

Effect of time on the biosorption was determined by measuring dye adsorption at different time intervals from 0 to 120 min. The pH, adsorbent dosage and dye concentration were kept constant for each dye in these experiments.

3.3.3 Effect of adsorbent dosage:

The effect of adsorbent dosage i.e. the amount of biomass, on the adsorption of dye was recorded by varying the amount from 50 to 500 mg. The dye concentration, pH, agitation time and agitation speed were kept constant depending on the dye under consideration.

3.3.4 Effect of adsorbate concentration:

In order to understand the effect of dye concentration on biosorption by green seaweeds, initial dye concentration was varied from 50 to 1000 mg/L. The pH, adsorbent dosage, agitation time and speed were kept constant for each dye.

3.3.5 Effect of agitation speed:

The process of biosorption also responds to the speed of agitation of the rotary shaker. Hence optimum speed was determined by recording biosorption at various speeds from 50 to 250 rpm. Dye concentration, biomass dose, pH and time were kept constant in these experiments.

3.3.6 Effect of particle size:

By using the biomass of different particle size, the process of biosorption of different dyes was recorded. Particles having fine (0.1 to 0.84 mm), medium (0.84 to 2 mm) and coarse (above 2 mm4) texture were used. Other parameters were kept constant which varied according to the adsorbent and adsorbate under consideration.

3.4 Determination of dye removal efficiency:

The percent removal of dye during biosorption process was determined using the following formula.

\[
\text{Dye Removal percent} = \left(\frac{C_i - C_e}{C_i}\right) \times 100
\]

Uptake of dye during the biosorption process was calculated as follows

\[
q_e = \left(\frac{C_i - C_e}{V}\right) \times \frac{M}{V}
\]
Where
C_j is initial concentration of dye (mg/L)
C_e is final concentration of dye (mg/L)
M is weight of adsorbent (mg)
V is volume of adsorbate (ml)
q_e is amount of dye adsorbed (mg/g)

All batch experiments were arranged in three replicates and results were analyzed for standard deviation using classical method.

3.5 Adsorption Isotherms:

Adsorption isotherm represents the amount of dye adsorbed per unit adsorbent as a function of equilibrium concentration. It gives the distribution of adsorbate between the liquid and solid phase at equilibrium state of adsorption process. A plot of C_e versus q_e is used to obtain the adsorption isotherm. The shape of the isotherm plot indicates whether the adsorption is favourable or not.

For analysis of adsorption data, Langmuir and Freundlich models of isotherms are widely used.

3.5.1 Langmuir Isotherm:

According to Langmuir (1916), biosorption occurs on a homogenous surface by monolayer sorption with interaction between adsorption molecules at equilibrium state. The Langmuir isotherm model is expressed by the equation.

\[
q_e = \frac{q_m K_L C_e}{1 + K_L C_e}
\]

The above equation can be rearranged into linear form as

\[
\frac{C_e}{q_e} = \frac{1}{q_m K_L} + \frac{C_e}{q_m}
\]

Where
q_e is amount of dye adsorbed (mg/g)
q_m is maximum amount of dye adsorbed (mg/g)
C_e is Final concentration of dye (mg/L)
K_L is sorption equilibrium constant (L/mg)

Langmuir isotherm is obtained by plotting C_e/q_e verses C_e. The values of slope of straight line (1/q_m) and intercept (1/ K_Lq_m) are calculated from this plot. The Langmuir constant (b) is determined from these values.
Further analysis of Langmuir isotherm is made on the basis of dimensionless equilibrium parameter \( R_L \). It is also called as separation factor which is represented in the following relationship.

\[
R_L = \frac{1}{1 + K_L C_e}
\]

The value of \( R_L \) indicates the nature of sorption process. Adsorption is favourable when \( R_L \) value lies between 0 and 1.

- If \( R_L > 1 \) adsorption is unfavourable
- If \( R_L = 1 \) adsorption is linear
- If \( R_L = 0 \) adsorption is irreversible.

