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Biodecolorization of azo dye Acid Blue 92 (AB92) by *Ceratophyllum demersum* L.: process optimization using Taguchi method and toxicity assessment

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Biodecolorization of azo dye Acid Blue 92 (AB92) by *Ceratophyllum demersum* L.: process optimization using Taguchi method and toxicity assessment

Abstract: This study evaluated the ability of the submerged aquatic plant *Ceratophyllum demersum* to remove the Acid Blue 92 (AB92) dye. The effect of some operational parameters such as the reaction time, initial dye concentration, initial plant biomass, and pH, on dye removal efficiency was studied. Based on Taguchi's results, the optimized conditions for dye removal were time 7 days, dye concentration 20 mg l^{-1}, initial plant biomass 4 g, and initial pH 5. Fourier-transform infrared spectroscopy (FTIR) results confirmed the interaction between dye molecules and plants. Based on the results of this study, *C. demersum* had a reusability to remove the dye, this fact confirming the mechanism of biodegradation in the dye removal process. Also, the effect of AB92 on the physiological responses of *C. demersum* was investigated. Minimum relative growth rate, tolerance index, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids at a concentration of 20 mg l^{-1} of AB92 were observed. The concentration of cyanidin glycoside, lipid peroxidation, and antioxidant activity increased in both concentrations of 10 and 20 mg l^{-1}. It can be concluded that both concentrations of AB92 induced antioxidant activity and the risk of oxidative stress for *Ceratophyllum*.

Key words: azo dye; Acid Blue 92; bioremediation; *Ceratophyllum demersum*; oxidative stress

Biorazbarvanje azo barvila Acid Blue 92 (AB92) z navadnim rogolistom (*Ceratophyllum demersum* L.): optimizacija Taguchijeve metode in ocena strupenosti

Izvleček: V raziskavi je bila ovrednotena sposobnost navadnega rogolista (*Ceratophyllum demersum* L.) za odstranjevanje barvila Acid Blue 92 (AB92). Preučevan je bil učinek parametrov kot so reakcijski čas, začetna koncentracija barvila, začetna biomasa rastline in pH na odstranjevanje barvila iz vode. Na osnovi Taguchijeve metode so bili najboljši pogoji za odstranitev barvila 7 dni pri koncentraciji barvila 20 mg l^{-1}, začetni masi rastlin 4 g in začetnem pH 5. Fourierjeva transformacijska infrardeča spektroskopija (FTIR) je potrdila interakcijo med molekulami barvila in rastlino. Na osnovi te raziskave je bilo ugotovljeno, da ima navadni rogolist sposobnost biodegradacije pri odstranitvi barvila iz vode. Preučevan je bil tudi učinek barvila na fiziološke procese v rogolistu. Pri koncentraciji 20 mg l^{-1} barvila so bile opažene minimalne vrednosti relativne prirasti, tolerančnega indeksa, vsebnosti klorofila a, klorofila b, celokupnega klorofila in celokupnih karotenoidov. Koncentracija cianidin glikozida, peroksidacija maščob in antioksidacijska aktivnost so se povečale pri obeh koncentracijah barvila,10 in 20 mg l^{-1}. Ugotovljeno je bilo tudi, da sta obe koncentraciji barvila vzpodbudili antioksidacijsko aktivnost in, da sta predstavljali nevarnost za oksidacijski stres v rogolistu.

Ključne besede: azo barvilo; Acid Blue 92; bioremediacija; *Ceratophyllum demersum*; oksidacijski stres

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1 INTRODUCTION

Discharge of colored effluents into rivers and lakes leads to reduced water quality, reduced oxygen transfer to water, and decreased solubility of gases (Pillai et al., 2014; Varjani et al., 2021). Dyes used in the textile industry are macromolecules not completely decomposed by conventional wastewater treatment processes due to their structure and nature. The dye "AB92" is from the group of mono-azo dyes and is in the category of anionic dyes. One of the largest and oldest classes of industrial dyes is Azo dyes, which contain about 70 % of textile dyes (Lang et al., 2013). Azo dyes have numerous desirable factors, making them widely useful not only for dyeing textiles and leather but also for application in new technologies. These factors include easy production, high molar absorption coefficient, good stability against light and moisture, and a wide range of colors (Singh & Arora, 2011). Phytoremediation, a term for natural technologies based on using plants to purify the environment and the final refining step after the initial treatments, is highly considered today. It is a relatively new technology that, in addition to being environmentally affable is considered economical, suitable, and particular (Bhat et al., 2022). Aquatic plants are more efficient in phytoremediation than terrestrial plants due to faster growth, higher biomass production, and a higher ability to absorb contaminants. They are also more effective in purification due to direct contact with contamination (Phillips et al., 2015; Sharma et al., 2015). The aquatic plant *C. demersum* has been introduced as a high-efficiency plant species for phytoremediation (Gałczyńska et al., 2019). The genus *Ceratophyllum* is globally distributed and is one of the most important and predominant aquatic plants in rivers and wetlands in Iran (Chorom et al., 2012; Mohan et al., 2017). Physical traits of this plant such as thin cuticle, specific leaf structure, and a lack of roots, facilitate the uptake of xenobiotics through the large surface of this plant without dependence on the root-to-stem transfer system (Rezania et al., 2016). Although *Ceratophyllum* has been very successful in the bioremediation of heavy metals (Krems et al., 2013; Nabi, 2021; Qadri et al., 2022), research on its ability to purify organic contaminants such as synthetic dyes and its effect on the physiological parameters of this plant is very limited. In previous studies, the biomass potential of this plant in bioremediation of synthetic dyes, Basic Blue 41 and Methylene Blue was observed at 94 % (Keskinkan & Lugal Göksu, 2007) and 96 % (Ewadh, 2020) respectively. In the present study, the ability of the submerged aquatic plant *Ceratophyllum demersum* to remove the monoazo dye (AB92) from polluted water has been investigated. To optimize the biological removal process, the effect of different conditions such as test time, the fresh mass of the plant, initial concentration of the dye, and pH was investigated at four levels simultaneously by the Taguchi test. Using the Taguchi test, all the existing interactions between different factors were investigated and finally, the most effective level of each factor and the most effective factor in the biorefining of AB92 by this plant were identified. By using FTIR analysis, the possible interaction between the dye and the functional groups of the plant was identified. Also, the effect of dye on some physiological variables including the content of relative growth rate (RGR), tolerance index (TI), photosynthetic pigments, the content of non-enzymatic antioxidants including carotenoid concentration and anthocyanidin glycoside concentration, free radical scavenging capacity (DPPH) and amount malondialdehyde (MDA) production was investigated.

