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Accumulation of Malachite Green and Crystal Violet Dye from Synthetic Effluents Using Single Cell Microalgae *Chlamydomonas reinhardtii*

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In present investigation, *Chlamydomona reinhardtii* organism was found as novel microalgae used for the bioaccumulation of dye in short period of time within 48 h. The percentage removal and uptake capacity of malachite green and crystal violet using *C. reinhardtii* with alive biomass was studied under optimized conditions. The survival of organism was studied using the chlorophyll content of the organism in the presence of dye. The accumulation of malachite green and crystal violet dyes using *C. reinhardtii* was studied as the function of initial pH (1-10) and the initial dye concentration (10 to 400 mg/L). The optimum pH value for both growth and accumulation of the dye was determined as 7. It was observed that *C. reinhardtii* was capable of removing crystal violet and malachite green with a maximum specific uptake capacity of 173.24 and 183.68 (mg/g), respectively for a dye concentration of 400 mg/L. Higher percentage removal of dye was found at lower concentrations of dye.

Keywords: *Chlamydomonas reinhardtii*, Bioaccumulation, Malachite green, Crystal violet.

INTRODUCTION

Most of the industries use synthetic dyes to color their final products[1-2], which includes the textile and dyestuff industries. Dyes are largest and most diverse compounds which are used in a number of industries such as textiles, food, cosmetics, pharmaceutical, leather and paper printing. The dyestuff usage has been increased day by day because of tremendous increase of industrialization and man's urge for colour[1]. The textile industries used most of the dyes for coloring the fabrics. The textile sector alone consumes about 60 to 70 % of total dye production for coloration of various fabrics[3]. The textile industry utilizes about 10000 different dyes and pigments[4]. The worldwide annual production of dyes is obtained as 7×10^5 tons[5-7]. The colored effluents produced by these industries are not found to be eco-friendly. So the removal of these dyes from the effluent is of great importance[8]. Dye usually has a synthetic origin and complex aromatic molecular structure that makes them more stable and more difficult to biodegrade[9]. The dye is generally recalcitrant to biodegradation due to their xenobiotic nature. Decolorizations of these dyes are possible by using several methods such as adsorption, oxidation, coagulation, flocculation, chemical degradation and photo degradation which fall under the broad classification of physical or chemical methods[10]. Most of the above methods are not found to be economical and hence biological methods are used to reduce the cost[11]. However microorganisms, being highly versatile, have developed with higher biosorption and bioaccumulation capacity for the removal of textile dyes from the effluent under certain environmental conditions.

Removal of textile dyes was established by various strains of bacteria *e.g.*, *Escherichia coli*[12], *Pseudomonas luteola*[13], *Aeromonas hydrophila*[14], *Kurthia species*[15]; fungi: *Aspergillus niger*[16], *Phanerochaete chrysosporium*, *Aspergillus terricola*[17], *P. chrysosporium*[18]; yeast: *Saccharomyces cerevisiae*, *Candida tropicalis*, *C. lipolytica*[19]; algae: *Spirogyra species*[20], *Chlorella vulgaris*[21], *C. sorokinian*[22], *Lemna minuscule*[23], *Scenedesmus obliquus*, *C. pyrenoidosa* and *Closterium lunula*[24], *Oscillatoria*[25] and *Chorella pyrenoidosa* and *Chlorella vulgaris*[21].

Algae are photosynthetic organisms which are found in all the parts of the world and in all kinds of habitats. Alga can remove number of dyes. It was reported that many dyes were removed by *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Oscillateria tenuis*[24]. Despite the fact that some dyes have high toxicity, they do not significantly inhibit the algal growth, like *Selenastrum capricornutum*.

Research work reported in the literature is concerned with dead algal biomass for the removal of textile dyes[26]. Only few studies on accumulation of dyes using alive algal biomass are

available. The advantage of using alive biomass for the removal of textile dyes is to neglect the necessities of using separate biomass synthesis process for instant activation, harvesting, drying, size reducing, separating and storage when compared to usage of dead microbial biomass so bioaccumulation mechanism is used in the removal of textile dyes by using alive algal biomass.

