Highly efficient magnetic phosphoric acid modified defatted Chlorella vulgaris algae (MDCV/Fe₃O₄) as a novel biosorbent for methylene blue removal

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Abstract

Background:

A novel biosorbent based on defatted *Chlorella vulgaris* (DCV) as a by-product of the biofuel industry was considered as an economical and inexpensive biosorbent in the form of magnetic modified defatted *Chlorella vulgaris* (MDCV/Fe3O4) for methylene blue (MB) removal. The lipid extraction was performed on raw *Chlorella vulgaris* (RCV). Phosphoric acid was selected as a DCV modifier. During acid modification, the variables affecting the biosorption capacity and the residual algae such as temperature (30-70 °C), the contact time of DCV with acid (3-9 hr), the concentration of acid (2-6 mol/L), and the ratio of acid volume to DCV (30-70 mL/g) were investigated and optimized using Minitab-18 software. The modified defatted *Chlorella vulgaris* (MDCV) was prepared by acidification of DCV under optimal conditions. MDCV/Fe3O4 was prepared using the co-precipitation method for easy and low-cost separation of biosorbent. The XRD, FTIR, SEM, EDS, BET, and VSM analyses were performed to identify the structures and characteristics of RCV, DCV, MDCV, and MDCV/Fe3O4. Some experiments were designed using Minitab-18 software to investigate the effects of temperature (5-45 °C), contact time (30-90 min), biosorbent dosage (15-45 mg), initial concentration of MB (20-100 mg/L), and pH (5-9) on the biosorption capacity of MDCV/Fe3O4. The kinetic, isothermal and thermodynamic parameters were investigated on MDCV/Fe3O4.

Results:

The specific surface area of MDCV/Fe3O4 was 25.20 m²/g. RCV, DCV, and MDCV/Fe3O4 had a crystalline structure and MDCV had an amorphous structure. The data were most consistent with Pseudo-second-order and Freundlich models. The maximum biosorption capacity of MDCV/Fe3O4 was calculated in the amount of 32.44 mg/g. According to the positive values of ∆G and negative values of ∆H (-46.56 kJ/mol) and ∆S (-0.17 kJ/mol.K), the biosorption of MB on MDCV/Fe3O4 was non-spontaneous, exothermic with a decrease in irregularity.

Conclusions:

In this study, MDCV/Fe3O4 was applied as the MB biosorbent. Modifications such as lipid extraction, phosphoric acid modification and magnetization improved this biosorbent in its biosorption capacity and separation. Data were optimized in the acid modification and MB biosorption steps. Comparison of the characteristics of MDCV/Fe3O4 with unmodified species confirmed its high efficiency. Kinetic, isothermal and thermodynamic studies were also performed.

Keywords:
Chlorella vulgaris, Lipid extraction, Phosphoric acid modification, magnetic composite, Methylene blue biosorption

Background

Water is one of the main human needs that the development of urbanization and industry has polluted. Among organic pollutants, dyes are one of the important toxic with destructive effects, causing global attentions [1]. They have negative influences on the health of aquatic organisms and the passage of light into the water for plants’ photosynthesis. In addition, they are very carcinogenic due to their aromatic structure, which poses a serious threat to human and animal health [2]. Artificial dyes are used in various industries such as textiles, plastics, foods, medicines, etc. [3]. Methylene blue (MB) is a cationic dye with detrimental effects on organisms over a short time [4]. It has morbidities such as eye irritation, headache, nausea, dizziness, and excessive sweating [5]. Therefore, the treatment of wastewater containing this dye is very significant due to its harmful effects.

Hitherto, various physical and chemical methods such as filtration, coagulation, flocculation, irradiation, oxidation, catalytic ozonation, etc, have been used to separate organic pollutants from effluents [6-11]. Environmental hazards, high operating costs, more time consuming, and requiring special expensive equipment are some of the disadvantages of these methods [12]. So, it is important to develop efficient, inexpensive, environmentally friendly, and high-efficiency methods.

The biosorption methods using biochars due to environmental compatibility, non-toxicity, low price, availability, and abundance are suitable for removing organic pollutants [13]. Some studies have suggested the use of biosorbents such as banana, cucumber, and potato peel [14], sago [15], leaves [16], rice husk [17], egg shell [18], and diverse algae such as Sargassum dentifolium, Ulva fasciata [19]. Chlorella pyrenoidosa [20], Scenedesmus [21], and Spirulina [22]. These biosorbents are very efficient and inexpensive for the removal of organic pollutants from industrial effluents. However, the separation of these biosorbents is difficult and costly. Therefore, research was done on magnetizing the adsorbents as a cheap, effective, and easy separable material [23].

Chlorella vulgaris is biomass with high potential in biofuel production and as a by-product in the biofuel industry can be used for adsorbing many urban, industrial, and agricultural pollutants such as heavy metals, dyes, and pharmaceutical pollutants. This algae grows rapidly and its
structure is resistant to adverse conditions. They are also a source of substances such as lipids, carbohydrates, proteins, vitamins, pigments, and minerals. Growth conditions such as limited nitrogen and phosphorus sources, high levels of carbon dioxide, light intensity and temperature increase the level of lipids and starches [24]. The fatty acids in polar lipid cell membranes and non-polar lipids, as energy sources, are used in the biodiesel production process and directly affect its quality [25].

Some researchers have shown that the extraction of lipids from algae increases the specific surface area and its biosorption capacity [26, 27]. On the other hand, there are studies on the use of sulfuric acid, hydrochloric acid, phosphoric acid, boric acid, etc, as a pretreatment of algae to increase biosorption capacity [28, 29].

One of the important issues is the efficient and low-cost separation of the biosorbents after the biosorption process. For this purpose, there are some studies on magnetic biological materials from different sources such as magnetic *Sargassum horneri* [30], magnetic *Cymopolia barbata* [31], and magnetic activated carbon [32].

In this study extraction of the lipid from raw *Chlorella vulgaris* (RCV) and modification of defatted *Chlorella vulgaris* (DCV) was carried out to gain further MB biosorption. Then it was subjected to an acid modification to increase the specific surface area of DCV. Another goal is magnetizing the modified defatted *Chlorella vulgaris* (MDCV) for easy and low-cost separation. In the acid treatment step, the variables affecting the MDCV biosorption capacity and residual MDCV were modeled by Minitab-18 software, using the response surface methodology (RSM). The magnetic modified defatted *Chlorella vulgaris* (MDCV/Fe$_3$O$_4$) was prepared as the final biosorbent. In the end, the effect of variables on the biosorption capacity of MDCV/Fe$_3$O$_4$, was considered and modeled by Minitab-18 software. Finally, kinetic, isotherm, and thermodynamic parameters were studied.