2 MATERIALS AND METHODS

2.1 PLANT CULTİVATİON AND TREATMENT

Ceratophyllum aquatic plant was collected from Sustan wetland in Lahijan ("N, 50 ° 0'14" E, 37 ° 12' 26) and transferred to the laboratory. Samples were washed and disinfected with 0.5 % NaClO solution and transferred to plastic containers containing 10 % Hoagland medium (Hoagland & Arnon, 1950). Plastic containers were transferred to the culture chamber for better growth and were placed in basic conditions with a temperature of 25 ± 2 °C and a light-dark period of 16/8 hours. Treatment was done after one week (Movafeghi et al., 2016).

2.2 DYE ANALYSİS

Industrial dye AB92 [Mono azo, Anionic; C.I. number: 13390; Molecular Formula: $C_{26}H_{16}N_3Na_3O_{10}S_3$; Mw (g mol^{-1}): 698.58] was purchased from Alvan Sabet Company (Iran). The absorption spectrum of the dye was measured at wavelengths of 200 to 800 nm with a spectrophotometer (CamSpec M501 Single Beam UV/Visible, United Kingdom). AB92 dye has a maximum absorption at a wavelength of 571 nm. Different concentrations of dye were prepared, their adsorption was measured at maximum wavelength, and a calibration diagram was drawn. The amount of dye removal was calculated after the treatment period using Eq. (1). In bioremediation experiments for each treatment, a negative control (without plant) was considered to calculate the adsorption of dye to the wall of the test vessel and the effect of non-biological factors (physical-chemical) on dye removal. Finally, the percentage of net removal was calcu-

lated from the difference between the amount of removal in the presence of the plant and the conditions without the plant (Eq. (1)).

$$\text{Dye removal} = \frac{C0 - Cn}{C0} \times 100 \quad (1)$$

Cn: the final concentration of dye,
C0: initial concentration.

2.3 ORTHOGONAL ARRAY

Signal-to-noise ratio analysis was used to detect and obtain the optimal conditions for the experiment. The best level for each factor was introduced after performing this analysis. Signal refers to factors that can be controllable by the user, and noise refers to uncontrollable factors. S / N ratio analysis identifies the conditions in which the S / N ratio is the highest as the optimum condition (Silver, 1991). The signal-to-noise ratio was calculated based on Eq. (2).

$$\frac{S}{N} = \frac{-10\log\left(\frac{1}{Y_1^2}+\frac{1}{Y_2^2}+\frac{1}{Y_3^2}+\cdots+\frac{1}{Y_n^2}\right)}{n} \quad (2)$$

S: signal, N: noise, n: the number of experiments, Y: the result of each experiment

In this study, the effect of four parameters including time, plant biomass, initial dye concentration, and pH were investigated in the removal at four levels. The result of designing the experiment by the Taguchi method was a table with 16 experiments (L_{16}). The conditions of each experiment are shown in Table 1. All experiments were repeated three times. Other environmental conditions, including temperature (25 °C) and solution volume of samples (1 l), were considered constant factors.

2.4 INVESTİGATİON OF REUSABİLİTY OF *C. DEMERSUM* TO REMOVE DYE AB92

4 g of *C. demersum* were exposed to concentrations of 10 and 20 mg l^{-1} of AB92 and the percentage of dye removal was measured for 4 weeks. The culture medium containing the dye was changed weekly.

2.5 FTIR ANALYSIS

FTIR spectroscopy was used to investigate the interaction of acid blue dye 92 with *C. demersum*. For this purpose, 4 g of *C. demersum* was exposed to a dye solution at a concentration of 20 mg l^{-1} for 7 days. First, fresh plant tissue was homogenized with 3 ml of 2-propanol and 7 ml of diethyl ether. The reaction mixture was filtered, and distilled water was added and shaken for 20 seconds. After the separation of the two phases, the organic phase was collected. After evaporation of the solvent, samples were collected and used in FTIR spectroscopic analysis. All preparation steps for FTIR analysis were done for the control sample (dye concentration = 0 mg l^{-1}, fresh mass = 4 g, time = 7 days).

Table 1: Parameters and their values corresponding to their levels were studied in Experiments

	Level			
Parameter	1	2	3	4
A. Time (day)	1	3	5	7
B. Concentration (mg l^{-1})	5	10	15	20
C. Biomass (g)	0.5	1	2	4
D. pH	2.5	5	7	9.5

2.6 GROWTH ASSESSMENT

To evaluate plant growth rate, relative growth rate (RGR) was used based on plant fresh mass. The fresh mass of the plant was weighed after 20 days of treatment of plants with two concentrations of AB92 (10 and 20 mg l^{-1}), and the final mass of the samples was recorded, and the RGR was calculated using Eq. (3) (Radić et al., 2010).

$$\text{RGR (day}^{-1}) = [Ln(final\ mass) - Ln\ (initial\ mass)] / day \quad (3)$$

The tolerance index was calculated based on changes in relative growth rate in the presence of AB92 compared to control conditions by Eq. (4) (Forni et al., 2001).

$$\text{Tolerance index } (TI) = \frac{\text{RGR of treatment}}{\text{RGR of control}} \quad (4)$$

2.7 MEASUREMENT OF PHOTOSYNTHETIC PIGMENTS

The plant samples were treated with 0 (as control), 10, and 20 mg l^{-1} of AB92, and the quantity of some physiological parameters were measured after 7 days.