Malachite green and crystal violet are triphenylmethane dyes which are widely used for colouring purpose, among all other dyes of their categories[27]. These dyes have the property that makes it difficult to remove from aqueous solutions. If the solution containing malachite green discharged into receiving streams it will affect the aquatic life and cause detrimental effects in liver, gill, kidney, intestine and gonads. In human, it may causes irritation to gastrointestinal tract upon ingestion. Contact of malachite green and crystal violet with skin causes irritation and redness with pain. Upon contact to eye leads to permanent injury of human eyes and laboratory animals[28].

Chlamydomonas reinhardtii is one of the microalgal strain which has been used for the removal of textile dyes. In the present research work accumulation of textile dyes is established with the use of *C. reinhardtii*. It is easily and abundantly available biomass. Scope of the work is to study the growth and uptake capacity of *C. reinhardtii* as a function of initial pH and initial dye concentrations.

EXPERIMENTAL

The green algae *Chlamydomonas reinhardtii* (obtained from culture collections from University of Madras from the Department of Botany), was grown in 1L conical flask containing growth medium BBM (Bold Basal Medium) with pH value of 7, in order to get several stock cultures to be used during the experiments. Algal cells were cultivated in shaker at 100 rpm at 25 °C for a maximum period of 15 days. The pH of the medium was adjusted to 7 with dilute H_2SO_4 and NaOH solution. *C. reinhardtii* biomass was measured by counting the number of cell by light microscope using Neubauer Haemocytometer. The growth profile was established at 640 nm and various parameters like pH and initial dye concentration was optimized.

Algal biomass: Algal biomass was cultivated for 15 days. The algae was collected from the stock and centrifuged at 16,000 rpm for 15 min. The supernatant was discarded and pellet was washed thrice with double distilled water. The pellet was allowed to dry at 37 °C for 6 to 8 h and then weighed.

Dye: Malachite green and crystal violet are used in the study were purchased from CDH (Central Drug House) New Delhi. The stock solution was prepared by dissolving 1g of dye in 100 mL of double distilled water with dye solution concentration of 10,000 mg/L. Experimental solutions were prepared from the original stock solution. The malachite green and crystal violet dyes used in all experiments are basic cationic dyes which are widely used in textile industry. These dyes are also called as the triphenylmethane dyes. The chemical structure of the dye is shown in Fig. 1 and the physical properties are given in the Table-1.

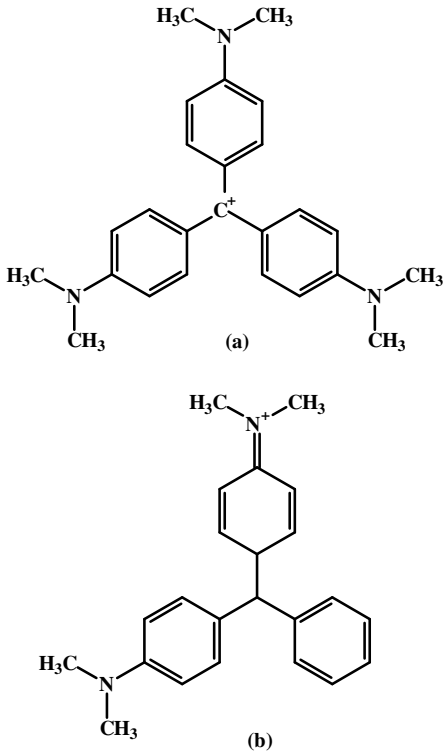

Fig. 1. Structure of the dye used for this study (a) crystal violet, (b) malachite green

