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Influence of heavy metal as co-contamination on biodegradation of dyes by free and immobilized *Scenedesmus obliquus*

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ABSTRACT

The textile industry consumes water and it produces huge amounts of contaminated water by dyes and some heavy metals that were used to fix textile dyes. The potential ability of the free and immobilized green alga *Scenedesmus obliquus* (*S. obliquus*) to degrade azo dyes (Methyl red 20 ppm and Congo red 20 ppm) contaminated with Cu^{2+} (0.01, 0.1, 0.5 and 1 mg L^{-1}) was studied. The degradation product after decolorization was identified and confirmed by spectroscopic analysis and Fourier transformed infrared spectroscopy analysis, determination of total phenol content and antioxidant content by DPPH. The decolorization ratio of Methyl red and Congo red by *S. obliquus* was 48.60% and 41.15%, respectively, while when media supplemented with Cu^{2+} (0.5 mg L^{-1}) were 46.38% and 40.43% after 10 days incubation. The decolorization of both dyes by the alga beads and alginate beads were 55.45% and 26.27% with Methyl red, 50.7% and 21.64% with Methyl red added to Cu^{2+}, 62.05% and 31.2% with Congo red 57.72% and 18.52% Congo red with Cu^{2+}, respectively after 10 d of incubation. Influence of heavy metals as co-contamination decreased the biodegradation of dyes not inhibitor for decolorization by *S. obliquus* suspension and immobilized. The alga beads are capable of adsorption of Cu^{2+} and dyes degradations more than the alga in suspension. In general, such a metallic compound appeared to decrease the biodegradation of azo dyes, and with increasing, the concentration of heavy metal there was a decrease in biodegradation efficiency.

Keywords: Degradation; Heavy metals; Azo dyes; *Scenedesmus obliquus*; Immobilization

1. Introduction

One of the major obstacles that humans are facing is the refurbishment of the contaminated environment. Near 10%–15% of dyes are mislaid from some industries such as food, paper printing, pharmaceutical, leather, cosmetics and the huge amounts of dyes are lost in the textile industry [1,2]. The textile dye is the most significant environment-polluting agents. Azo dyes are the main compounds of such effluence because of their huge applicability and usages [3]. Azo dyes are mutagenic to the human hepatoma cell line [4,5]. Amin et al. [6] reported that azo dyes are mutagenic and carcinogenic which causes various destructions to any organisms that exposed to it. Azo dye Methyl red is a dark red crystalline powder that causes mutagenic in nature, and most microbial degradation studies reveal the formation of N,N-dimethyl-p-phenylenediamine, a toxic and mutagenic aromatic amine [7]. Congo red is an azo dye complex

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chemical structure, high solubility in water and released into the natural environment [8]. In textile industries, heavy metals such as cobalt, chromium, copper, and nickel form parts of some dyes, meanwhile copper salts are also used to fix dyes and enhance the light fastness of neutral metalized dyes on nylon for some applications [9]. Heavy metals often used in different textile processes, as dyeing and printing [10]. The excess of copper causes toxicity which breakdown of all protein structures in the body such as hair, nails and inhibits an enzyme required for collagen synthesis. High tissue copper levels can cause a relative manganese deficiency, which causes anemia because manganese is important to stimulate hemoglobin formation [11]. Algae have availability live in both fresh and saltwater and also have possible bioremediation of dyes [12]. El-Sheekh et al. [13] studied the ability of green algae *Chlorella vulgaris* and *Volvox aureus* and also blue-green algae *Lyngbya lagerlerimi*, *Nostoc linckia* and *Oscillatoria rubescens* to decolorize and remove Methyl red and Congo red, they noticed that the ability of algae to decolorize dyes were differed according to the algae and dyes types. Guolan et al. [14] reported that *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Spirogyra* and *Oscillatoria tenuis* had the ability to degrade azo dyes. Both dead and fresh algae possess good efficiency of bioremediation dyes and also heavy metals due to the presence of proteins, lipids, and polysaccharides on their cells [15]. Immobilized algae are also used in bioremediation of heavy metals and other xenobiotics. For instance, immobilized *Spirogyra condensata* and *Rhizoclonium hieroglyphicum* on amberlite XAD-8 showed higher efficiency in removing chromium from tannery wastewater compared to suspension cultures [16]. Immobilized *C. vulgaris* in alginate beads has been shown to be useful in removing tributyltin by biosorption and biodegradation [17].