**Results and discussion**

**Phosphoric acid treatment and optimization of parameters**

In order to optimize variables of acid treatment, the experimental results of the proposed levels are reported in Table 1. Also, multiple regression analysis of experimental data with 95% probability
has been reported in Equations 1-2. Ineffective variables were removed to increase the suitability of the models with experimental data [33].

| Run no. | $X_1$, T | $X_2$, t | $X_3$, $C_A$ | $X_4$, V/W | $Y_1$, $q_{MDCV}$ | $Y_2$, $R_{P_{MDCV}}$ |
|---------|---------|---------|-------------|-----------|----------------|------------------|
| 1       | -1      | -1      | -1          | -1        | 8.2250         | 56.5             |
| 2       | 1       | -1      | -1          | -1        | 9.1465         | 45.5             |
| 3       | -1      | 1       | -1          | -1        | 9.8998         | 52.5             |
| 4       | 1       | 1       | -1          | -1        | 10.6434        | 31.0             |
| 5       | -1      | -1      | 1           | -1        | 10.4720        | 52.5             |
| 6       | 1       | -1      | 1           | -1        | 8.8975         | 22.5             |
| 7       | -1      | 1       | 1           | -1        | 9.3889         | 46.5             |
| 8       | 1       | 1       | 1           | -1        | 7.8629         | 10.0             |
| 9       | -1      | -1      | -1          | 1         | 6.8480         | 53.5             |
| 10      | 1       | -1      | -1          | 1         | 8.2671         | 49.5             |
| 11      | -1      | 1       | -1          | 1         | 7.7110         | 57.5             |
| 12      | 1       | 1       | -1          | 1         | 9.7640         | 30.0             |
| 13      | -1      | -1      | 1           | 1         | 10.4203        | 45.5             |
| 14      | 1       | -1      | 1           | 1         | 10.5270        | 20.5             |
| 15      | -1      | 1       | 1           | 1         | 9.9386         | 51.5             |
| 16      | 1       | 1       | 1           | 1         | 10.6725        | 9.5              |
| 17      | -1      | 0       | 0           | 0         | 9.5086         | 50.5             |
| 18      | 1       | 0       | 0           | 0         | 11.6263        | 23.0             |
| 19      | 0       | -1      | 0           | 0         | 11.3029        | 52.0             |
| 20      | 0       | 1       | 0           | 0         | 11.1090        | 46.0             |
\[ Y_1 = -5.02 + 0.4863X_1 + 2.047X_2 + 0.504X_3 - 0.1110X_4 - 0.004711X_1^2 - 0.1384X_2^2 - 0.01156X_1X_3 + 0.000898X_1X_4 - 0.0832X_2X_3 + 0.01603X_3X_4 \]  
(1)

\[ Y_2 = 3.97 + 2.433X_1 + 1.819X_2 + 2.041X_3 - 0.02264X_1^2 - 0.0599X_1X_2 - 0.1086X_1X_3 \]  
(2)

ANOVA results of recent regression are reported in Table 2. The calculated Fisher distributions of both models are greater than their critical values. Also, the P-values of them are less than 0.05. Therefore, the compatibility of the models with data is proven. The adjusted determination coefficients of models \( Y_1 \) and \( Y_2 \) are 91.34\% and 96.14\%, respectively. So, it can be concluded that no other effective variable has been ignored [33].

| Response | Variation source | df | SS   | MSS  | F_{calc.} | F_c | P-value | \( R_{adj}^2 \) |
|----------|------------------|----|------|------|------------|-----|---------|-----------------|
| MDCV biosorption capacity, \( Y_1 \) | Regression       | 10 | 71.07| 7.10 | 28.43      | 2.49| 0.00    | 91.34\%         |
|          | Error            | 16 | 4.00 | 0.25 | -          | -   | -       | -               |
|          | Total            | 26 | 75.0778 | - | - | - | - | - |
| Residual MDCV, \( Y_2 \) | Regression       | 6  | 4863.95 | 810.66 | 108.85 | 2.60 | 0.00 | 96.14\% |
|          | Error            | 20 | 148.95 | 7.45 | - | - | - | - |
|          | Total            | 26 | 5012.91 | - | - | - | - | - |
According to Table 3, the P-values of all coefficients of the effective variables in $Y_1$ analysis is less than 0.05. Since the coefficients of interactions of $X_2$ and $X_4$ with other variables are significant, they can not be ignored. Regarding the response of $Y_2$, all variables except $X_4$ are significant [33].

Table (3) - Estimated regression coefficients for the quadratic models of the MDCV biosorption capacity and residual MDCV

| Terms     | Uncoded coefficient | The standard error of the coefficient | t-value | P-value |
|-----------|---------------------|--------------------------------------|---------|---------|
|           | $Y_1$               | $Y_2$                                | $Y_1$   | $Y_2$   | $Y_1$   | $Y_2$   | $Y_1$   | $Y_2$   |
| constant  | -5.02               | 3.97                                 | 0.177   | 0.910   | 70.03   | 53.19   | 0.000   | 0.000   |
| $X_1$     | 0.4863              | 2.433                                | 0.118   | 0.643   | 2.35    | -19.43  | 0.032   | 0.000   |
| $X_2$     | 2.047               | 1.819                                | 0.118   | 0.643   | 1.36    | -5.48   | 0.193   | 0.000   |
| $X_3$     | 0.504               | 2.041                                | 0.118   | 0.643   | 3.88    | -10.54  | 0.001   | 0.000   |
| $X_4$     | -0.1110             | -                                    | 0.118   | -       | -0.33   | -       | 0.744   | -       |
| $X_1^2$   | -0.004711           | -0.02264                             | 0.274   | 1.11    | -6.88   | -8.13   | 0.000   | 0.000   |
| $X_2^2$   | -0.1384             | -                                    | 0.274   | -       | -4.55   | -       | 0.000   | -       |
| $X_1X_2$  | -0.0599             | -                                    | 0.682   | -       | -5.27   | -       | 0.000   | -       |
| $X_1X_3$  | -0.01156            | -0.1086                              | 0.125   | 0.682   | -3.70   | -6.37   | 0.002   | 0.000   |
| $X_1X_4$  | 0.000898            | -                                    | 0.125   | -       | 2.87    | -       | 0.011   | -       |
| $X_2X_3$  | -0.0832             | -                                    | 0.125   | -       | -3.99   | -       | 0.001   | -       |
| $X_3X_4$  | 0.01603             | -                                    | 0.125   | -       | 5.13    | -       | 0.000   | -       |

Based on Equations 1-2, $Y_1$ is a second-order function of $X_1$ and $X_2$. Growing of $X_3$ increases $Y_1$ and $X_4$ is almost ineffective. $Y_2$ increases by enhancement of $X_1$, $X_2$, and $X_3$. Also, $X_4$ is
completely ineffective. Therefore, it is important to determine the optimal values of variables to maximize the amounts of $Y_1$ and $Y_2$ to achieve the maximum biosorption capacity in economic conditions. The optimal values of the recent factors are reported in Table 4. The composite desirability of this optimization is 0.95.

Table (4) - Optimized factors affecting MDCV biosorption capacity and residual MDCV

| Optimized factor | Optimized response | Composite desirability |
|------------------|--------------------|------------------------|
| Temperature $(^\circ C)$ | Contact time (hr) | The concentration of phosphoric acid (mol. L$^{-1}$) | The ratio of acid volume to DCV (mL. g$^{-1}$) | MDCV biosorption capacity (mg. g$^{-1}$) | Residual MDCV (%) |
| 47.3 | 6.27 | 2 | 30 | 12.61 | 55.80 | 0.95 |

DCV was treated with phosphoric acid under optimized conditions, then it was used as a biosorbent for more experimental results. The experimental data were consistent with the predicted responses. So, optimized MDCV is used in the magnetization stage.

**Biosorbent characterization**

**X-Ray Diffraction (XRD)**

The XRD analysis of RCV, DCV, MDCV, and MDCV/Fe$_3$O$_4$ are shown in Fig. 1. In the analysis of RCV, the peaks located in the range of 20, 23.1, 29.5, 36, 39.5, 43.2, 47.7, 48.6 and 57.5 are attributed to the reflections of (012), (104), (110), (113), (202), (018), (116) and (122) respectively. They represent the magnesium calcite ($\text{Mg}_{0.03}\text{Ca}_{0.97}\text{(CO}_3\text{)}$) with the rhombohedral crystal structure in RCV [34]. It is also found in DCV. The plated distances in these two samples are almost the same [35]. The chart of MDCV has no sharp peaks, so its structure is amorphous. In this diagram, the peaks related to magnesium calcite have been removed. In the analysis of MDCV/Fe$_3$O$_4$, peaks in the range of 20, 30.1, 35.5, 43.1, 53.5, 57.1 and 62.7 related to reflections (220), (311), (400), (422), (511) and (440) which are shown the presence of iron oxide particles
(Fe$_3$O$_4$). Therefore, it can be concluded that the cubic crystal structure of iron oxide is well-formed [32, 36].