TABLE-1
PHYSICAL AND CHEMICAL PROPERTIES OF DYE

Property of dye	Crystal violet	Malachite green
Common Name	Crystal violet	Malachite Green
C.I. No	42535	42000
C.I. Name	Crystal Violet	Malachite Green
Molecular formula	$C_{25}H_{30}N_3Cl$	$C_{22}N_2H_{35}Cl$
Molecular weight	407.19	364.91
Water solubility	Soluble	Soluble
Ionization	Basic	Basic
Maximum absorption	590 nm	616 nm
Colour	Violet	Green

Study of chlorophyll content: The difference in the chlorophyll content of the algae (without the dye and with the dye) did not show any significant change at the lower initial dye concentration (10 and 20 mg/L). The chlorophyll content of the alga was initially observed as 3.15 mg/g in the control without the dye solution where as the chlorophyll content in the presence of the dye was observed as 3 mg g^{-1} when the dye was kept at the lower concentration of 50 mg/L. However, at the higher dosage of the dye concentration showed the loss of 10 % chlorophyll content. Similar reports were observed in the case of *Hydrilla verticillatae*[29].

Study of pH: The accumulation studies were conducted at 50, 100, 200, 300 and 400 mg/L of the dye concentration in 250 mL of culture in a conical flask along with the nutrient medium at a constant temperature of 25 ° C at 100 rpm in a temperature controlled shaker. The samples were centrifuged at 16,000 rpm for 15 min and analyzed using UV-spectrometer at a maximum absorbance. The results obtained from accumulation studies were used to calculate the specific uptake capacity (mg dye absorbed/g of biomass).

$$q = \frac{(C_o - C_c)V}{W}$$

where C_o is the initial dye concentration (mg/L) and C_e is the final dye concentration (mg/L), V is the volume of the sample (L) and W is the biomass (g). The initial pH was varied from 4 to 10 to study the effect of pH on uptake capacity of the dye.

Statistical analysis: The initial dye concentration was employed by 6 replicate. Equilibrium uptake capacity was determined in batch colour removal studies. Standard deviation was calculated for the 6 final dye concentration values using the statistical analysis and the values were observed to be less than 5 % of the mean value.

Spectral analysis: A volume of 5 mL was taken from 100 mL flask of experimental solution at 0 h before biological treatment. The dye solution of 5 mL at 96 h was withdrawn along with algae *C. reinhardtii*, centrifuged at 16,000 rpm for 15 min and the supernatant was taken for UV-spectroscopic analysis.

Fourier transformation infrared spectroscopy analysis: Before biological treatment, 5mL of dye solution was taken from 100 mL of experimental dye solution. The dye solution of 5 mL at 48 h (after inoculation) was withdrawn along with algae *C. reinhardtii* and centrifuged at 16,000 rpm for 15 min. The separated biomass was taken for lyophilization. Lyophilized samples were mixed with 200 mg of potassium bromide to get pellet under vacuum pressure at 250 atm for 10 min. Pellets were utilized for FTIR analysis.

Scanning electronic microscopic analysis: The biomass of the algae was collected in the presence and absence of the dye. The collected biomass was taken for SEM analysis to study the surface morphology of the algal biomass before and after bioaccumulation of textile dyes.

RESULTS AND DISCUSSION

The influence of textile dyes on the growth of algae and the bioaccumulation properties were studied. The dry cell biomass (X), specific growth rate of the organisms (μ), bioaccumulation dye concentration at any time (C_{acc}) and specific dye uptake capacity (q_m) were calculated.

The specific growth rate of *C. reinhardtii* was expressed as

$$\mu = \frac{1}{X}\frac{dX}{dt}$$

The specific growth rate was determined by the slope of the ln X *vs*. time (t) plot at the exponential growth phase.

The absorbance of the dyes was measured (with UV/Vis spectrometer) at maximum absorption. The colour removal experiments were performed in erlenmeyer flasks containing 100 mL of the synthetic dye solution and algal biomass at 100 rpm in a temperature controlled shaker. The colour removal was determined from the absorbance calibration curve of standard solutions. The efficiency of color removal was expressed as the color removal (%).

$$\text{Colour removal (\%)} = \frac{(C_0 - C)}{C_0} \times 100$$

where C_o is the initial concentration of dye (mg/L) and C is the concentration of dye at time t.