The objective of this work was to develop and test a more direct and quantitative measure of the inhibitory effects of metals on the microbial degradation of dyes, and the influence of heavy metals as co-contamination on biodegradation of dyes by free and immobilized *Scenedesmus obliquus*.

2. Materials and methods

2.1. Alga preparation

The green microalga *Scenedesmus obliquus* was obtained from the Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

2.2. Immobilization of Scenedesmus obliquus

Alginate and alga beads were synthesized according to methods of [18]. After 10 d, *Scenedesmus obliquus* (*S. obliquus*) culture was centrifuged at 4,000 rpm for 10 min and the cells residue were washed by distilled water three times. The concentrated alga was 1×10^6 cells ml^{-1}. The alga was mixed with sodium alginate suspension with stirring to give the final concentration 4%. The alga beads (diameter 1.5 ± 0.2 mm) were acquired by adding drop-wise of alginate alga suspension through a 3 ml syringe into sterile 2.5% $CaCl_2$ solution with ambient temperature. The alginate beads were carried by the same method without alga as a control. The resulting beads were washed by sterile distilled water to remove any calcium chloride residues from the surface of beads and stored for 12 h at 4°C in distilled water to stabilized and harden the beads.

2.3. Alga and growth conditions

The nutrient medium Bold's Basal [19] was used for the growth of alga maintained at 25°C ± 1 and pH 7.4 under continuous light with the intensity of 80 μE m^{-2} s^{-1}.

2.4. Dyes

Methyl red and Congo red were obtained from Sigma-Aldrich, Germany (Fig. 1).

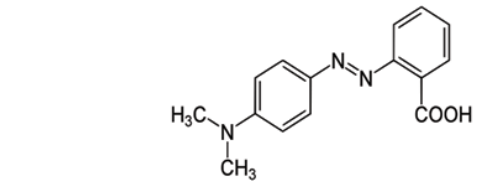
2.5. Decolorization study and spectroscopic analysis

The experiments were conducted in sterilized 250 ml Erlenmeyer flask containing 100 ml of the sterile medium with 20 ml of algal culture, 100 algal beads, 100 alginate beads and the azo dye (Methyl red - Congo red) at concentration 20 ppm. The culture was incubated at 25°C for 10 d. The degradation ratio was determined after 3, 5, 7 and 10 d of incubation by measuring the absorbance of the cell-free supernatant of the sample at the maximal absorption wavelength 430 and 496 nm for Methyl red and Congo red, by Spectro UV-Vis Dual Beam UVS-2700, respectively. The percentage of decolorization was calculated by using the equation according to [20–22].

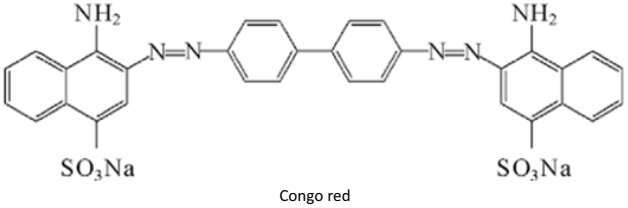
$$\text{Decolorization}\,(\%) = \frac{\left(\begin{array}{l}\text{Initial absorbance} -\\ \text{Final absorbance}\end{array}\right)}{\text{Initial absorbance}} \times 100 \quad (1)$$

2.6. Dry weight estimation

Aliquots of 100 ml was filtered through a pre-dried (24 h at 80°C) and pre-weight Whatman GF-52 filter (47 mm



Methyl red



Congo red

Fig. 1. Chemical structure of the used dyes Methyl red and Congo red.

diameter). After filtration, the cells were washed with deionized water and dried in an oven at 80°C until constant weight. After cooling to room temperature in desiccators, the filters with algal cells were weight again and the dry weight was calculated and expressed as mg L^{-1} [23].