To measure the content of photosynthetic pigments, 500 mg of fresh plant tissue was homogenized in 80 % acetone. The samples were kept in the dark for 24 hours at a temperature of 4 °C and then centrifuged for 10 minutes at 4500 rpm. The absorbance at 470, 645, and 662 nm was

read by the spectrometer. The amounts of photosynthetic pigments were determined based on the method of Lichtenthaler (1987) and were reported in μg g^{-1} FM.

2.8 MEASUREMENT OF MEMBRANE LİPİD PEROXİDATİON

The amount of peroxidation of membrane lipids was measured based on the concentration of malondialdehyde (MDA). 500 mg of fresh plant tissue was homogenized with 1 % trichloroacetic acid (TCA). The obtained extract was centrifuged at 4500 rpm for 5 minutes. Then 20 % TCA solution containing 0.5 % thiobarbituric acid (TBA) was added. The reaction mixture was first heated in a water bath at 100 °C for 30 minutes and then immediately cooled on ice and centrifuged at 4500 rpm for 7 minutes. The absorbance of the sample (MDA + TBA) was read at 532 nm. To calculate the concentration of MDA, the extinction coefficient of 155 $\mu mmol^{-1}\, cm^{-1}$ was used and finally, the amount of MDA was calculated using the following formula (5) and expressed as $nmolg^{-1}$ FM (Heath & Packer, 1968).

$$MDA\ (nmol / g\ FM) = A / \varepsilon B \qquad \text{Eq. (5)}$$

$$A = A600 - A532$$

A600: absorption of non-specific aldehydes at 600 nm, A532: absorption at 532 nm, B: cuvette width (1 cm), ε: extinction coefficient (155 $\mu mmol^{-1}\, cm^{-1}$).

2.9 CYANİDİN GLYCOSİDE ASSAY

500 mg of the fresh mass of the plant was homogenized in acidic methanol (including methanol and hydrochloric acid in a ratio of 99 : 1). After that, the resulting extract was centrifuged for 15 minutes at 10,000 rpm, and the absorbance was read at 550 nm. The concentration of cyanidin glycoside was calculated using the extinction coefficient of 33,000 $mol^{-1}\, cm^{-1}$ and was reported in μmol g^{-1} FM (Wagner, 1979).

2.10 MEASURİNG THE FREE RADİCAL SCAVENGİNG ABİLİTY

The free radical scavenging ability was measured based on the electron-donating ability of the extract to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical (Sampath & Vasanthi, 2013). 100 mg of plant sample were homogenized in 80% methanol and then centrifuged at 10,000 g for 10 min. The reaction mixture included 3 ml of methanolic extract and 1 ml of DPPH ethanolic solution (0.1 **μM**). After placing the samples in the dark for 30 minutes, the absorbance of DPPH ethanolic solution without plant extract was measured at 517 nm as a control solution against 80 % methanol as a blank. Using the following equation, the percentage of DPPH free radical scavenging was calculated Eq. (6).

$$DPPHsc\ \% = ((A_0 - A_1)) / A_0 \times 100 \qquad \text{Eq. (6)}$$

A_0: absorbance of control, A_1: absorbance of sample.

2.11 STATİSTİCAL ANALYSİS

Minitab 15 software and the Taguchi test were used to design the experiments. The experiments were performed in a completely randomized design with three replications. Compare means were done at a 95 % confidence level using one-way ANOVA and Duncan test. Deviation from the mean of the data was indicated by the standard error (SE). SPSS software (version 21) was used for statistical analysis of data, and Microsoft Excel software (2016) was used to draw graphs.

3 RESULTS AND DISCUSSION

3.1 BIODECOLORIZATION OF AB92

Bioremoval of AB92 in the aquatic media via *C. demersum* was investigated. Analysis of plant remediation was carried out on the four factors including time, initial concentration of dye, plant biomass weight, and pH. The results of decolorization obtained from 16 treatments under the influence of the four factors obtained from the Taguchi test are presented in Table 2. The highest amount of refinement was obtained in experiment 13 with an average of 58.83 % and the signal/noise rate was 35.39.

Based on the results of quality analysis, the highest dye removal efficiency was obtained at level 4 of treatment time (7 days), plant biomass (4 g), level 4 of initial concentration of dye (20 $mg\ l^{-1}$), and level 2 pH (pH = 5) (Fig. 1).

The importance of the variables in the AB92 dye decolorization process by *C. demersum* has been shown in Table 3. The results based on S/N showed "time" as the most effective factor and "pH" as the least influential factor in this process.

C. demersum showed a significant ability to remove AB92 dye from the culture medium, which was proven by successive experiments. With increasing treatment

Table 2: Experimental layout using the L16 orthogonal array and experimental results for percent of dye removal

	A	B	C	D	Dye removal (%)				
Experimental number					1	2	3	Mean	S / N
1	1	1	1	1	9.7	9.23	8.82	9.25	19.30
2	1	2	2	2	12.73	11.97	12.46	12.38	21.85
3	1	3	3	3	13.75	14.06	13.76	13.85	22.83
4	1	4	4	4	15.83	14.65	15.36	15.28	23.66
5	2	1	2	3	23.36	23.97	24.13	23.82	27.53
6	2	2	1	4	22.76	21.23	21.96	21.98	26.83
7	2	3	4	1	27.56	26.89	26.17	26.87	28.58
8	2	4	3	2	30.32	29.98	30.73	30.34	29.63
9	3	1	3	4	37.45	36.1	37.32	36.95	31.35
10	3	2	4	3	44.09	43.86	43.79	43.91	32.85
11	3	3	1	2	42.18	42.77	43.63	42.86	32.63
12	3	4	2	1	39.21	39.64	39.95	39.66	31.95
13	4	1	4	2	59.9	58.12	58.48	58.83	35.39
14	4	2	3	1	56.97	57.08	56.37	56.80	35.08
15	4	3	2	4	52.33	54.79	51.83	52.98	34.47
16	4	4	1	3	53.13	53.56	52.49	53.06	34.49