Effect of pH: The effect of pH on maximum algal biomass concentration, specific growth rate and maximum specific uptake capacity of the dyes are presented in Table-2. It was observed from the (Table-2) that the specific growth rate of the *C. reinhardtii* and the biomass concentration was found to be increased with increase in pH value from 4 to 7 and the maximum uptake of malachite green and crystal violet dyes were determined as 224.71 and 189.70 mg/g, respectively at pH 7. The uptake capacity of basic dye was found to be more at pH 7 when compare to all other pH studied. The accumulation of charged dye groups on to the algae was influenced by the surface charge of adsorbent of the algae which is influenced by the pH of dye solution. With pH value of 7 the electronegativity of algae increases due to the deprotonation of functional groups. Therefore the increase in electrostatic attraction between positively charged cations to the negatively charged algal biomass. It was well documented and results were established.

At lower pH, the negatively charged ions present in the dye solution are being adsorbed by the positively charged cell surface because of the electrostatic attraction at lower pH[1,26,30-32].

Effect of initial dye concentration: Malachite green and crystal violet dye solutions of different concentrations (10, 20, 50, 100, 200, 300 and 400 mg/L) from the stock solution were prepared and the solution pH was adjusted to the optimal value of 7. Bioaccumulation experiments were carried out with *Chlamydomonas reinhardtii* and the results are presented in the Table-3.

The specific growth rate and the maximum algae concentration was found to be decreased with the increase in initial dye concentration .This was due to the inhibition effect of the dyes at higher concentrations on the growth of the *C. reinhardtii*. In the absence of the dye the maximum specific growth rate and algae concentration were determined as 0.0301 (1/h) and 0.4632 (g/L), respectively.

TABLE-2
EFFECT OF INTIAL pH ON THE *Chlamydomonas reinhardtii* MAXIMUM GROWTH RATE ACID, MALACHITE GREEN AND CRYSTAL VIOLET MAXIMUM UPTAKE PER GRAM OF DRIED ALGAE

pH	Malachite Green			Crystal Violet		
	$\mu \times 10^{-2}$ (1/h)	$X_m \times 10^{-2}$ (g/L)	q_m (mg/g)	$\mu \times 10^{-2}$ (1/h)	$X_m \times 10^{-2}$ (g/L)	q_m (mg/g)
4	0.18 ± 0.02	18.8 ± 0.5	56.19 ± 0.8	0.17 ± 0.02	20.2 ± 0.3	52.02 ± 0.4
5	0.64 ± 0.02	24.6 ± 0.2	81.30 ± 0.2	0.60 ± 0.02	24.6 ± 0.4	80.30 ± 0.2
6	0.8 ± 0.02	28.6 ± 0.3	145.6 ± 0.2	0.70 ± 0.04	26.3 ± 0.4	132.3 ± 0.2
7	1.2 ± 0.02	35.6 ± 0.5	224.7 ± 0.2	1.30 ± 0.04	32.4 ± 0.4	189.7 ± 0.2
8	0.7 ± 0.03	20.0 ± 0.4	148.5 ± 0.2	0.68 ± 0.04	24.0 ± 0.4	122.7 ± 0.2
9	0.62 ± 0.02	32.2 ± 0.3	31.05 ± 0.3	0.58 ± 0.04	30.2 ± 0.2	48.06 ± 0.2
10	0.2 ± 0.03	28.4 ± 0.2	17.60 ± 0.2	0.30 ± 0.04	28.2 ± 0.4	19.05 ± 0.2

TABLE-3
COMPARISON OF THE ALGAL CULTURE GROWTH RATE, MAXIMUM DRIED ALGAL BIOMASS, BIOACCUMULATED MALACHITE GREEN AND CRYSTAL VIOLET DYE CONCENTRATIONS AND MAXIMUM SPECIFIC UPTAKE CAPACITY OF MALACHITE GREEN AND CRYSTAL VIOLET OBTAINED AT DIFFERENT CONCENTRATIONS (TEMPERATURE = 25°C, pH = 7 AND INOCULUMS VOLUME = 10 %)