2.7. Dry weight of algal cells in beads

Beads were washed with distilled water to remove any algal cells present in exterior beads. A known volume of 0.1 M sodium citrate was added to beads with stirring, sodium citrate chelates Ca^{2+} and disrupts the gel structure, the resulting solution was centrifuged and the algal pellets were washed by deionized water, and algal pellets were dried in an oven at 80°C until constant weight.

2.8. Total phenolic content

The dry alga was extracted with methanol for consecutive three times, and methanol extractions were collected and concentrated. The total phenolic contents were determined by Folin–Ciocalteu's methods [24]. The color strengths were measured at 765 nm by spectrophotometer (UV-200-RS LW) and concentrations of phenol contents were determined by using a gallic acid standard curve.

2.9. Determination of antioxidant capacity (DPPH assay)

The percentage of antioxidant capacity of methanol extracts of the dry weight of the alga and alga beads after 10 d of incubations were determined by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay [25].

2.10. Effect of heavy metal ions

To inspect the influence of metal ions such as Cu^{2+} on decolorization activity, experiments were carried out in the presence of the metal at different concentrations (0.01, 0.1, 0.5 and 1 mg L^{-1}). Metal ions from stock solutions were added to the cell suspension and incubated for 15 min, and then flasks were added to the dye. Decolorization was monitored at 25°C for different time intervals [26].

2.11. Percentage reductions of metal in microbial degradation rate

Percentage reductions in microbial degradation rate coefficients were calculated based on comparisons with control sediment.

% Reduction = [(control – treatment/control] × 100 [27] the copper sulphate was determine by atomic absorbance spectrophotometer (PerkinElmer 2380, USA).

2.12. Infrared measurements (IR)

The infrared (IR) analysis technique used to identify the structural variation of the alga biomass in beads that grown with dyes and grown with dyes and Cu^{++} to determine the ability of alga cells to sorb some of these dyes. For this purpose, IR measurements were carried out using (PerkinElmer) IR data station (1430 Ratio-Recording IR Spectrophotometer) [28].

2.13. Statistical and data analysis

All the experiments were performed in triplicate (n = 3) and the results were expressed as the mean value ± standard error (st-er). Significant differences between the means were carried out using Duncan's multiple range tests (P = 0.05). All analyses were determined using SPSS software (Version 17), also data were analyzed using 2-way-ANOVA (analysis of variance) to test for the effects of alga, alga beads, alginate beads, with and without supplemented copper with time.

3. Results and discussion

Azo dye is the major group of dyes, with a-N=N as a chromophore with an aromatic system. There are monazo, disazo, trisazo, tetrakisazo and polyazo dyes the difference according to the number of the azo-groups present. Azo pigments are colorless particles which have been colored using an azo compound [29].

3.1. Azo dyes reduction in media supplemented with Cu^{2+}

Fig. 2a denoted that the Methyl red reduction was caused by the fresh alga, alga beads and alginate beads. The alga beads were more effective in Methyl red reductions following by fresh alga and alginate beads, respectively. Increasing the concentrations of Cu^{2+} decreased dye reductions in all treatments.

The results shown in Fig. 2b indicated that copper caused a gradual decrease of biodegradation of Congo red at increasing concentrations of Cu^{2+} and this due to that