Figure (1) – XRD analysis for a) RCV, b) DCV, c) MDCV and d) MDCV/Fe$_3$O$_4$

**Fourier Transform Infrared (FTIR)**

The results of FTIR analysis for RCV, DCV, MDCV, and MDCV/Fe$_3$O$_4$ are shown in Fig. 2. In all samples, the broad peak was observed in the range of 3400 cm$^{-1}$. It shows the tensile vibrations of the hydroxyl bond (O-H), the amide groups (N-H), and the presence of the water molecules [29]. The peaks around 2926 cm$^{-1}$ are associated with symmetrical and asymmetric tensional vibrations C-H in aliphatic groups such as CH, CH$_2$, and CH$_3$ [29]. The intensity of this peak is highest in RCV and almost the same in other samples. In RCV, a peak was observed in 2855 cm$^{-1}$, indicating CH$_2$ and CH$_3$ bonds in fatty acids. It confirms the presence of lipids in the structure of RCV that was removed after the lipid extraction process due to the removal of fatty acids [37]. The peak of 1730 cm$^{-1}$ in RCV indicates tractions in carbonyl ester groups. It was removed by lipid extraction. Then, by acidification and magnetization, two peaks were formed again in the range of
1730 cm\(^{-1}\) and 1726 cm\(^{-1}\), which are due to the formation of carboxyl groups. The peaks in the range of 1650 cm\(^{-1}\) for all samples are related to the presence of C=O an amide (I) of proteins and the carboxyl functional groups. The peaks in 1549 cm\(^{-1}\), 1540 cm\(^{-1}\), 1537 cm\(^{-1}\), and 1535 cm\(^{-1}\) related to RCV, DCV, MDCV and MDCV/Fe\(_3\)O\(_4\) respectively, indicate the presence of amide (II) of proteins. Peaks in the region of 1400 cm\(^{-1}\) are associated with C-H bonds of lipids and proteins. The peaks in the region of 1260 cm\(^{-1}\) are related to C=O bonds in carbonyl amide (II) of proteins and P=O nucleic acid, phosphoryl, and phosphorylate groups. The intensities of these peaks are almost the same in all samples and indicate the preservation of these groups during the process. Also, wide peaks are observed in the range of 1200-900 cm\(^{-1}\), which is related to the C-O-C and C-O bonds present in carbohydrates and polysaccharides [37, 38]. Similar results have been reported by Kristina Maliutina et al. [39]. In RCV and DCV, peaks in 871 cm\(^{-1}\) and 875 cm\(^{-1}\) are observed respectively, which are related to the presence of CO\(_3\)\(^{2-}\) mineral compounds such as carbonates. In general, small peaks in the wavelength regions below 600 cm\(^{-1}\) are related to the tensile vibrations of organic and mineral halogen compounds such as KCl [40]. About the sample of MDCV/Fe\(_3\)O\(_4\), a wide peak is observed in the region of 571 cm\(^{-1}\), which indicates the Fe-O vibrations, so the magnetic property of this sample is confirmed [32].
Figure (2) - FT-IR analysis for a) RCV, b) DCV, c) MDCV, and d) MDCV/Fe$_3$O$_4$

**Scanning Electron Microscopy (SEM)**

The results of SEM analysis of RCV, DCV, MDCV, and MDCV/Fe$_3$O$_4$ can be seen on the scale of 1 $\mu$m and 200 nm in Fig. 3. As shown in Fig. 3a, the crystalline structure of the RCV is heterogeneous. According to Fig. 3b, no significant changes in algae structure occurred after lipid extraction. Chloroform and methanol can enter cell tissue without serious damages to the cell wall [41]. Based on Fig. 3c, phosphoric acid modification has caused severe structural changes. The crystalline structure of DCV has been transformed into an amorphous structure with a uniform and relatively homogeneous surface. Also, the particle size has increased compared to the previous ones. The formation of cavities and bulges on the surface of MDCV is an important factor in increasing the biosorption of dye pollutants [41, 42]. Fig. 3d shows the uniform coverage of the MDCV surface by iron oxide particles and the formation of a spherical crystalline structure [43].
Figure (3) - SEM analysis for a) RCV, b) DCV, c) MDCV, and d) MDCV/\text{Fe}_3\text{O}_4
Energy-Dispersive X-ray Spectroscopy (EDS)

The EDS analysis was performed to obtain the elements of RCV, DCV, MDCV, and MDCV/Fe$_3$O$_4$ that is shown in Fig. 4. Percentages of elements are reported in Table 5. The highest weight percentages in RCV and DCV are related to carbon and oxygen. Other elements such as nitrogen, sodium, magnesium, aluminum, calcium, phosphorus, sulfur, chlorine, potassium, and silicon are also found with a lower weight. According to the results, after magnetization, about 38% of the weight of MDCV/Fe$_3$O$_4$ is related to the iron elements. Therefore, the weight ratio of the MDCV: Fe$_3$O$_4$ (1:1) is almost confirmed.

Table (5) - Elemental composition of a) RCV, b) DCV, c) MDCV, and d) MDCV/Fe$_3$O$_4$

| Element | Weight percentage (%) |
|---------|-----------------------|
|         | RCV       | DCV       | MDCV      | MDCV/Fe$_3$O$_4$ |
| C       | 40.76     | 47.1      | 49.4      | 18.12           |
| N       | 10.65     | 13.38     | 17.83     | 7.41            |
| O       | 34.95     | 30.43     | 29.3      | 35.26           |
| Na      | 1.14      | 0.7       | 1.08      | 0.21            |
| Mg      | 0.67      | 0.65      | -         | -               |
| Al      | 0.26      | 0.31      | -         | -               |
| Si      | 1.02      | 0.54      | 1.29      | 0.71            |
| P       | 1.46      | 1.4       | -         | -               |
| S       | 0.62      | 0.67      | 0.53      | 0.25            |
| Cl      | 1.04      | 1.43      | 0.57      | 0.19            |
| K       | 0.63      | 0.68      | -         | -               |
| Ca      | 3.41      | 2.71      | -         | -               |
| Ni      | 3.39      | -         | -         | -               |
| Fe      | -         | -         | -         | 37.85           |
Figure (4) - EDS analysis for a) RCV, b) DCV, c) MDCV, and d) MDCV/Fe₃O₄
Brunauer–Emmett–Teller (BET)

The BET analysis was performed for RCV, DCV, MDCV, and MDCV/Fe$_3$O$_4$. Features such as specific surface area, average pore size, and porosity volume of samples were investigated in Table 6. The specific surface area of DCV and MDCV increased about 2 and 15 times, respectively, compared to RCV. The decrement in specific surface area of MDCV/Fe$_3$O$_4$ compared to its non-magnetic type was due to the coverage of some of its pores with iron particles. Nevertheless, the specific surface area of MDCV/Fe$_3$O$_4$ compared to RCV has increased about 11 times. Thus, the appropriate modification process is confirmed on *Chlorella vulgaris* algae to prepare an easily separable biosorbent while increasing its specific surface area [43]. Fig. 5 shows the nitrogen adsorption-desorption isotherm for the samples. According to the classification of the International Union of Pure and Applied Chemistry (IUPAC), adsorption isotherms are defined in four groups called H1, H2, H3, and H4. The adsorption isotherms of RCV, DCV and MDCV are in H3, and MDCV/Fe$_3$O$_4$ is in the H1 category. H1 represents materials with a porous structure that have spherical or cylindrical porous channels. H3 also represents materials with a soft and plate-like structure and porous slits [44, 45].