C_o (mg/L)	$\mu \times 10^{-2}$ (1/h)	$X_m \times 10^{-2}$ (g/L)	C_{accm} (mg/L)	$q_{m\,(mg/g)}$
Malachite green				
0	3.01 ± 0.00	46.32 ± 0.00	NIL	NIL
10	2.45 ± 0.02	40.12 ± 0.5	40.24 ± 0.10	36.55 ± 0.3
20	2.07 ± 0.02	36.62 ± 0.4	36.13 ± 0.10	40.40 ± 0.3
50	1.13 ± 0.02	27.30 ± 0.2	38.24 ± 0.10	63.32 ± 0.2
100	0.92 ± 0.04	24.36 ± 0.4	32.42 ± 0.10	108.80 ± 0.5
200	0.75 ± 0.04	20.43 ± 0.5	30.34 ± 0.10	140.18 ± 0.2
300	0.57 ± 0.04	17.54 ± 0.5	28.45 ± 0.10	168.92 ± 1.0
400	0.46 ± 0.02	15.24 ± 0.5	17.34 ± 0.10	183.68 ± 2.0
Crystal violet				
0	3.01 ± 0.00	46.32 ± 0.0	NIL	NIL
10	1.45 ± 0.02	30.62 ± 0.5	17.41 ± 0.10	46.53 ± 0.5
20	0.92 ± 0.02	27.23 ± 0.4	27.14 ± 0.10	63.11 ± 0.5
50	0.78 ± 0.01	20.37 ± 0.3	35.22 ± 0.10	88.46 ± 0.5
100	0.56 ± 0.01	30.54 ± 0.4	37.83 ± 0.10	121.96 ±1.0
200	0.43 ± 0.01	35.23 ± 0.3	30.23 ± 0.10	143.07 ± 2.0
300	0.28 ± 0.02	22.34 ± 0.4	25.44 ± 0.10	162.28 ± 3.0
400	0.15 ± 0.02	10.43 ± 0.4	14.56± 0.10	173.24 ± 4.0

Initially the dye concentration strongly influenced the growth and the specific (malachite green and crystal violet) dye uptake capacity of *C. reinhardtii*. The specific growth rate was found to be decreased from 0.0245 to 0.0046 (1/h) (malachite green) and from 0.0145 t0 0.0015 (1/h) (crystal violet) when the dye concentrations were increased from 10 to 100 mg/L, respectively. With 400 mg/L the maximum specific uptake capacity was obtained from the bioaccumulation experiment as 183.68 mg/L (malachite green) and 173.24 mg/L (crystal violet).

C. reinhardtii is unicellular green algae and used for the removal of dye which is available abundantly in the environment. They are capable of growing in darkness in acetate provided medium as an alternative carbon source. *C. reinhardtii* was selected because of its availability and easy to culture in a simple growth medium. Hence, the removal of textile dyes using *C. reinhardtii* is considered as important.

Algal growth and dye accumulation: Growth and dye accumulation profiles of *C. reinhardtii* was analyzed by inoculating algae in 10, 20, 50, 100, 200, 300 and 400 mg/L initial dye concentrations of malachite green and crystal violet. It was observed that with the increase in the dye concentration the maximum uptake capacity was found to be increased due to the increase in the number of collisions between the dye cations and algae which enhances the accumulation process. The maximum uptake capacity of malachite green was found to be 36.55, 40.40, 63.32, 108.80, 140.18, 168.92 and 183.68 mg/g for initial dye concentrations of 10, 20, 50, 100, 200, 300 and 400 mg/L, respectively.