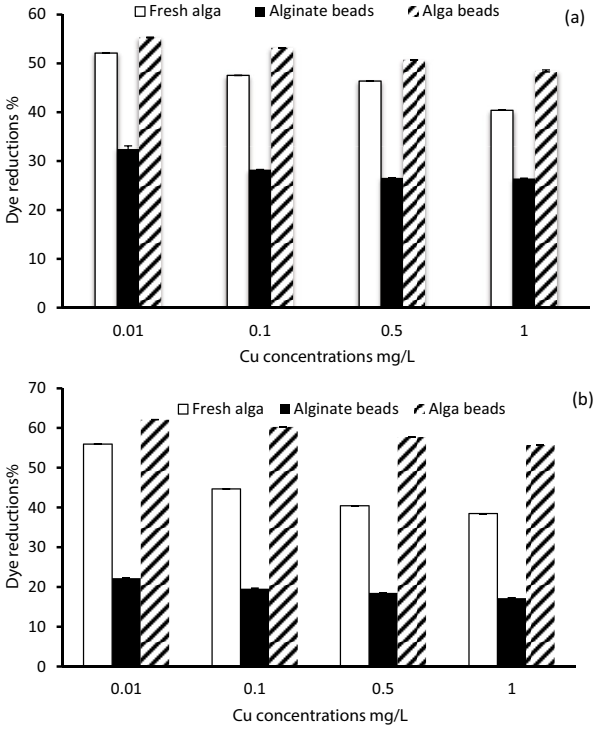


Fig. 2. Reduction of Methyl red (a) and Congo red (b) supplemented with various concentrations of Cu^{++} by fresh and immobilized green alga *S. obliquus* and alginate beads.

copper caused a gradual slow decline of growth rate at increasing concentrations but had a significant effect only on its highest level of 1.0 mg L^{-1} clearly suppressing the growth rate [30]. Chevalier and de la Noue [31] reported that immobilized algae are advantageous for wastewater treatment, especially for the removal of nitrogen, phosphorus and heavy metals. Such systems are more attractive compared to suspension cultures as they solve the problem of cell harvesting. In addition, the immobilized cells are more stable than free cells as they are protected from direct exposure to the toxic substances present in the medium. Higher nutrient removal efficiency has been recorded for the immobilized algae compared to the suspension cultures of the same algal species [32].

Fig. 3a shows the effect of *S. obliquus* on Methyl red degradation with supplemented Cu decreased than without supplemented by copper [33] reported some of the diazotrophs (filamentous Cyanobacteria) can fix atmospheric nitrogen and thus the utilization of algae for wastewater treatment is an efficient and cheap cost process.

Fig. 3b shows that *S. obliquus* tolerance to Cu^{2+} and degraded Congo red with Cu^{2+} 39.9%. Hording and Whitton [34] reported that heavy metal tolerance according to algae appearing in polluted sites is considered either metal tolerant or metal resistant species. Several green algal species are tolerant or resistant to Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} [35].

Fig. 4 shows that there is a gradual increase in growth by increasing incubation time and this is concomitant with an increase in the degradation ratio, and Cu ions had negative effects of the alga growth. El-Sheekh et al. [13] reported that different concentrations of industrial pollutants such as dyes significantly decreased the dry weight production of different algae by increasing incubation time.

Fig. 5a shows the decolorization of Methyl red by immobilized *S. obliquus* was 49.2% at 3 d and increased by 52.22% and 55.44% after 5 and 7 d of incubation, respectively. The decolorization of the same dye with Cu^{2+} by *S. obliquus* was 44.1% after 3 d and this value increased to 44.36%, 46.66%, 50.7% respectively, after 5, 7 and 10 d. Aksu and Tezer [36] concluded cells immobilized in 1% κ-carrageenan were able to remove higher percentage color from Supranol Red 3BW and Lanaset Red 2GA than those in 2% alginate.

The results in Fig. 5b shows that decolorization of Congo red by *S. obliquus* beads were affected by the algal growth and incubation period, where 58.10% was degraded after 3 d that increased to 60.01%, 61.2% and 62.05% after 5, 7 and 10 d of incubation. And also denoted that the decolorization of Congo red that supplemented with Cu^{2+} by *S. obliquus* beads was 38.52% after 3 d that increased to 38.8% after 5 and at 7 d was 39.1% and also the alga beads was more effective in decolorizations of dyes than alginate beads. The present results agree with the previous findings where the presence of dyes imparts an intense color to effluents, which lead to environmental problem and algae undoubtedly have the potential to rapidly, efficiently and effectively remove dyes to low concentrations and less toxic compounds [37].

The results in Fig. 6 revealed that the dry weight of alga after beads thawing at 3, 5, 7 and 10 d of grown with Methyl red and Congo red increased with days and also when supplemented with Cu^{2+} but alga grown with dyes and Cu^{2+} was less dry weight than alga grown in the only dye.