A high value of correlation coefficient (\( R^2 \)) obtained from graph indicates a good agreement between the parameters and confirms the monolayer adsorption with the adsorbent surface.

3.5.2 Freundlich Isotherm:

According to Freundlich (1906), the dye uptake occurs on a heterogonous surface by multilayer adsorption and that amount of adsorbate adsorbed infinitely with an increase in concentration. It is used to estimate the adsorption intensity of the adsorbent towards the adsorbate. The stronger binding sites are occupied first and the binding strength decreases with an increasing degree of site occupation. Mathematically it is characterized by the heterogeneity factor \( 1/n \).

The Freundlich equation is expressed as

\[
q_e = K_f C_e^{1/n}
\]

Arivoli and Thenkuzhali (2008) converted this equation to linear form by taking the logarithm of both sides.

\[
\log q_e = \log K_f + \frac{1}{n} \log C_e
\]

Where,

- \( q_e \) is amount of dye adsorbed (mg/g)
- \( C_e \) is final concentration of dye (mg/L)
- \( K_f \) and \( n \) are Freundlich constants

Freundlich isotherm model is obtained by the plot of \( \log C_e \) verses \( \log q_e \). Nature of plot is linear. \( K_f \) and \( n \) are constants calculated from intercept and slope of the graph respectively.
3.6 Adsorption dynamics:

Kinetic models are always used to explain the mechanism of biosorption of dye. The mechanism of adsorption depends on the physical and chemical characteristics of the adsorbent as well as on the mass transfer process. The kinetic parameters which are helpful for the prediction of adsorption rate, give information for designing and modeling the adsorption processes. In this study, two kinetic based models were applied to the experimental data to judge the favourable fitting.

3.6.1 Pseudo first order kinetics:

Lagergren’s pseudo first order kinetic equation is given by Ho and McKay (1999) which is as follows

\[ \frac{d(q_t)}{dt} = K_1 (q_e - q_t) \]

The integrated form of given equation becomes

\[ \log (q_e - q_t) = \log q_e - \left( \frac{K_1 t}{2.303} \right) \]

Where

- \( q_e \) and \( q_t \) are amount of dye adsorbed (mg/g) at equilibrium and time \( t \) (min)
- \( K_1 \) is rate constant of pseudo first order adsorption process.

The plot of \( \log (q_e - q_t) \) verses \( t \) should give a linear relationship. \( K_1 \) and \( q_e \) are calculated from slope and intercept of the plot respectively.

3.6.2 Pseudo second order kinetics:

This model is proposed by Ho (1995) and Ho and McKay (2000). It assumes that the rate of occupation of adsorption sites is proportional to the square of number of unoccupied sites. The equation of pseudo second order kinetics is written as

\[ \frac{d(q_t)}{dt} = K_2 (q_e - q_t)^2 \]

Where \( K_2 \) is rate constant of pseudo second order adsorption (g mg\(^{-1}\) min\(^{-1}\)) for the boundary condition \( t=0 \) to \( t=1 \) and \( q_t=0 \) to \( q_t=q_e \). The integrated form of equation becomes

\[ \frac{1}{q_e - q_t} = \frac{1}{q_e} + K_2 t \]

This equation can be rearranged to the following linear form

\[ \frac{t}{q} = \frac{1}{K_2(q_e)^2} + \frac{t}{q_e} \]

Where

- \( q_e \) is the amount of dye adsorbed (mg/g)
- \( K_2 \) is rate constant of pseudo second order kinetics
The values of rate parameters $K_2$ & $q_e$ are directly obtained from the intercept and slope of plot $t/q_t$ versus $t$.