Based on the Barrett-Joyner-Halenda (BJH) diagram in Fig. 6, the structure of all samples contains micropores, mesopores, and macropores. While the largest distribution of the size of the pores belongs to the group of mesopores with a radius in the range of 2-25 nm [46].

| Sample                    | Specific surface area (m$^2$/g) | Average pore size (nm) | Porosity volume (cm$^3$/g) |
|---------------------------|---------------------------------|------------------------|---------------------------|
|                           | BET                | BJH       | Langmuir |                           |                          |
| RCV                       | 2.30               | 2.91      | 2.08     | 23.84                     | 0.013                     |
| DCV                       | 5.27               | 7.73      | 3.45     | 16.21                     | 0.021                     |
| MDCV                      | 34.95              | 36.2      | 43.89    | 4.38                      | 0.119                     |
| MDCV/Fe$_3$O$_4$           | 25.20              | 34.12     | 26.93    | 13.88                     | 0.087                     |
Figure (5) – Nitrogen adsorption-desorption isotherm for a) RCV, b) DCV, c) MDCV, and d) MDCV/Fe$_3$O$_4$
Figure (6) – BJH pore size distribution for a) RCV, b) DCV, c) MDCV, and d) MDCV/Fe$_3$O$_4$

Vibrating Sample Magnetometer (VSM)

Based on Fig. 7, the magnetization hysteresis loops of Fe$_3$O$_4$ and MDCV/Fe$_3$O$_4$ appeared S-like with no remanent magnetization and coercivity. It indicated the strong magnetic response to the different magnetic fields in paramagnetic materials. Therefore, they were easily separable by an external magnetic field and did not stick together when the magnetic field was removed [47]. The saturation magnetization of Fe$_3$O$_4$ and MDCV/Fe$_3$O$_4$ were 78.64 emu/g and 38.89 emu/g, respectively, which were suitable compared to the magnetic adsorbent derived from Chlorella
vulgaris reported by M. Govarthanan [48]. The decrease in MDCV/Fe₃O₄ magnetization relative to Fe₃O₄ was due to changes in particle size, irregular bonding of particles, and surface spin disorientation [49, 50].

Figure (7) – VSM analysis for a) MDCV/Fe₃O₄, b) Fe₃O₄

**Biosorption analysis**

Experimental results for biosorption analysis are reported in Table 7. Based on Equation 3, the multiple regression analysis of experimental data with 95% probability was used to study the effect of each parameter on the biosorption capacity of MDCV/Fe₃O₄, kinetics, isotherms, and thermodynamic models. Ineffective variables were removed to increase the suitability of the models with experimental data.
Table (7) - Experimental results of biosorption analysis from CCD proposed levels

| Run no. | $X_1$, T | $X_2$, t | $X_3$, m | $X_4$, $C_0$ | $X_5$, pH | $Y$, $q_{\text{MDCV/Fe}_3\text{O}_4}$ |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------------------------|
| 1       | -1              | -1              | -1              | -1              | 1               | 10.7565                          |
| 2       | 1               | -1              | -1              | -1              | -1              | 7.8790                           |
| 3       | -1              | 1               | -1              | -1              | -1              | 8.7164                           |
| 4       | 1               | 1               | -1              | -1              | 1               | 8.8619                           |
| 5       | -1              | -1              | 1               | -1              | -1              | 3.8118                           |
| 6       | 1               | -1              | 1               | -1              | 1               | 3.6523                           |
| 7       | -1              | 1               | 1               | -1              | 1               | 4.0791                           |
| 8       | 1               | 1               | 1               | -1              | -1              | 3.4669                           |
| 9       | -1              | -1              | -1              | 1               | -1              | 23.1100                          |
| 10      | 1               | -1              | -1              | 1               | 1               | 27.4232                          |
| 11      | -1              | 1               | -1              | 1               | 1               | 26.2010                          |
| 12      | 1               | 1               | -1              | 1               | -1              | 25.9489                          |
| 13      | -1              | -1              | 1               | 1               | 1               | 13.4475                          |
| 14      | 1               | -1              | 1               | 1               | -1              | 12.5616                          |
| 15      | -1              | 1               | 1               | 1               | -1              | 11.5723                          |
| 16      | 1               | 1               | 1               | 1               | 1               | 13.3958                          |
| 17      | -1              | 0               | 0               | 0               | 0               | 11.3094                          |
| 18      | 1               | 0               | 0               | 0               | 0               | 9.3598                           |
| 19      | 0               | -1              | 0               | 0               | 0               | 12.7255                          |
| 20      | 0               | 1               | 0               | 0               | 0               | 12.3278                          |
\[ Y = -3.72 - 0.0411X_1 - 0.2702X_2 - 0.1062X_3 + 0.0994X_4 + 5.72X_5 + 0.002240X_2^2 + 0.001158X_4^2 - 0.386X_5^2 + 0.000664X_1X_4 - 0.003177X_3X_4 \]  

(3)

The compatibility of the models with data is proven by the ANOVA results of recent regression. Based on Table 8, the calculated Fisher distributions is greater than its critical value. Also, the P-value is less than 0.000. The adjusted determination coefficient of model Y is 98.35% [51].

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 21 | 0 | 0 | -1 | 0 | 0 | 13.2300 |
| 22 | 0 | 0 | 1  | 0 | 0 | 6.0006  |
| 23 | 0 | 0 | 0  | -1| 0 | 6.1024  |
| 24 | 0 | 0 | 0  | 1 | 0 | 18.6266 |
| 25 | 0 | 0 | 0  | 0 | -1| 8.5650  |
| 26 | 0 | 0 | 0  | 0 | 1 | 9.3695  |
| 27 | 0 | 0 | 0  | 0 | 0 | 11.5809 |
| 28 | 0 | 0 | 0  | 0 | 0 | 11.5809 |
| 29 | 0 | 0 | 0  | 0 | 0 | 10.2133 |

Table (8) - ANOVA of the quadratic models of the MDCV/Fe\textsubscript{3}O\textsubscript{4} biosorption capacity

| Response source | df | SS   | MSS  | F\textsubscript{calc} | F\textsubscript{c} | P-value | R\textsubscript{adj} |
|-----------------|----|------|------|------------------------|------------------|---------|----------------|
| Regression      | 10 | 1207.00 | 120.70 | 168.20                 | 2.19             | 0.00    | 98.35%         |
| Error           | 18 | 12.92  | 0.718 | -                      | -                | -       | -              |
| Total           | 28 | 1219.92 | -     | -                      | -                | -       | -              |