The color of DPPH is reduced when reacting with the antioxidant compound because it can donate hydrogen that reduces the color [38]. The results in Table 1 show that the

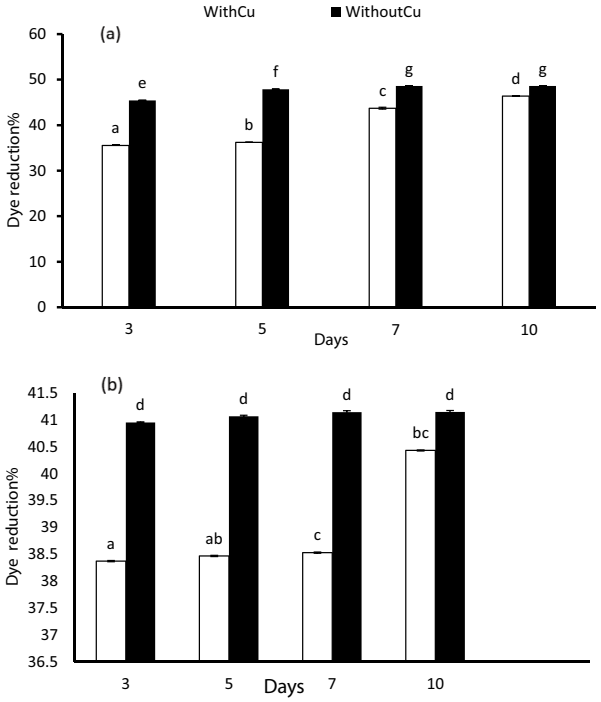


Fig. 3. Effect of *S. obliquus* on Methyl red (a) and Congo red (b) degradation with and without supplemented by copper ions 0.5 gm L^{-1} with days.

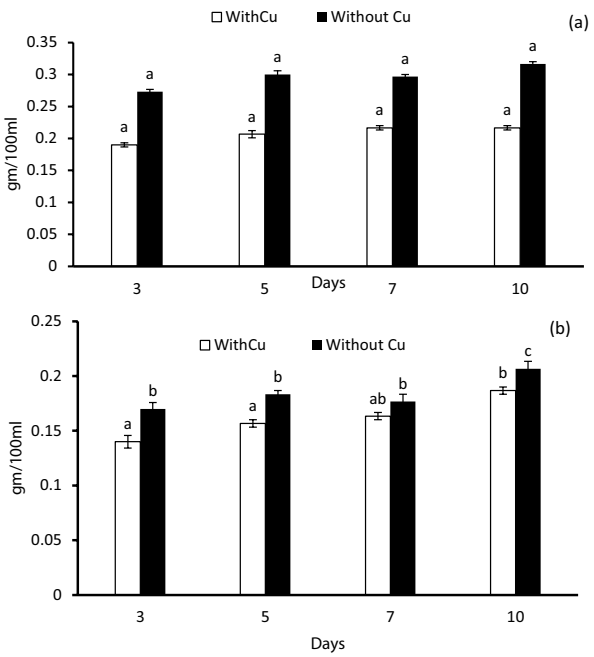


Fig. 4. Dry weight of *S. obliquus* grown on Bold's Basal medium supplemented by Methyl red (a) and Congo red (b) with and without copper ions.