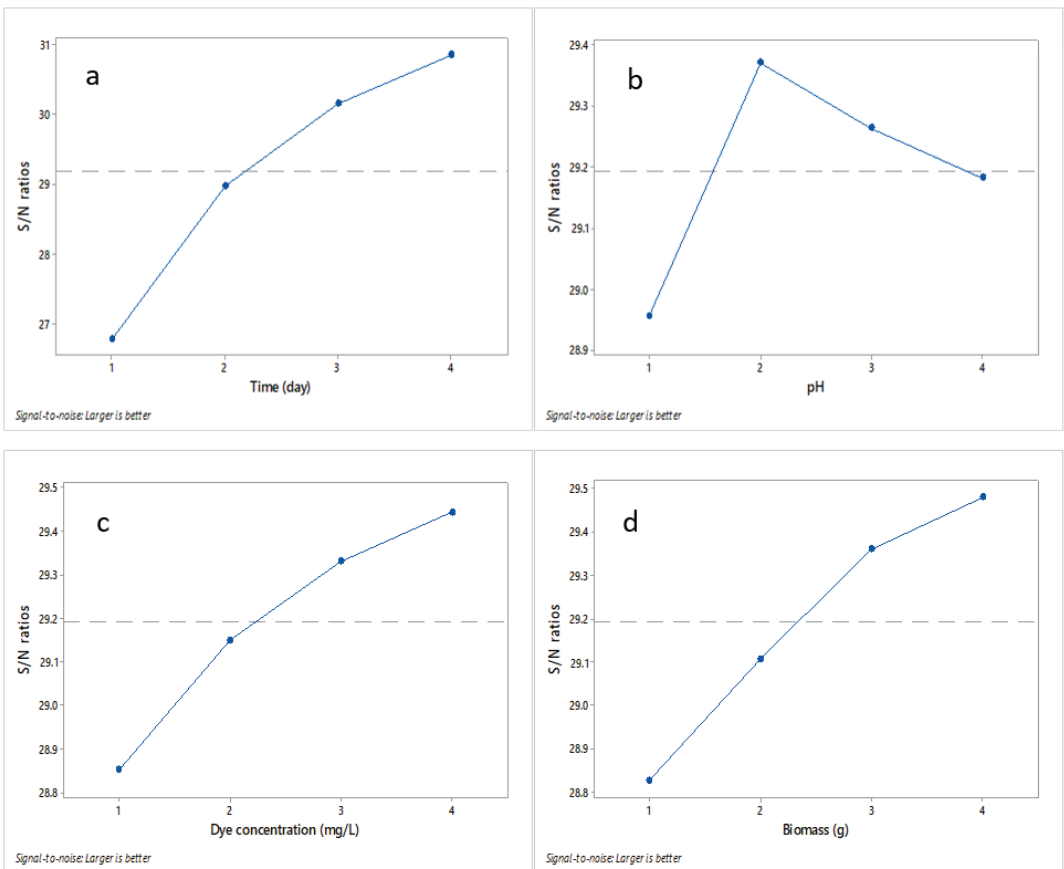

Figure 1: Effect of time (a), pH (b), dye concentration (c), and biomass (d) on dye removal

Table 3: Response to the Taguchi analysis of dye removal data

Parameter	Mean S / N ratio					
	Level 1	Level 2	Level 3	Level 4	Delta	Rank
A	21.91	28.15	32.2	34.86	12.95	1
B	28.4	29.16	29.63	29.94	1.54	3
C	28.32	28.95	29.73	30.12	1.81	2
D	28.73	29.88	29.43	29.08	1.15	4

time, the removal rate of AB92 dye increased significantly. The data obtained from the Taguchi test showed that the time factor had the highest effect on the decolorization process of AB92 dye by *C. demersum* compared to the other factors. Increasing the fresh mass of the plant by providing more surface to remove the dye increases the contact surface of the plant with the dye and consequently increases the efficiency of the process of adsorption and biodegradation of the dye. In submerged aquatic plants, stems play an important role in nutrient uptake; therefore, increasing the decolorization with increasing fresh mass seems logical. Increased decolorization efficiency due to increasing the mass of samples treated with dyes has been reported in previous studies (Daneshvar et al., 2007; Dhote & Dixit, 2009; Khataee et al., 2010). According to the results of the Taguchi test, with increasing the concentration of AB92 dye, its removal by *C. demersum* increased. It seems that two factors are involved in increasing the uptake of the dye by increasing the initial concentration. The first factor, increasing the concentration of the dye, provides the driving force needed to overcome the mass transfer resistance between the solid and liquid phases of the plant. In other words, higher concentrations facilitate the diffusion of the dye. Another factor is that increasing the concentration of the dye increases the likelihood of physical contact and collision between the molecules of the dye and the plant surface, and increases the number of available molecules of the dye at the plant binding site, resulting in increased decolorization (Aravindhan et al., 2007; Daneshvar et al., 2007). In the present study, among the 4 experimental pH ranges, the highest percentage of dye removal was determined at acidic pH of 5. The pH of the environment affects the rate of ion absorption by plants by controlling ionization and mobility. Many factors are involved in this, but the most important factor can be the molecular structure of the dye and the structure of the cell wall of plants. The pH of the environment also affects the solubility of the dye (Solís et al., 2012). AB92 dye is an anionic dye and most ionization occurs at acidic pH. On the other hand, most cell wall molecules have hydroxyl groups that are protonated at acidic pH, thus the electrostatic interactions of cell wall molecules with AB92 anion molecules increase at acidic pH (Ena et al., 2007). The ability of different organisms used in the purification of dyes following their sequential use is considered one of the most important factors in their selection for bioremediation (Ihsanullah et al., 2020).