The percentage colour removal of crystal violet with the initial dye concentration of 10, 20, 50, 100, 200, 300 and 400 mg/L was found to be 46.53, 63.11, 88.46, 121.96, 143.07, 162.28 and 173.24 mg/g, respectively. From the above result, it was observed that the percentage colour removal of dye using *C. reinhardtii* was observed to be more for crystal violet when compared to malachite green. A similar type of pattern was previously reported for the removal of dyes using various species. The maximum percentage of colour removal of malachite green was reported as 92.4 % using *Cosmarium sp.*[9]. However in the present investigation it has been observed that the percentage of colour removal of malachite green was 95.2 % using *C. reinhardtii*. Different dyes has different physical and chemical properties, consequently algae capable of removing colour of one dye may have different capacities then other dyes.

Spectral analysis: UV spectroscopy analysis was conducted to establish the change in the absorption spectrum of the dye before and after the treatment of the dye in the presence of the *C. reinhardtii*. The spectrum was observed and the intensity of the dye was found to be decreased in the presence of the alga. From Fig. 2, it was observed that the absorbance value was found to be decreased from the initial value of the dye with increase in the time of contact with the algae. The absorbance value of the treated dye with alga after 24 h was found to be less when compared to the initial value of absorbance. The dye solution absorbance value after 48 h was found to be least when compared to the other entire absorbance peak in the Fig. 2 at λ_{max} of 590 nm.

FTIR analysis: FTIR study was carried out for the algal biomass in the presence and absence of dye to confirm the presence of amines, carboxyl and phosphate groups. The FTIR spectroscopy as indicated in Figs. 3 and 4 from a range of 4500 to 500 cm^{-1} for malachite green and algal biomass, respectively. It was observed from Fig. 3 the assignment of major infrared bands obtained with the dye at 0 h (control) and after 24 h incubation with the algae differs. The FTIR of malachite green showed some new specific peaks in the region of 4500-500 cm^{-1} for the mono-substituted and *para* disubstituted benzene rings which are supporting to the peak at 1542 cm^{-1} for the C = C stretching of the benzene ring. The peak 1051 cm^{-1} indicates aromatic C -N stretching vibrations. Furthermore at, 2928 cm^{-1} showed C-H asymmetric stretching and free -NH_2

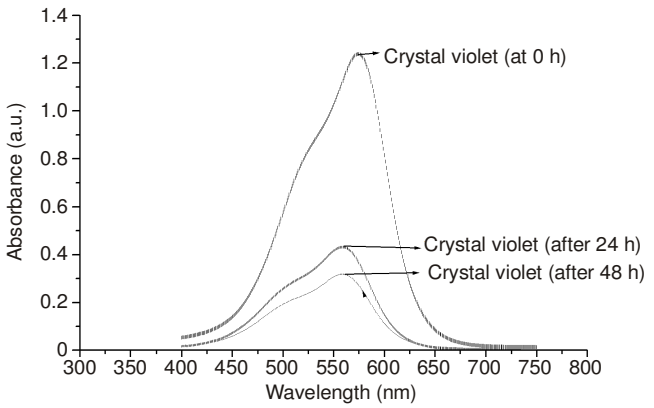

Fig. 2. UV spectrometeric analysis of crystal violet dye with the concentration of 100 mg/L in the presence of the algae

group showed amide antisymmetric stretching at 3413 cm^{-1}. In addition, the peak 770 cm^{-1} was observed for ring hydrogen. The sharp peak at 732 cm^{-1} corresponds to concerted rocking of all of the CH_2's as shown in Table-4.