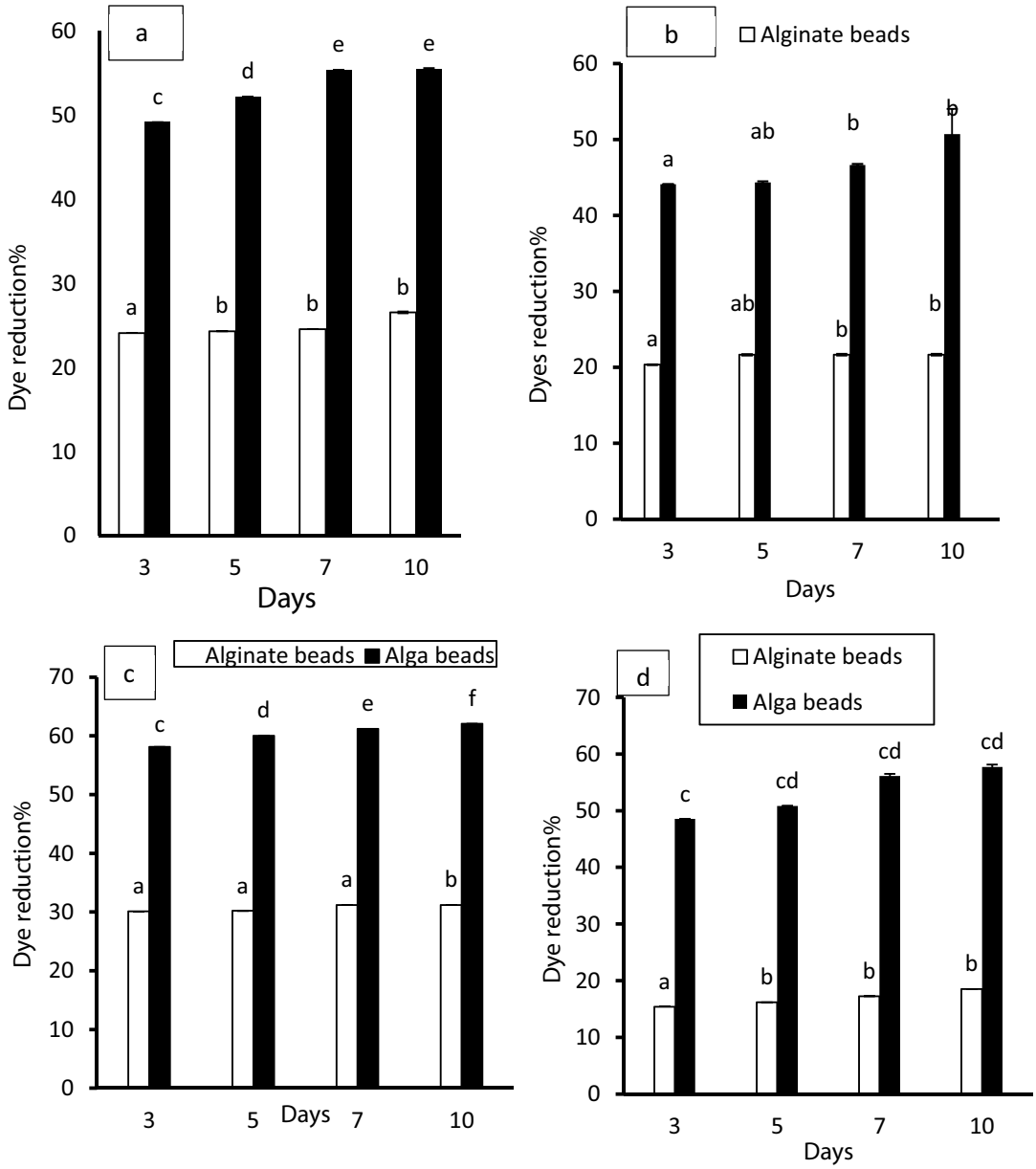


Fig. 5. Effect of immobilized *S. obliquus* and alginate beads on Methyl red degradation without copper (a) and with copper (b) and Congo red degradation without supplemented with copper (c) and with copper (d).

influence of Azo dye (Methyl red and Congo red) on the radical scavenging activities of *S. obliquus* alga free and immobilized, when grown in Bold"s Basal supplemented with and without copper ions. Radical scavenging activities increase with stress conditions (azo dyes and copper ions) in comparison with control, no treated alga. The resulted in Table 1 indicated that phenol content increased in all treatments in comparison to the untreated alga. The results in Table 1 investigated the positive relationship between total phenol contents and antioxidant capacity of treated alga. The phenolic content significantly contributed to the antioxidant capacity of microalgae [39,40]. The algae are adapted toward stress conditions and accumulated several organic materials that include phenolic and antioxidant compounds [41]. The previous studies reported that under salt stress the phenolic content of algae increases followed by antioxidant activity [42]. This result is disagreeing with [43] who revealed that nutrient stress is not an impact strategy to enhance overall antioxidant content in microalgae. The accumulation of phenol content was achieved under copper stress in *Dunaliella tertiolecta* [44]. Manivannan et al. [45] revealed that the antioxidant activity of algal methanolic extract grown in the presence of copper is elevated than control. Srivastava et al. [46] reported that the anti-oxidative defense system is encouraged in *A. doliolum* by cultivation with copper.