3.2 INTERACTION BETWEEN PARAMETERS

The results of the interaction between the time factor and other factors (dye concentration, plant biomass, and pH) are shown in (Fig. 2 a-c). In all cases, the lowest mean of refinement (less than 20 %) was observed on the first day and the highest refinement (more than 50 %) was observed on the seventh day of treatment. The results of the interaction between the two factors of biomass and dye concentration showed an inverse ratio between these two factors, so the highest amount of refinement was observed at high concentrations of dye and low values of plant biomass (Fig. 2 - d).

3.3 REUSABILITY EXPERIMENTS

The results obtained from both concentrations of the dye indicated that this plant had an acceptable ability to purify the dye and its ability to refine in the fourth stage was better than in the first stage. Thus, the minimum percentage of purification related to the experiment on the seventh day of the first week, which was obtained at concentrations of 10 and 20 mg l^{-1}, was 51 % and 40 %, respectively. The maximum percentage of purification related to the seventh day of the fourth week at concentrations of 10 and 20 mg l^{-1} were 56 % and 42 %, respectively. In addition, during the decolorization process in the four stages, no morphological changes were observed in the plants due to the accumulation of the dye (Fig. 3).

C. demersum showed an acceptable ability to be reused to remove the dye AB92, which can confirm the occurrence of the biodegradation process in removing the pollutant and distinguish it from other processes, especially adsorption. Because in the adsorption process

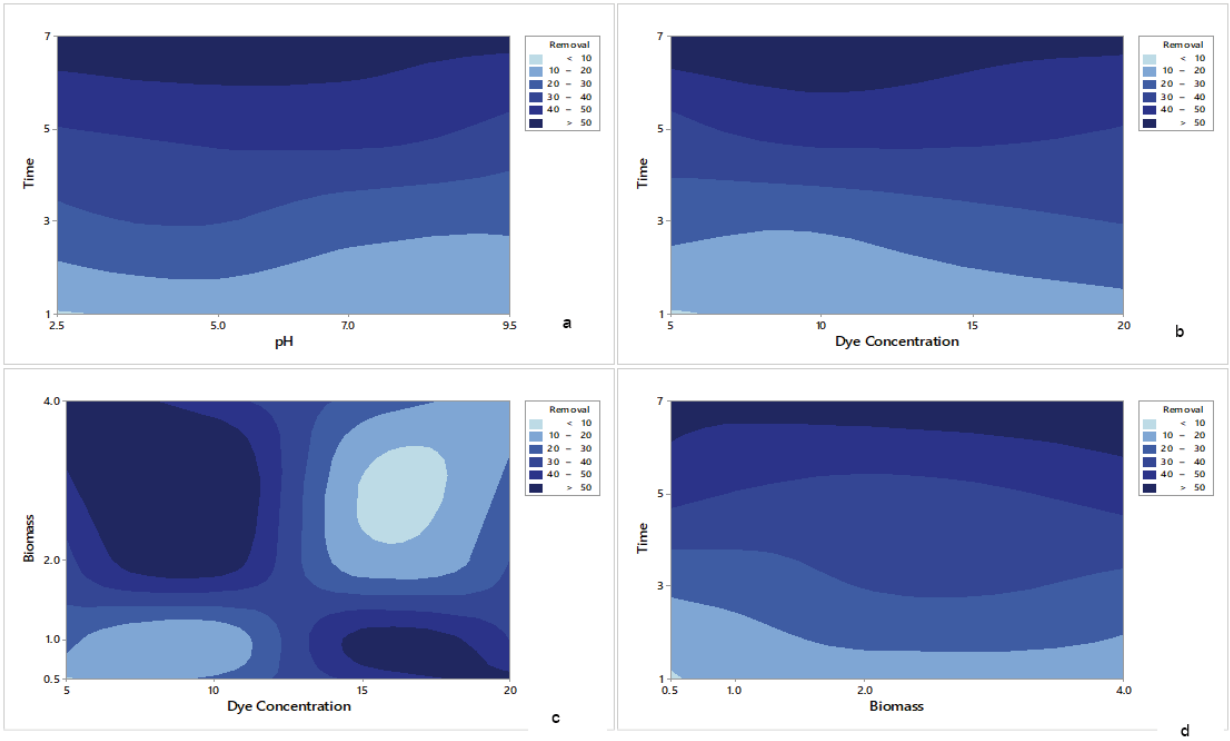

Figure 2: Interaction (a) time and concentration of dye (b) time and pH (c) time and plant biomass (d) plant biomass and dye concentration

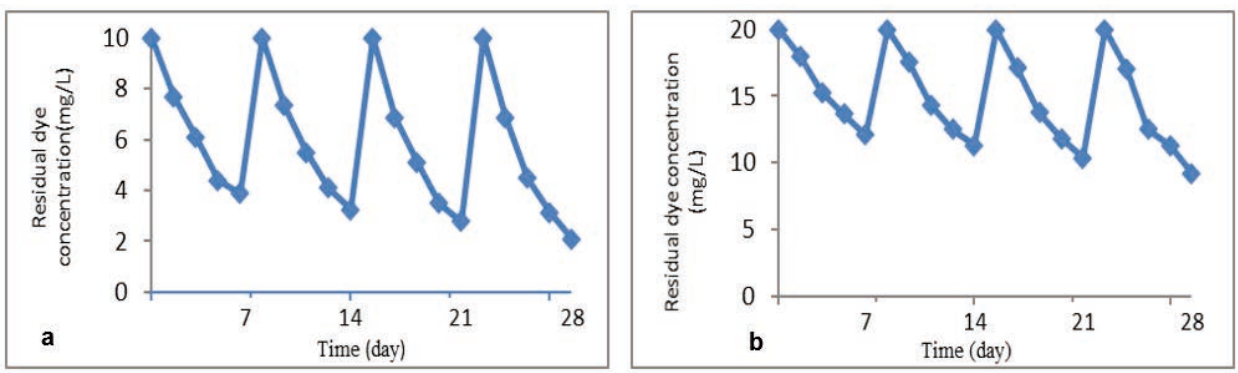

Figure 3: Biological decolorization profiles during repeated batch operations. T = 25 ◦C; [AB92] = a 10 m l^{-1} and b 20 ml^{-1}; [Biomass] = 4g; pH = 7