### 3.6.3 Intra particle diffusion model:

The adsorbates are most probably transported from the bulk of the solution into the solid phase through intra particle diffusion or transport process which is often the rate limiting step in many adsorption processes. The possibility of intra-particle diffusion was studied by using the intra particle diffusion model (Weber and Morris, 1963; Ho & McKay, 1998; Dogan et al., 2004). The most commonly used technique for identifying the mechanism involved in the adsorption process is by fitting the adsorption data into an intra particle diffusion plot according to the following equation

$$q_t = K_{id} t^{1/2} + C.$$  

Where

- $C$ is a Constant
- $K_{id}$ is Intra particle diffusion rate constant (mg/g min$^{1/2}$)
- $q_t$ is the amount of dye adsorbed at a time (mg/g)
- $t$ is time (min)

The $K_{id}$ is determined from the slope of linear gradients of the plot $q_t$ versus $t^{1/2}$.

Intra particle diffusion process is controlled by the diffusion of ions within the adsorbent. If the intra particle diffusion is involved in the adsorption, then the plot of $q_t$ versus $t^{1/2}$ would result in a linear relationship and the intra particle diffusion would be the controlling step, if the line passes through the origin. When line does not pass through the origin, it indicates some degree of boundary layer control. Further it suggests that the intra particle diffusion is not the only rate limiting step, but other processes also exist that may control the rate of adsorption (Alzaydien, 2009).

### 3.7 Reutilization of adsorbent:

Efficiency of reutilization of adsorbent was studied by analyzing dye loading capacity and studying the effect of protonation.

#### 3.7.1 Dye loading capacity:

After completion of batch experiment, the adsorbent was removed from the dye solution by centrifugation and gently washed with water to remove any unadsorbed adsorbate. Regeneration of adsorbate from dye loaded adsorbent was carried out using water as the desorbing medium. The dye loaded adsorbent was...
mixed with 50 ml. distilled water in a 250 ml. Erlenmeyer flask and kept for shaking at optimum agitation speed for optimum time on a rotary shaker. Then the adsorbent was collected by centrifugation, and dried at room temperature. This biomaterial was utilized again for the second cycle of batch experiment. The process was repeated four to five times keeping all the parameters at optimized condition which varied according to the adsorbent and adsorbate under consideration.

3.7.2 Effect of protonation:

For enhancing the biosorption efficiency, process of protonation was employed wherein adsorbent was treated with 0.1N HCl for three hours and then dried in shade. The protonated biomass was used for batch experiment at optimized experimental conditions.

3.8 Confirmation of biosorption:

The confirmation of adsorption was done by characterization of adsorbent using following techniques.

3.8.1 Fourier Transform Infrared Spectroscopy:

In order to explore the biosorption mechanism, it is essential to identify the functional groups present on adsorbent that are involved in the biosorption process. FTIR spectroscopy offers important information related to the nature of the bonds and allows identification of different functional groups on the cell wall structure. The extent of band shifting in natural (pure) and dye-loaded (treated) biomass gives an indication of the degree of interaction of functional groups with dye cations (Murphy et al., 2007).

The sample was mixed with KBr and then ground in an agate mortar to prepare a mixture. The mixture was pressed at 10 tons of pressure for 5 minutes to obtain a pellet. The pellet was used to record the spectrum on FTIR spectrophotometer (Brooker Tensor-37 SPIRATR) within the range of wave number 400-4000cm\(^{-1}\).

FTIR analysis of biomass was done before and after the biosorption process in the present study.

3.8.2 Scanning Electron Microscopy with Energy Dispersive X-ray Analytical System:

Scanning Electron Microscopy (SEM) is a powerful technique which can be used to investigate surface morphology of adsorbent before and after biosorption of
dye ions (Murphy et al., 2007). This technique allows evaluation of morphological changes occurring in the cell wall structure before and after the process of biosorption.

With SEM, EDX technique is used in combination which provides a valuable information regarding the distribution of various elements on the adsorbent surface (Figueira et al., 1999).

Dried biomass of alga was coated with a thin layer of KBr in Auto Fine Coater (JFC-1600) and then observed under scanning electron microscope. The energy (voltage) used was in the range of 20 kv. SEM and EDX analysis were performed simultaneously on model JEOL-JSM-6360A for pure and dye-loaded biosorbents of different algal species.