Fig. 7 shows that there was stretching vibration of the azo bond diminish within the range 1,630–1,575 cm^{-1} (shaded

parts in the Figs) and the curve C decreases the intensity of the peak with Cu^{++}. The results denoted that the alga beads grown with dyes were more absorbed dyes than alga beads that grown with dyes and supplemented with copper ions in both two dyes were tested.

The results obtained in Table 2 represented that the reduction of Methyl red by alga increased significantly with times and with or without copper. Time (3, 5, 7 and 10 d),(2-way-ANOVA: F = 67.99, P = 0.000). The reduction of Congo red no interaction effect between times and with or without copper (2-way-ANOVA: F = 1.133, P = 0.365). No interaction effect between times and beads type (immobilized alga and alginate beads) in related to the reduction of Methyl red supplemented with copper and without Cu. Meanwhile interaction effects at the same treatments in the case of Congo red.

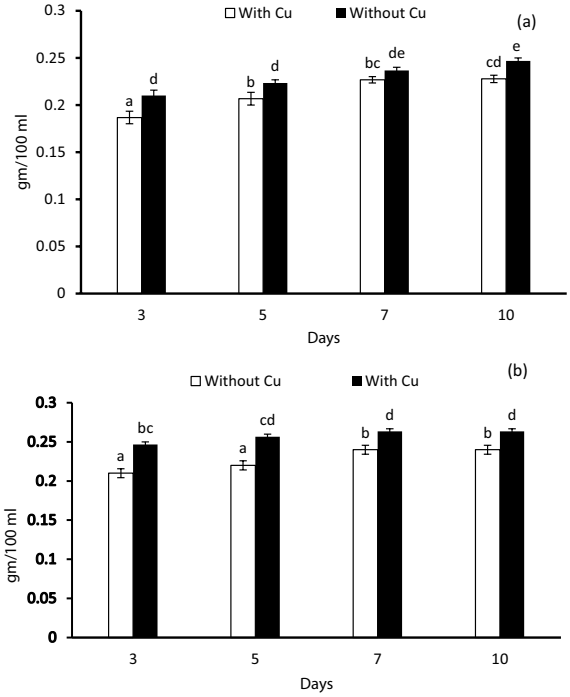
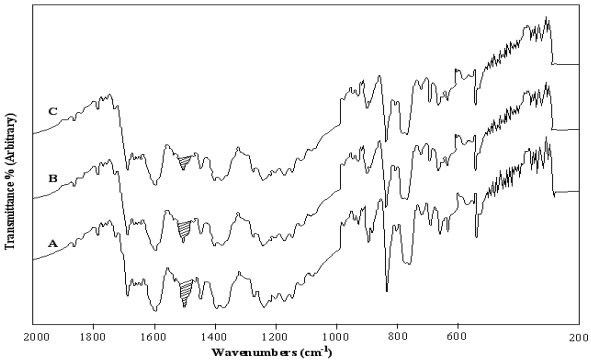


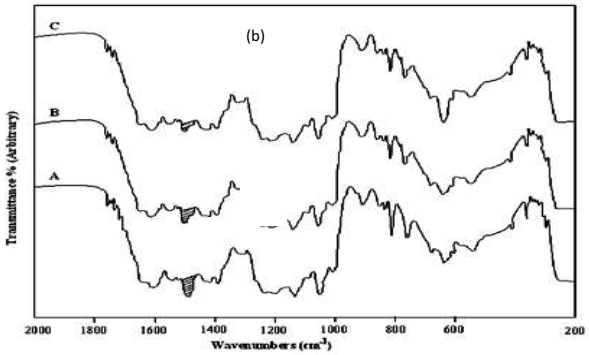
Fig. 6. Dry weight of immobilized *S. obliquus* grown in Methyl red (a) and Congo red (b) with and without supplemented copper and control.