due to surface capacity limitation, the gradient of the contaminant concentration is quickly balanced and the continuous addition of dye to the environment will not increase the adsorption efficiency (Khataee et al., 2013; Srinivasan & Viraraghavan, 2010). Also, this could have happened due to the positive relative growth rate of *Ceratophyllum* in the fourth week. The positive effect of plant mass on purification efficiency was also confirmed based on the Taguchi test analysis (Table 3). It is also possible that mechanisms such as increasing the antioxidant defense system and increasing the activity of enzymes effective in color decomposition have contributed to increasing the refining capacity of the *Ceratophyllum* plant in the fourth week, and the proof of this requires a more detailed study. In previous studies, the ability of aquatic plants *Nasturtium officinale* Aiton (Torbati et al., 2015), *Hydrocotyle vulgaris* L. during successive purification with acidic blue dye 92 and the aquatic plant *Spirodela polyrrhiza* (L.) Schleid. (Movafeghi et al., 2016) exposed to azo dye Direct Blue 129 has been reported.

3.4 SPECTRAL ANALYSIS OF IR

The IR spectrum of the AB 92 showed some peaks, which correspond to functional groups (Fig. 4a). The peak observed at 3425 cm^{-1} can be related to O-H stretching as in R-OH compounds or N-H stretching as in amines and amides and 2922 cm^{-1} for asymmetric –CH3 stretching vibrations. The peak at 1618 cm^{-1} for N = N stretching confirms the azo nature of the dye. Peaks at 1566 cm^{-1} correspond to C-N stretching as in amides, 1454 cm^{-1} for C–H in plane C–H bend, 1400 cm^{-1} for C-H deformation as in cis-alkene, 1340 cm^{-1} for O-H stretching of phenols. Peaks at 1127 Cm^{-1} for disubstituted benzene ring. This confirms the aromatic nature of the dye, 1046 cm^{-1} for S–O stretching as in sulphonic acids. The IR spectrum plant before and after decolorization has been shown in Fig. 4. In the plant before treatment (control plant) several peaks were observed at 3424, 2920, 1725, 1628, 1464, and 1117 cm^{-1}. The peaks at 3424 cm^{-1} can be related to NH_2 stretching of amino acids or O-H stretching, 2920 cm^{-1} for C–H stretching of CH_2. The peaks at 1725 cm^{-1} for C = O stretching, 1628 cm^{-1} for N–H deformation of primary amines, 1464 cm^{-1} for C–H stretching of alkane CH_3, and 1117 cm^{-1} for C-N vibrations in aliphatic amines. The IR spectrum plant after treatment with AB92 dye, the transfer of peaks related to plant functional groups from 3424, 2920, 1725, 1464, and 1117 cm^{-1} to 3417, 2922, 1726, 1460, and 1154 cm^{-1}, respectively. Also, the appearance of additional peaks at 1649, 1544, 1381,1327, 1154, and 1077 cm^{-1} representing N = N and stretching, C = C vibration of aromatic homocyclic compounds, CH_3 deformation of alkanes, C-N stretching vibrations in aromatic tertiary amines, O-H stretching as in alcohols, and for S = O stretching as in $R–SO_3H$ compounds (Fig. 4).

The results of the FTIR spectrum showed that the control plant has various functional groups including amine, hydroxyl, and carboxyl groups. The displacement of these peaks in the spectrum of the treatment plant may have been due to the formation of interactions between the *Ceratophyllum* plant and the molecules of the AB92 dye. Studies have shown that the functional groups of amines, hydroxyl, carbonyl, and carboxyl in plants can play an important role in the interaction between plants and dye molecules (Liu & Wang, 2023; Sah et al., 2022). This could indicate the occurrence of the first phase of