According to Table 9, the P-values of all coefficients of the effective variables in Y analysis are less than 0.05. Since the coefficients of interactions of X\textsubscript{2}\textsuperscript{2} and X\textsubscript{1}X\textsubscript{4} are significant, X\textsubscript{1} and X\textsubscript{2} can not be ignored [51].
Table (9) - Estimated regression coefficients for the quadratic model of the MDCV/Fe$_3$O$_4$ biosorption capacity

| Terms  | Uncoded coefficient | The standard error of the coefficient | t-value | P-value |
|--------|---------------------|----------------------------------------|---------|---------|
| constant | -3.72                | 0.274                                  | 38.26   | 0.000   |
| $X_1$    | -0.0411              | 0.200                                  | -0.13   | 0.901   |
| $X_2$    | -0.2702              | 0.200                                  | -0.22   | 0.827   |
| $X_3$    | -0.1062              | 0.200                                  | -22.30  | 0.000   |
| $X_4$    | 0.0994               | 0.200                                  | 31.99   | 0.000   |
| $X_5$    | 5.72                 | 0.200                                  | 3.22    | 0.005   |
| $X_2^2$  | 0.002240             | 0.504                                  | 4.00    | 0.001   |
| $X_4^2$  | 0.001158             | 0.504                                  | 3.68    | 0.002   |
| $X_5^2$  | -0.386               | 0.504                                  | -3.06   | 0.007   |
| $X_1X_4$ | 0.000664             | 0.212                                  | 2.51    | 0.022   |
| $X_3X_4$ | -0.003177            | 0.212                                  | -9.00   | 0.000   |

**Effect of contact time**

The biosorption capacity of MDCV/Fe$_3$O$_4$ was plotted in Fig. 8a. The biosorption capacity is zero at the beginning of the process. It reaches the maximum levels during the first five minutes. Then, it decreases over time and reaches equilibrium mode. This phenomenon is called repulsion. It occurs when large biosorption surface areas are available and rapidly covered by MB molecules in the first few minutes [52-54]. Eventually, the system reaches equilibrium after a while of biosorption and desorption. In the first minutes, the higher MB concentration, due to the high driving forces of mass transfer, increase the biosorption capacity. So the highest MB removal efficiency appears in the first minutes before the time of equilibrium. After reaching equilibrium, the removal efficiency of MB by MDCV/Fe$_3$O$_4$ at initial concentrations of 20, 60, and 100 mg/L are about 63%, 37% and 37%, respectively.
Effect of pH

The pH of the solution significantly affects the relationship between biosorbent and adsorbate. On the other hand, the interactions of hydrogen ions, Van der Waals forces, and the propagation of cavities, also affect the biosorption rate. As can be seen in Fig. 8b, the amounts of biosorption capacity in pH 3 to 7 increased from 17.6 to 25 mg/g and then it decreased to 13 mg/g as the pH increased to 9. The cell wall of *Chlorella vulgaris* contains a large number of functional groups, carboxylic acid. Therefore, at low pH, cell wall ligands are protonated and the $H^+$ also increases. As a result, the biosorption capacity decreased due to the competition between $H^+$ and cationic molecules of MB. As the pH increased and $OH^-$ formed, more ligands, such as the amino and carboxyl functional groups, were exposed to the attraction between $OH^-$ and MB cationic molecules. The competition between $H^+$ and MB also decreased, so the biosorption capacity of MDCV/Fe$_3$O$_4$ increased [55, 56]. At higher pHs, the presence of ions, MB cationic molecules, and the interactions of charges increased the forces between MB monomer molecules. This phenomenon had led to the formation of MB dimer molecules, which were probably larger than the size of surface holes. This difference in sizes reduced the number of active surface cavities and caused less biosorption capacity [57].

Effect of biosorbent dosage

Based on Fig. 8c, the development of biosorbent amount increases the removal efficiency of MB due to the increment of active sites for dye biosorption. While the reduction in biosorption capacity by increasing the amount of biosorbent is due to the gradient of the biosorbent and adsorbate concentration. The factors influencing biosorption are investigated based on the biosorbent mass unit. As a result, phenomena such as aggregation and condensation cause many active levels to remain unused, thereby reducing biosorption capacity. It is observed that the biosorption capacity and removal efficiency of MB in the biosorbent amounts of 15 to 45 mg has changed from 25 mg/g and 37% to 12 mg/g and 55%, respectively [58, 59].

Effect of the initial concentration of MB

Changes in the biosorption capacity and removal efficiency of MB are calculated according to the changes in the initial concentration of MB and reported in Fig. 8d. By changing the initial concentration of MB from 20 to 100 mg/L, the biosorption capacity has increased from 8.5 to 25
mg/g, due to mass transfer-driving forces. As mentioned in the previous section, the removal efficiency of MB is directly related to the number of active sites of the biosorbent. Therefore, at a constant biosorbent dosage, with increasing the initial concentration of MB, the active sites of the biosorbent decrease and lead to the reduction in removal efficiency from 63% to 37% [60].

Effect of temperature

As mentioned in the ANOVA analysis section, the temperature does not significantly affect the MDCV/Fe$_3$O$_4$ biosorption capacity. According to Fig. 8e, with increasing temperature from 5 to 45℃, the biosorption capacity of MDCV/Fe$_3$O$_4$ with initial MB concentration of 60 mg/L did not change much and with initial MB concentrations of 20 mg/L and 100 mg/L was associated with a decrease of 12% and an increase of 4%, respectively. Similar results were observed in recent research by Konig-Peter Aniko et al. [61]. These differences in the behavior of MDCV/Fe$_3$O$_4$ biosorption capacity relative to temperature indicates that the biosorption process is entropically rather than enthalpically driven [62].
Figure (8) - Effect of a) contact time (T=25°C, m=15 mg, pH=7), b) pH (T=25°C, t=60 min, m=15 mg, C0=100 mg/L), c) biosorbent dosage (T=25°C, t= 60 min, C0=100 mg/L, pH=7), d) initial concentration of MB (T=25°C, t=60 min, m=15 mg, pH=7), and e) temperature (t= 60 min, m=15 mg, pH=7) on biosorption of MB by MDCV/Fe₂O₄
**Biosorption kinetics**

Kinetic trends and their parameters are reported in Fig. 9 and Table 10. Due to the downward trend in biosorption capacity over time, the pseudo-first-order model cannot be examined [63]. The determination coefficients of the pseudo-second-order model in all initial concentrations of MB are greater than 0.99. Also, the equilibrium biosorption capacities calculated in this model are very close to the experimental amounts. Therefore, this model is a good predictor of the kinetics of MB biosorption on the MDCV/Fe₃O₄ and more compatible than the intraparticle diffusion model [63]. Based on the intraparticle diffusion model, increasing the value of surface biosorption (intercept, C) with increasing temperature indicates a decrease in the rate of diffusion of MB from the external surface to the internal surfaces of biosorbent [64].

![Figure (9) - Plots of a) pseudo-second-order, b) intraparticle diffusion kinetic models for MB biosorption onto MDCV/Fe₃O₄ (T=25°C, m=15mg, pH=7)](image-url)
Table (10) - Kinetic parameters for MB biosorption onto MDCV/\(Fe_3O_4\)

| Parameter   | Concentration (mg/L) |
|-------------|-----------------------|
|             | 20        | 60        | 100       |
| \(q_e,\text{exp} (\text{mg}\cdot\text{g}^{-1})\) | 8.46      | 14.90     | 25.05     |
| \(t_e (\text{min})\)             | 60        | 60        | 60        |
| Pseudo-second-order          |           |           |           |
| \(q_e,\text{cal} (\text{mg}\cdot\text{g}^{-1})\) | 7.90      | 14.20     | 24.21     |
| \(k_2 (\text{g}\cdot\text{mg}^{-1}\cdot\text{min}^{-1})\) | -0.0234   | -0.0193   | -0.0170   |
| \(R^2\)                     | 0.99      | 0.99      | 0.99      |
| Intraparticle diffusion      |           |           |           |
| \(k_{id} (\text{mg}\cdot\text{g}^{-1}\cdot\text{min}^{0.5})\) | -1.2947   | -1.2947   | -1.2947   |
| \(C (\text{mg}\cdot\text{g}^{-1})\)          | 17.952    | 24.391    | 34.536    |
| \(R^2\)                     | 0.98      | 0.98      | 0.98      |