The FTIR spectra of biomass before and after dye accumulation are showed in Fig. 4. The comparison of FTIR spectra

TABLE-4
ASSIGNMENTS OF THE FTIR SPECTRAL PEAKS OF MALACHITE GREEN BEFORE AND AFTER TREATMENT WITH *C. reinhardtii*

	Wavelength (cm^{-1})	Functional group or component
Malachite green	3836 – 3736	O-H stretch Hydroxyl groups and water
	2928 – 2267	C- H groups
	1658-1542	C=C stretch Aromatic ring
	1051	C–N stretch Tertiary amine
	770	Ring hydrogen
Malachite green (after 24 h)	3413	Free $–NH_2$ group anti symmetric stretching vibration.
	2147	C-H groups
	1644	C=C stretch Aromatic ring
	1050	C–N stretch Tertiary amine
	732	Concerting rocking of all CH_2's.

of pure biomass and dye loaded biomass displayed significant changes in some of the peaks. The shift of the peak 3418 cm^{-1} to 3367 cm^{-1} was seen in *Chlamydomonas reinhardtii* reflects the changes in the strength of the -OH groups. The change in the peak of 1657 to 1651 cm^{-1} indicates the C=O chelate

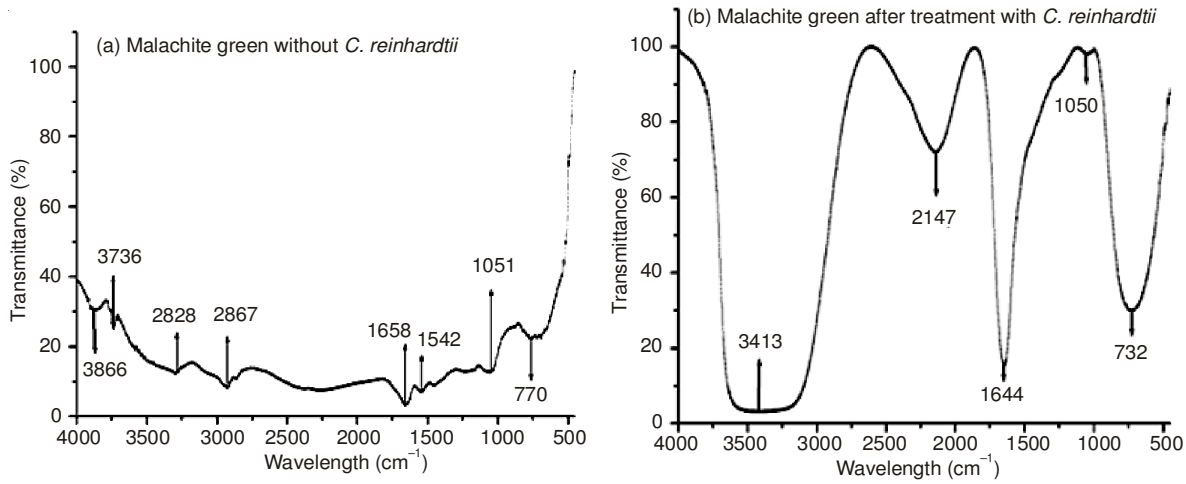

Fig. 3. (a) FTIR of malachite green at 0 h (control) (b) FTIR of malachite green was obtained after 24 h of bioaccumulation by *Chlamydomonas reinhardtii*