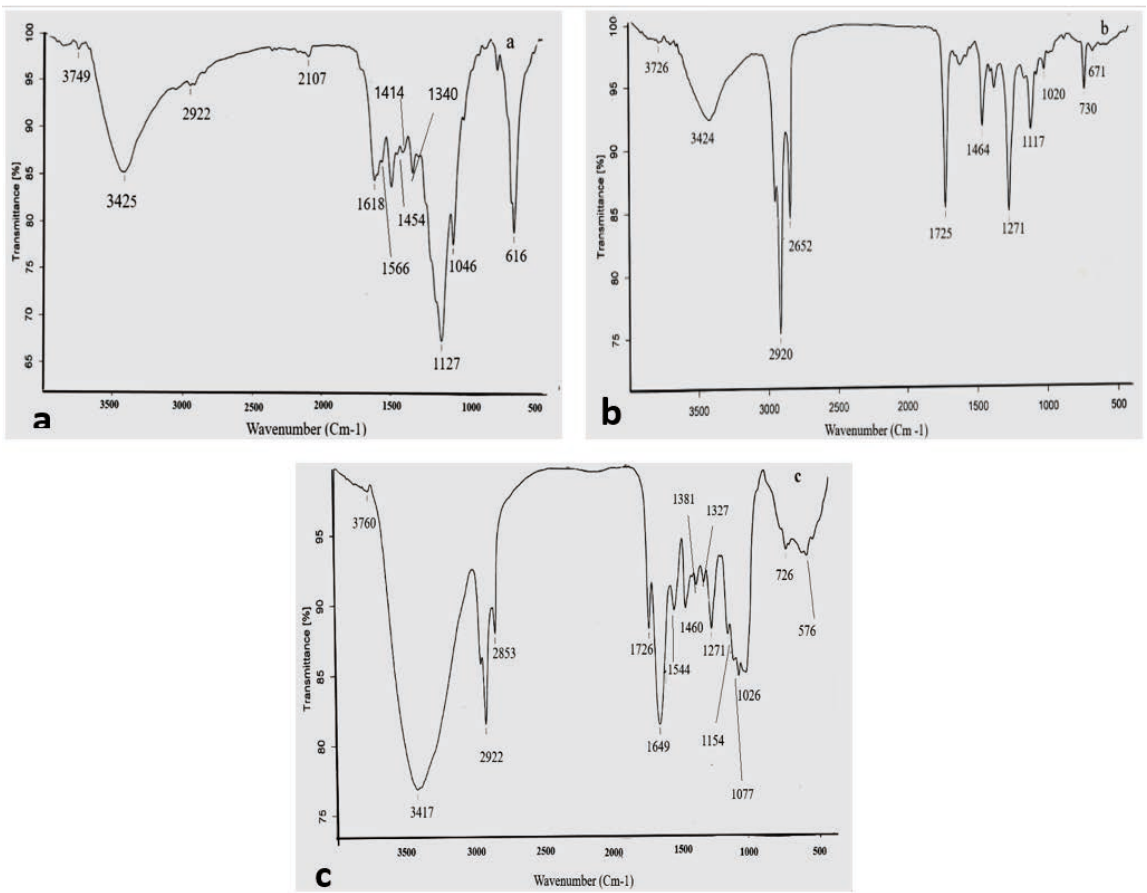

Figure 4: FTIR spectrum of (a) dye AB92, (b) *C. demersum,* (c) *C. demersum* after treatment with AB92

detoxification, i.e. the activation phase of the dye. In this process, organic non-biomolecules obtain a hydrophilic functional group such as hydroxyl, amino, carboxyl, etc. because of enzymatic transformations of oxidation, reduction, hydrolysis, etc. These functional groups increase the reactivity and polarity of the molecule, as well as increase the susceptibility of the contaminant molecule to enzymes and accelerate the change of the contaminant (conjugation or oxidation) (Kvesitadze et al., 2006). Similar results were observed for the adsorption of Basic Red 46 dye (Mahmoodi et al., 2010) and cation dye by biosorbents (Zhang et al., 2013). The IR spectrum of the plant after dye removal significantly differed from that of the AB92 dye and of the control, like the disappearance of the peaks at 1618, 1566, 1414, 1340, 1127, and 1046 cm^{-1} in the treated plant, which was present in the spectra of the dye. Also, the appearance of several new peaks at 1649, 1649, 1544, 1381, 1327, 1154, and 1077 cm^{-1}, supports the biotransformation of the dyes within the *Ceratophyllum*. Thus, it can be suggested that the plant could play the expected role in dye biodegradation. Such bioremediation can be consistent with previous research (Khataee et al., 2013; Vafaei, et al., 2012).

3.5 GROWTH ASSESSMENT

With increasing the concentration of AB92 dye, the relative growth rate and tolerance index showed a significant decrease compared to the control. The tolerance index reached a minimum of 0.26 at a concentration of 20 mg l^{-1} (Table 4).

The relative growth rate is an important parameter to observe the physiological effects of the toxicity of chemicals (Duman & Koca, 2014). The results showed that by increasing the concentration of AB92 dye, the growth rate of *C. demersum* decreased. Previous studies have shown that organic and inorganic xenobiotics can accelerate the aging process and stimulate premature plant death (Parent et al., 2008). This can be a plant defense response to persistent stressors because in these conditions' stressors are stored in the old organs and by separating these areas, toxic compounds are removed from the living parts of the plant. In previous studies, the toxic effects of other environmental pollutants had been shown on the growth of *C. demersum*. For example, reduced growth of *C. demersum* versus increased concentrations of the heavy metals nickel and cadmium have been previously reported (Chorom et al., 2012). In previous studies, a decrease in the growth of this plant against high concentrations of non-ionic surfactant 4-tert-octylphenol (OP) has been reported (Chiu & Wu, 2017). Also, reduced growth of aquatic plants *Nasturtium officinale* (Torbati et al., 2015), *Lemna minor* L. (Khataee et al., 2012), and aquatic fern *Azolla filiculoides* Lam. (Khataee et al., 2013) with increasing concentration of acidic dye 92 were reported.

Table 4: Effects of different concentrations of AB92 (0 – 20 mg l^{-1}) on relative growth rate (RGR) and Tolerance index (TI) in *C. demersum* treated for 7 days (mean ± standard error, n = 3)

AB92 (mg l^{-1})	RGR	TI
0	2.98± 0.09 [a]	1 [a]
10	1.94± 0.27 [b]	0.65± 0.09 [b]
20	0.78 ± 0.06 [c]	0.26± 0.02 [c]

3.6 PHOTOSYNTHETIC PIGMENTS CONTENTS

The effect of different concentrations of AB92 on the concentration of chlorophyll a and chlorophyll b is shown in Table 5. The amount of chlorophyll a and chlorophyll b showed a significant decrease only at the concentration of 20 mg l^{-1} of the dye compared to the control. The results showed that with the increase in the dye concentration, the total chlorophyll concentration decreased significantly. At a concentration of 20 mg l^{-1}, the concentration of total chlorophyll decreased by 16.88 % compared to the control (Table 5). At a concentration of 10 mg l^{-1}, the total carotenoid concentration decreased by 21.4 %, and at a concentration of 20 mg l^{-1}, it decreased by 45 % compared to the control (Table 5).