4. Conclusion

The present study revealed that *Scenedesmus obliquus* alga was capable of degrading the azo dyes such as Methyl red and Congo red and also capable of absorption of Cu^{2+}. The ability of *S. obliquus* degradation of both dyes decreased with increasing concentrations of Cu^{2+}. The alga beads were more capable of degradation of both dyes than fresh alga and alginate beads. The ability of alga beads, alginate beads, and fresh alga to biodegrade both dyes was decreased when the media supplemented with Cu^{2+}. Meanwhile, the alga beads



Methyl red (A), alga beads grown with Methyl red (B),alga beads grown with Methyl red and Cu^{++} (C)



Congo red (A), alga beads grown in congo red (B), immbolized alga grown in G.R with Cu^{++} (C)

Fig. 7. Infrared spectrum of Methyl red (a) and Congo red (b) effect on *S. obliquus* with different treatments.

Table 1
Influence of Methyl red and Congo red on antioxidant activity determined by DPPH radical scavenging activities and total phenol content mg gallic acid/gm dry wt. produced by free and immobilized *Scenedesmus obliquus* grown in Bold's Basal medium supplemented with and without copper ions

Treatments	Influence	Control	Methyl red		Congo red	
			with Cu^{++}	without Cu^{++}	with Cu^{++}	without Cu^{++}
Alga	Antioxidant activity	35 ± 2.02	51.89 ± 2.02	47.64 ± 4.88	52.71 ± 0.32	52.99 ± 2.97
	Total phenol content	1.7 ± 0.2	3.93 ± 0.10	4.09 ± 0.53	3.04 ± 0.09	3.74 ± 0.54
Alga beads	Antioxidant activity	39 ± 1.04	58.07 ± 1.64	62.17 ± 2.62	47.37 ± 1.85	51.49 ± 1.98
	Total phenol content	2.1 ± 0.23	4.96 ± 0.55	5.58 ± 0.25	3.18 ± 0.36	3.39 ± 0.10

±St error.

Table 2
2-way-ANOVA on the reduction of the dye by free and immobilized *S. obliquus* with and without Cu for different time intervals

Response	Factor	*df*	*F*	Significant
Methyl red reduction	Time	3	410	0.0000
by alga	Cu	1	5.951ES	0.000
	Time × Cu	3	67.99	0.000
	Error	16		
Congo red reduction	Time	3	16.867	0.000
by alga	Cu	1	561.8	0.000
	Time × Cu	3	1.133	0.365
	Error	16		
Methyl red reduction	Time	2	3.623	0.038
by beads with Cu	Beads type	1	1.257E3	0.000
	Time × beads type	3	1.498	0.165
	Error	15		
Methyl red reduction	Time	3	6.717	0.004
by beads without Cu	Beads type	1	734.832	0.000
	Time × beads type	3	1.705	0.206
	Error	16		
Congo red reduction	Time	3	60.878	0.000
by beads with Cu	Beads type	1	1.008E4	0.000
	Time × beads type	3	17.517	0.000
	Error	16		
Congo red reduction	Time	3	406.777	0.000
by beads without Cu	Beads type	1	2.584E5	0.000
	Time × beads type	3	92.759	0.000
	Error	16		

Beads type represents immobilized green alga *S. obliquus* and alginate beads time at 3, 5, 7, and 10 d.

were more effective to biodegrade Methyl red than Congo red when media supplemented with Cu^{2+}. The alga biomass decreased when media supplemented with Cu^{2+}. Antioxidant activity and phenolic content of the alga and alga beads treated with dyes and dyes with copper ions were increased in comparison with control. The infra-red spectroscopy revealed that the alga beads were more effective to absorb dyes than alga beads supplemented media with Cu^{2+}.

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