**Biosorption isotherms**

Comparing the results of Fig. 10 and Table 11, Freundlich isotherm has the highest compatibility with data compared to Langmuir and Temkin. The equilibrium parameters of the Langmuir isotherm \((R_L)\) are in the range of \(0 < R_L < 1\). Also, in the Freundlich isotherm, the values of \(1/n\) are in the range of \(0 < 1/n < 1\). So, the biosorption of MB onto MDCV/\(Fe_3O_4\) is desirable [65, 66]. The values of \(K_f\) also indicate a growth in biosorption capacity with decreasing temperature. The decreasing of \(b_t\) with increasing temperature in Temkin isotherm, indicates an exothermic biosorption [67].
Figure (10) - Plots of a) Langmuir, b) Freundlich, c) Temkin isotherm models for MB biosorption onto MDCV/Fe₃O₄
### Table (11) - Isotherm parameters for MB biosorption onto MDCV/Fe<sub>3</sub>O<sub>4</sub>

| Parameter | Temperature (°C) | 5 | 25 | 45 |
|-----------|------------------|---|----|----|
| Langmuir  |                  |   |    |    |
| K<sub>L</sub> (L mg<sup>-1</sup>) | 0.014 | 0.010 | 0.007 |
| q<sub>m</sub> (mg g<sup>-1</sup>) | 46.08 | 56.50 | 75.18 |
| R<sub>L</sub> | 0.40-0.69 | 0.48-0.75 | 0.58-0.82 |
| R<sup>2</sup> | 0.74 | 0.67 | 0.55 |
| Freundlich |                  |   |    |    |
| K<sub>F</sub> ((mg g<sup>-1</sup>)(mg L<sup>-1</sup>)<sup>1/n</sup>) | 1.79 | 1.38 | 1.05 |
| n | 1.65 | 1.48 | 1.33 |
| R<sup>2</sup> | 0.94 | 0.95 | 0.96 |
| Temkin    |                  |   |    |    |
| b<sub>t</sub> (J g mol<sup>-1</sup>, mg<sup>-1</sup>) | 242.78 | 234.72 | 226.74 |
| K<sub>T</sub> (L mg<sup>-1</sup>) | 0.15 | 0.13 | 0.11 |
| R<sup>2</sup> | 0.87 | 0.88 | 0.88 |

**Biosorption thermodynamic**

The Van’t Hoff plot of ln K<sub>c</sub> versus 1/T is shown in Fig. 11. The calculated thermodynamic parameters are reported in Table 12. Positive values of ΔG indicate the non-spontaneous biosorption of MB onto MDCV/Fe<sub>3</sub>O<sub>4</sub>, which grows with increasing temperature [50]. The result of changes in the energy of the biosorption and desorption has led to a negative amount of ΔH. As a result, the biosorption process of MB on MDCV/Fe<sub>3</sub>O<sub>4</sub> is exothermic [68]. Based on previous studies and considering the enthalpy value (-46.56 kJ/mol), the biosorption process of MB onto MDCV/Fe<sub>3</sub>O<sub>4</sub>, is considered as almost physical biosorption [69, 70]. The negative value of ΔS
also indicates a reduction in randomness and irregularity of MB particles when they are placed on the active sites of biosorbent [71].

![Van't Hoff diagram for the estimation of thermodynamic parameters](image)

**Figure (11)** - Van't Hoff diagram for the estimation of thermodynamic parameters

**Table (12) – Thermodynamic parameters for MB biosorption onto MDCV/Fe₃O₄**

| Parameter       | Temperature (K) | R²  |
|-----------------|-----------------|-----|
|                 | 278             | 298 | 318 |
| K_c             | 0.4167          | 0.1230 | 0.0328 |
| ΔH (kJ.mol⁻¹)   | -46.56          |     |     |
| ΔS (kJ.mol⁻¹.K⁻¹) | -0.17          |     |     |
| ΔG (kJ.mol⁻¹)   | 1.92            | 5.41 | 8.90 |

**Comparison of MDCV/Fe₃O₄ with other biosorbents**

The maximum biosorption capacity of the biosorbents depends on the modification method and the type of dyes. The maximum biosorption capacity of MDCV/Fe₃O₄ is 32.44 mg/g at temperature (45°C), contact time (5 min), biosorbent dosage (15 mg), initial concentration of MB (100 mg/L), and pH (7). Comparison of MDCV/Fe₃O₄ with other biosorbents for MB biosorption was
performed in Table 13. The results show that the maximum biosorption capacity of MDCV/Fe$_3$O$_4$ is good. Although this amount is lower than some biosorbents such as biochars and their activated carbons, it is promising and even higher compared to some green, red, and brown algae with similar modifications. On the other hand, few studies have been done on the modified dry Chlorella vulgaris. Therefore, this study offers a useful approach in MB biosorption with maximum removal efficiency during the shortest time.

| Biosorbent                  | Modification                                | Maximum biosorption capacity (mg/g) | Ref. |
|-----------------------------|---------------------------------------------|-------------------------------------|------|
| Enteromorpha spp.           | -                                           | 274                                 | [72] |
| Nizamuddinia zanardinii     | -                                           | 863.4                               | [73] |
| Magnetic wakame biochar     | Magnetization Loading nickel on biochar      | 479.49                              | [74] |
| Ultrasonicated activated carbon of empty fruit bunch | Ultrasonication                           | 435                                 | [71] |
| Activated carbon of Empty Fruit Bunch | -                                           | 400                                 | [71] |
| Aegagropila Linnaei         | -                                           | 250                                 | [75] |
| Chemical modified Aegagropila Linnaei | Extracted functionalized cellulose | 100                                 | [75] |
| EDTA-modified bentonite     | Modification with EDTA                      | 160                                 | [76] |
| Gracilaria parvispora       | -                                           | 83.08                               | [73] |
| Palm leaflets               | -                                           | 72.3                                | [77] |
| Palm frond base             | -                                           | 70.87                               | [77] |
| Cystoseira barbatula        | -                                           | 38.61                               | [78] |
| Raw wet Chlorella vulgaris  | -                                           | 10.142                              | [79] |
Annona squamosa seed: Sulfuric acid activation - 8.52 [80]

S. dimorphus: - 6 [81]

Defatted algal biomass of S. dimorphus (DAB): Lipid extraction - 7.73 [81]

Sulfuric acid pretreated DAB: Sulfuric acid pretreatment - 7.80 [81]

Raw posidonia oceanica fibres: - 5.56 [82]

Caulerpa racemosa var. cylindracea: - 5.23 [83]

This work: Lipid extraction - 32.44 [83]

Phosphoric acid modification

Magnetization

**Conclusion**

In this work, *Chlorella vulgaris* algae was examined to remove MB from wastewater. This study showed that lipid extraction could provide a good opportunity to use algae residues after the esterification process as an economic biosorbent. Optimization of the variables in the phosphoric acid modification process ensured the maximization of the biosorption capacity and residual defatted algae. Finally, the modified defatted algae were magnetized for easy and low-cost separation of the biosorbent. The specific surface area of the magnetic modified defatted algae (MDCV/Fe$_3$O$_4$) reached 25.2 m$^2$/g. So a novel magnetic biosorbent was considered to remove MB. Parameters affecting the biosorption were investigated to increase the biosorption capacity of MDCV/Fe$_3$O$_4$. The pseudo-second-order kinetic model was well matched to the data. Equilibrium data were well described by the Freundlich model. MB biosorption on the selected biosorbent was non-spontaneous and exothermic. This process was associated with decreasing irregularity and randomness at the interface of dye and biosorbent. This study proved that *Chlorella vulgaris* can be used as a cheap and available source to remove the MB dye pollutant.
Methods

Materials

*Chlorella vulgaris* algae were obtained from the Biotechnology Laboratory, Faculty of Chemical Engineering, University of Tehran, Tehran, Iran. Chloroform (CHCl₃, 98%), methanol (CH₃OH, 99.5%), ortho-phosphoric acid (H₃PO₄, 85%) and sodium carbonate anhydrous (Na₂CO₃, 99.9%) were purchased for extraction of lipids and acid treatment from Merck. For magnetization, iron (II) sulfate heptahydrate (FeSO₄·7H₂O, 99%), iron (III) chloride hexahydrate (FeCl₃·6H₂O, 99%), and ammonia (NH₄OH, 25wt%) was purchased from Merck. Methylene blue Powder (C₁₆H₁₈ClN₃S, ≥82%), sodium hydroxide (NaOH, 98%), and hydrochloric acid (HCl, 37%) were purchased from Merck for the biosorption process. All chemicals are in the analytical grade.