According to the results of this study, probably, at the concentration of 20 mg l^{-1}, the production and accumulation of free radicals increased and caused damage to the photosynthetic apparatus, and subsequently caused the reduction of photosynthetic pigments. Previous

Table 5: Effects of different concentrations of AB92 (10–20 mg l^{-1}) on chlorophylls and total carotenoid content (μg g^{-1} FM) in the *C. demersum* treated for 7 days (mean ± standard error, n = 3)

AB92	Chlorophyll a	Chlorophyll b	Total chlorophyll	Total carotenoid
0	0.52 ± 0.0080 [a]	0.24± 0.0066 [a]	0.77 ± 0.0118 [a]	0.20 ± 0.0107 [a]
10	0.50 ± 0.0067 [a]	0.21 ± 0.1369 [ab]	0.73 ± 0.0092 [b]	0.16 ± 0.0076 [b]
20	0.44 ± 0.0036 [b]	0.20 ± 0.0097 [b]	0.64 ± 0.1268 [c]	0.11 ± 0.0135 [c]

studies have shown that chlorophylls are more unstable than carotenoids and are easily subjected to oxidative decomposition by singlet oxygen from photosynthesis (Weinberg et al., 2003). Carotenoids can quickly receive the energy from triplet chlorophyll excitation and thus prevent the formation of singlet oxygen and protect chlorophyll from oxidative degradation (Li et al., 2009; Santabarbara et al., 2007). The increase of AB92 showed a similar effect on the carotenoid concentration of aquatic plants *Hydrocotyle vulgaris* (Torbati et al., 2015) and *Azolla filiculoides* (Khataee et al., 2013). The reduction of carotenoids in the *Ceratophyllum* plant under flurochloridone treatment has been previously reported (Zhou et al., 2020).

3.7 LIPID PEROXIDATION ASSAY

The extent of oxidative damage was calculated based on the concentration of malondialdehyde (MDA) as a product of lipid peroxidation. MDA concentration increased by 39.9 % at a concentration of 10 mg l^{-1} of AB92 and 69.5 % at a concentration of 20 mg l^{-1} compared to the control (Table 6). Polyunsaturated fatty acids are exposed to attack by reactive oxygen species, which results in the production of small hydrocarbon fragments such as ketones and malondialdehyde. For this reason, malondialdehyde is considered an indicator of lipid peroxidation. Lipid peroxidation causes damage to the cell by reducing the fluidity of the membrane and increasing the leakage of substances. This research also confirmed this study. Similar results were observed in the treatment of aquatic plants *Azolla filiculoides* (Khataee et al., 2013) and *Nasturtium officinalle* (Torbati et al., 2015) exposed to AB92 and in *Spirodela polyrrhiza* (L.) Schleid. exposed to Direct Blue92 (Movafeghi et al., 2016). Also, an increase in the production of reactive oxygen species has been observed in the treatment of *Ceratophyllum* plants with heavy metals cadmium, lead, and zinc (Hak et al., 2020; Mishra et al., 2008; Mishra et al., 2006).

3.8 THE EFFECT OF AB92 ON ANTHOCYANIDIN GLYCOSIDE CONCENTRATION

With increasing the concentration of AB92, the amount of anthocyanidin glycosides increased. At a concentration of 10 mg l^{-1} of the dye, the amount of anthocyanidin glycosides increased by 33.6 %, and at a concentration of 20 mg l^{-1}, it increased by 81.8 % compared to the control (Table 6). Glycoside anthocyanidins are one of the most effective scavengers for most types of oxidizing molecules, including free radicals (Kong et al., 2003). Previous studies have shown that glycoside anthocyanidins produced in plants have more antioxidant activity than alpha-tocopherol (El-Alfy et al., 2005). An increase in the amount of anthocyanidin glycosides has been reported in the aquatic fern *Azolla filiculoides* (Masoudian et al., 2020) and *Lemna minor* (Masoudian et al., 2022) under oxidative stress conditions.

3.9 THE EFFECT OF AB92 ON FREE RADICAL SCAVENGING ABILITY

By increasing the concentration of AB92, the free radical scavenging ability of the plant increased. The free radical scavenging ability increased by 6.88 % and 14.29 % in the concentration of 10 mg l^{-1} and 20 mg l^{-1} of the dye, respectively (Table 6). The increase in antioxidant activity could probably be due to the increase in the anthocyanidin glycoside. It seems that the presence of AB92 in the culture medium of the plant has caused oxidative stress. The mentioned plant has tried to reduce stress by raising the oxidant defense system, and the increase in free radical scavenging activity confirms this.

4 CONCLUSIONS

Due to its cost-effectiveness and low side effects, phytoremediation technology is one of the most useful methods in pollutant purification. To use this technology, it is essential to identify plant species capable of removing various pollutants. This research showed that the aquatic plant *C. demersum* can significantly remove AB92 from polluted water. The results based on S/N showed treatment time had the most effect and pH factor had the least effect among the investigated factors Also, the highest percentage of removal of dyes was observed at level 4 of treatment time (7 days), level 4 of plant biomass (4 g), level 4 of initial concentration of dye (20 mg l^{-1}), and level 2 pH (pH = 5). The reusability of the plant in four consecutive periods confirmed the process of biological degradation of the dye. The FTIR results confirmed the possible interaction between the dye molecules and the plant's functional groups. Both dye concentrations caused negative effects on growth parameters and damage to the membrane of *C. demersum* through oxidative stress. The stress was more intense in the concentration of 20 mg l^{-1}, compared to 10 mg l^{-1}. *C. demersum* increased non-enzymatic antioxidant (anthocyanidin glycoside) content to defend against oxidative stress.

Table 6: The changes of MDA (μmol g^{-1} FM), anthocyanine glycoside (μmol g^{-1} FM), and antioxidant activity (%), in the *C. demersum* treated by 0, 10, and 20 mg l^{-1} of AB92 (mean ± standard error, n = 3)

AB92	MDA	Anthocyanidin glycoside	Antioxidant activity (%)
0	14.84 ± 0.3871 [c]	19.19 ± 0.2626 [c]	22.72 ± 0.9709 [c]
10	20.77 ± 0.5161 [b]	25.64 ± 0.2523 [b]	29.60 ± 0.6277 [b]
20	25.16 ± 0.3413 [a]	34.89 ± 0.4276 [a]	37.01 ± 0.7191 [a]

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