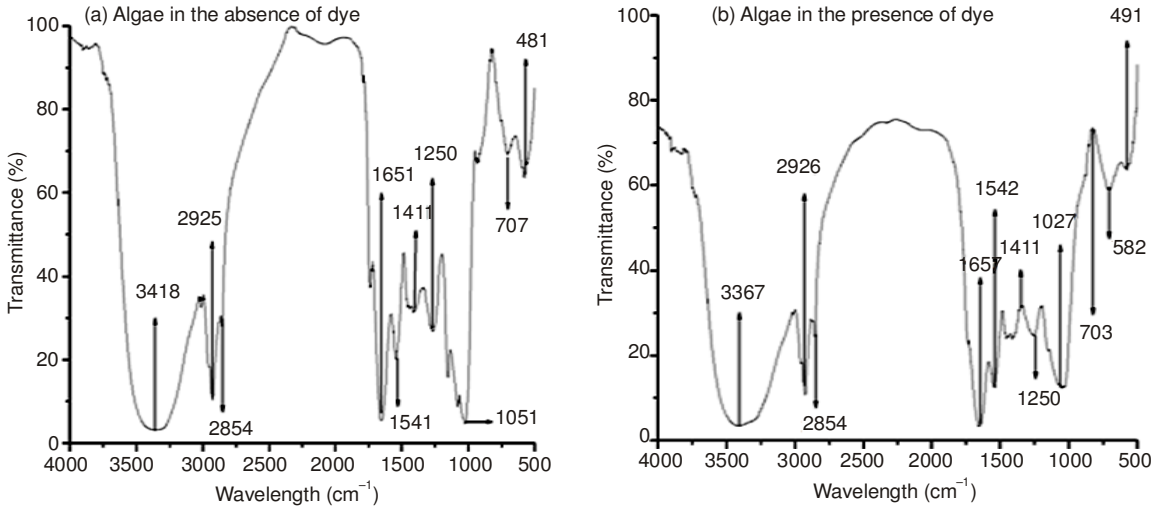

Fig. 4. (a) FTIR of the biomass of algae *Chlamydomonas reinhardtii* (b) FTIR of the biomass of algae *Chlamydomonas reinhardtii* after bioaccumulation of the dye

stretching, the shift of the peak 1542 to 1541 cm^{-1} indicates the amide bonds. Whereas the peak 582 cm^{-1} found in the algal biomass with dye malachite green indicates about the avability of C-N-S as dye component. This confirms the bioaccumulation of dye in algae.

SEM imaging: The surface structure of the *Chlamydomonas reinhardtii* before accumulation of the dye and after accumulation of the dye was analyzed by the scanning electron microscope (Fig. 5). From the Fig. 5 it was seen that the uneven surface was found to be more in *Chlamydomonas reinhardtii*. This uneven surface on the microorganism was found to be highly homogenous. Thus the uneven surface property was considered as a factor for providing more surface area on the sorbent. The increase in surface area will lead to the increase in the uptake capacity of the dye.

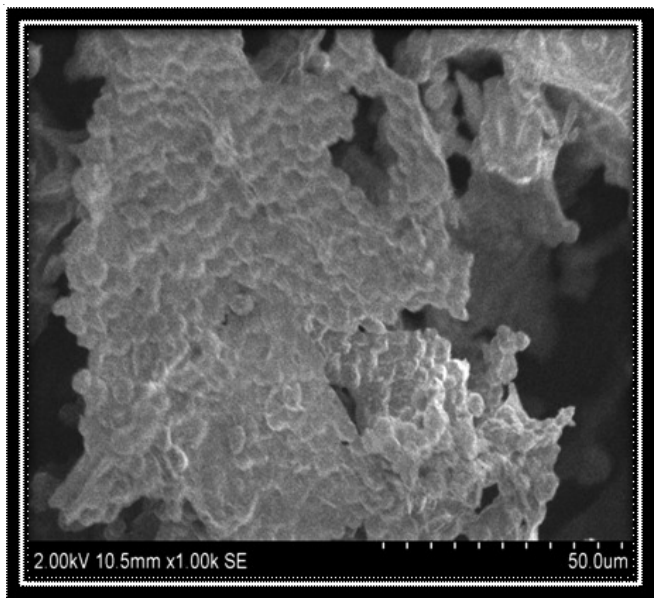

Fig. 5. SEM image of the algal culture

Conclusion

Chlamydomonas reinhardtii was found to be effective in the bioaccumulation of malachite green and crystal violet dye present in the aqueous solution during the growth of the algae. Maximum uptake capacity was obtained at pH 7 and at the dye concentration of 400 mg/L. Specific growth rate was found to be more in the absence of the dyes. Increase in the dye concentration reduces the biomass concentration. The uptake capacity of the dye was found to be increased with the increase in the dye concentration. *C. reinhardtii* appears to be useful as living biosorbent for the removal of the textile dyes present in the synthetic effluents. The maximum uptake capacity of crystal violet using algal biomass was found to be more when compared to malachite green.

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