**Biosorbent preparation**

Raw *Chlorella vulgaris* algae (RCV) was first washed several times with distilled water to remove impurities and dried at 45°C for 24 hr. Then it was powdered with mortar.

**Lipid extraction**

Lipid extraction of RCV was performed by ultrasonic cell destruction and Soxhlet extraction setup [84]. First, 10 g of RCV was mixed with 10 mL of methanol. Then it was sonicated with the frequency of 28 kHz at room temperature for 40 minutes. The sonicated RCV was then transferred to a cartridge and placed inside the Soxhlet setup. As a solvent mixture, 285 mL of chloroform:methanol (1:2 v/v) was poured into the flask according to Bligh & Dyer method [85]. Extraction was carried out for 24 hr at 65°C. The defatted *Chlorella vulgaris* (DCV) was washed several times with distilled water to remove the remaining solvent and dried at 50°C for 24 hr. Dry DCV was powdered with a mortar and used for the next steps.

**Phosphoric acid treatment and optimization of parameters**

DCV was exposed to a dilute phosphoric acid solution under specified conditions. The central composite design (CCD) was used by the response surface methodology (RSM) to design experiments. Coded & uncoded levels of variables such as the reaction temperature (X₁), the contact time (X₂), the concentration of phosphoric acid (X₃), the ratio of acid solution volume to
DCV ($X_4$), MDCV biosorption capacity ($Y_1$), and residual MDCV ($Y_2$), determined by the design of experiments, are shown in Table 14. The number of 27 experiments including 8 axial points ($\alpha = 1$) and 3 central points were considered in CCD. About 0.1 g of DCV was treated under each designed experimental conditions. Finally, MDCV was filtered and neutralized with deionized water and 0.1 M sodium carbonate. It was then dried at 50$^\circ$C for 24 hr and crushed into fine particles. The MDCV biosorption capacities and the residual MDCV were considered as experimental design responses. The residual of MDCV was calculated using Equation 4:

$$Residual \ MDCV \ (%) = \frac{m_2 - m_1}{m_1} \times 100 \tag{4}$$

Where $m_1$ and $m_2$ are the mass of DCV (mg) before and after acid treatment, respectively.

Table (14) - Levels of coded and uncoded variables for the MDCV biosorption capacity and residual MDCV

| Variable | Name                                      | Range of levels |
|----------|-------------------------------------------|-----------------|
| $X_1$    | The temperature of the reaction, T, °C    | 30  50  70      |
| $X_2$    | The contact time of DCV with acid, t, hr  | 3   6   9       |
| $X_3$    | The concentration of acid, $C_A$, mol/L   | 2   4   6       |
| $X_4$    | The ratio of acid volume to DCV, $V/W$, mL/g | 30  50  70      |
| $Y_1$    | MDCV biosorption capacity, $q_{MDCV}$, mg/g |                |
| $Y_2$    | Residual MDCV, $R_{P_{MDCV}}$, %         |                |

Considering the last responses, the regression, and the variance analysis (ANOVA) were done using Minitab-18 software. Optimal conditions were calculated in the maximum amount of both responses and DCV was modified in those conditions [33].

**MDCV/Fe$_3$O$_4$ preparation**

Synthesis of MDCV/Fe$_3$O$_4$ particles were performed by the co-precipitation method [86, 87]. To do that, about 0.1 g of MCDV was stirred with 100 mL of deionized water for 5 minutes and then sonicated with the frequency of 28 kHz at room temperature for 30 minutes. Sonicated solution was added to 100 mL of an aqueous solution containing FeSO$_4$.7H$_2$O and FeCl$_3$.6H$_2$O with a 1:2 M ratio. The mixture was stirred under a nitrogen atmosphere at 85$^\circ$C and 250 rpm. After 15
minutes, about 10 mL of ammonia was added dropwise to the system for setting the pH about 9. The reaction continued for an hour. The MDCV/Fe$_3$O$_4$ particles were separated using an external magnet and washed several times with deionized water to reach pH 7. Finally, MDCV/Fe$_3$O$_4$ particles were dried at 50℃ for 24 hr and then powdered by mortar [32, 88].

**Biosorption analysis**

The biosorption of MB on MDCV/Fe$_3$O$_4$ was performed in a batch using an incubator at 200 rpm. The effect of variables such as temperature ($X_1$), contact time ($X_2$), biosorbent dosage ($X_3$), initial concentration of MB ($X_4$), and pH ($X_5$) were investigated. NaOH and HCl 0.1M were used to regulate the pH. The experiments were performed using the CCD by RSM involving 29 runs with 3 central points and 10 axial points ($\alpha = 1$). The coded and uncoded conditions specified by the experimental design are shown in Table 15.

| Variable | Name | Range of levels |
|----------|------|-----------------|
| $X_1$    | Temperature, T, °C | 5 25 45 |
| $X_2$    | Contact time, t, min | 30 60 90 |
| $X_3$    | Biosorbent dosage, m, mg | 15 30 45 |
| $X_4$    | Initial concentration of MB, $C_0$, mg/L | 20 60 100 |
| $X_5$    | pH | 5 7 9 |

| $Y$ | biosorption capacity of MDCV/Fe$_3$O$_4$, $q_{MDCV/Fe_3O_4}$, mg/L |

The final concentration of MB was obtained using a UV-Visible spectrophotometer at $\lambda_{max} = 665$ nm [89]. Biosorption capacity (mg/g) and removal percentage (%) were calculated based on the following Equations:

$$q_t = \frac{(C_0-C_t)\times V}{m}$$

(5)

$$R = \frac{(C_0-C_t)\times 100}{C_0}$$

(6)
Where $C_0$ and $C_t$ are the initial and final concentrations of MB (mg/L), respectively. $V$ is the volume of the dye pollutant (L) and $m$ is the biosorbent dosage (g).

Regression, and variance analysis (ANOVA) of the biosorption capacity of MDCV/Fe$_3$O$_4$ were done using Minitab-18 software.

**Statistical analysis**

The design of the experiments was done by using the central compound design (CCD) of the response surface methodology (RSM). The responses were considered based on the quadratic functions with the interaction of the variables affecting them according to Equation 7. Also, the levels of variables are based on Equation 8 [90].

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_i x_i^2 + \sum_{1 \leq i < j} \beta_{ij} x_i x_j + \varepsilon$$

(7)

$$x_i = \left(\frac{z_i - z_i^0}{\Delta z_i}\right) \beta_d$$

(8)

Where $\beta_i$ shows the coefficients of the parameters of the quadratic function, $z_i$ and $x_i$ are real and coded values, respectively. $\Delta z_i$ is the distance between the value of the center point and the next or previous levels. $\beta_d$ is the major coded value for each variable. $z_i^0$ is the real value of the center point.

The relation between the total sum of the square ($SS_{Total}$), the sum of the square due to regression ($SS_{Reg}$), and errors ($SS_{Error}$) is based on Equation 9. The equations for calculating the sum of squares and the mean sum of squares of the data are shown in Table 16. The matching of the regression model and experimental data is determined by using the calculated Fisher distribution ($F_{calc}$), the critical Fisher distribution ($F_c$), the probability value (P-value), and the adjusted determination coefficient ($R^2_{adj}$), as shown in Equations 10-13 [90]. The statistical calculations are also based on the probability of 95% ($\alpha = 0.05$).

$$SS_{Tot} = SS_{Reg} + SS_{Error}$$

(9)

$$F_{calc} = \frac{MSS_{Reg}}{MSS_{Error}}$$

(10)

$$F_c = F_{\alpha, df_{Model}, df_{Error}}$$

(11)
\[ P - value = 2 \times \int_{F_c}^{\infty} f(F) df \]  
(12)

\[ R^2_{adj} = 1 - \frac{SS_{Error}/(n-p)}{SS_{Total}/(n-1)} \]  
(13)

Where \( n \) and \( p \) are the numbers of observations and parameters of the model, respectively.

Table (16) - Analysis of variance for a fitted mathematical model to an experimental data using multiple regression [90]

| Variation source | Sum of the square | Degree of freedom | Mean sum of the square |
|------------------|-------------------|------------------|-----------------------|
| Regression       | \( SS_{Reg} = \sum_i \sum_j (\hat{y}_{ij} - \bar{y})^2 \) | p-1              | \( MSS_{Reg} = \frac{SS_{Reg}}{p-1} \) |
| Error            | \( SS_{Error} = \sum_i \sum_j (y_{ij} - \bar{y}_{i})^2 \) | n-p              | \( MSS_{Error} = \frac{SS_{Error}}{n-p} \) |
| Total            | \( SS_{Total} = \sum_i \sum_j (y_{ij} - \bar{y})^2 \) | n-1              | -                     |

\( n \): Number of observations, \( p \): Number of the parameters of the model, \( \hat{y}_{ij} \): Estimated value for the level \( i \) by the model, \( \bar{y} \): Mean of overall data, \( y_{ij} \): Replication in each level, \( \bar{y}_{i} \): Mean of replicated data in the same conditions

**Biosorption kinetics**

Kinetic studies were performed to evaluate the reaction rate, equilibrium time, equilibrium capacity, and biosorption process behavior. For this purpose, the models such as pseudo-second-order and intraparticle diffusion were used according to Equations 19-20, which are shown in Table 17.

Table (17) - Kinetic models using in this study

| Model             | Equation                  | Plots                  | Parameters                        | Ref. |
|-------------------|---------------------------|------------------------|-----------------------------------|------|
| Pseudo-second-order | \[ \frac{t}{q_t} = \frac{1}{k_2q_e^*} + \frac{t}{q_e} \] (19) | \( \frac{t}{q_t} \) vs. \( t \) | Calculating \( q_e \) and \( k_2 \) through the slope and intercept | [91] |
| Intraparticle diffusion | \( q_t = k_{id}t^{0.5} + C \) (20) | \( q_t \) vs. \( t^{0.5} \) | Calculating \( k_{id} \) and \( C \) through the slope and intercept | [92] |

**Biosorption isotherms**
The trend of equilibrium biosorption capacity \( (q_e) \) according to the equilibrium concentration of MB \( (C_e) \) is investigated using biosorption equilibrium isotherms. The datasets were calculated in different initial concentrations of MB at constant temperatures over time. In each step, \( q_e \) was calculated using the biosorption capacity of MDCV/Fe\(_3\)O\(_4\). Then \( C_e \) was calculated using \( C_0 \) and \( q_e \) at each run. Langmuir, Freundlich, and Temkin isotherms were examined using Equations 21-24 reported in Table 18 [67, 93, 94].

**Table (18) - Isotherm models using in this study**

| Model   | Equation                                                                 | Plots                        | Parameters                                      | Ref. |
|---------|--------------------------------------------------------------------------|-------------------------------|-------------------------------------------------|------|
| Langmuir| \( \frac{C_e}{q_e} = \frac{1}{K_L q_m} + \frac{C_e}{q_m} \) (21)        | \( C_e \) vs. \( C_e \)      | Calculating \( q_m \) and \( K_L \) through the slope and intercept | [95] |
|          | \( R_L = \frac{1}{1+K_L C_0} \) (22)                                    | \( R_L \) vs. \( C_0 \)       | \( R_L \)                                       | [92] |
| Freundlich| \( \log q_e = \log K_F + \frac{1}{n} \log C_e \) (23)                   | \( \log q_e \) vs. \( \log C_e \) | Calculating \( n \) and \( K_F \) through the slope and intercept | [96] |
| Temkin  | \( q_e = \frac{RT}{b_T} \ln(K_T) + \frac{RT}{b_T} \ln(C_e) \) (24)     | \( q_e \) vs. \( \ln(C_e) \)  | Calculating \( b_T \) and \( K_T \) through the slope and intercept | [97] |

**Biosorption thermodynamics**

The thermodynamics of biosorption of MB on MDCV/Fe\(_3\)O\(_4\) were studied at temperatures of 278, 298, and 317 K. Gibbs free energy change \( (\Delta G) \), enthalpy change \( (\Delta H) \), and entropy change \( (\Delta S) \) were calculated according to the following Equations:

\[
\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ
\] (25)

\[
\Delta G^\circ = -RT \ln(K_c)
\] (26)

\[
\ln(K_c) = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT}
\] (27)

\[
K_c = \frac{K_F \rho}{1000} \left( \frac{10^6}{\rho} \right) (1-\frac{1}{n})
\] (28)
In the above equations, \( R \) is the gas constant (J/mol.K), \( T \) is the temperature of the process (K), \( \rho \) is water density (g/mL), \( K_F \) is the Freundlich constant \((\text{mg/g})(\text{mg/L})^{1/n}\), \( n \) is the Freundlich constant too, and \( K_c \) is the equilibrium constant. Equation 27 refers to the Van't Hoff plot of \( \ln K_c \) versus \( 1/T \) [98, 99].

**Biosorbent characterization**

Several analyses were performed to determine the morphology and specifications of four samples of RCV, DCV, MDCV, and MDCV/Fe\(_3\)O\(_4\). The X-ray Diffraction Patterns (XRD) was done by PW1730, PHILIPS, Netherland with Cu-ka radiation and wavelength of 1.54056 Å, at 40 kV in the range of \( 10 - 80^\circ \) with a scanning speed of 0.05°/s. Fourier Transform Infrared Spectroscopy (FTIR) AVATAR, Thermo, USA was used in the range of 400 to 4000 cm\(^{-1}\) to identify functional groups. Phase identification, percentage of elements, and morphology of samples were performed using Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) analyses by MIRA III, TESCAN, Czech Republic. Brunauer-Emmett-Teller (BET) analysis was performed using the liquid nitrogen adsorption-desorption process at 77 K by BELSORP MINI II, BEL, Japan to determine the porosity of the structure. Vibrating Sample Magnetometer (VSM) analysis by Meghnatis Daghhigh Kavir Co., Iran at -15000 to 15000 Oe was used to study magnetic properties. The UV-Visible spectrophotometer was also used to determine the MB concentration.

**Abbreviations**

RCV: Raw *Chlorella vulgaris*, DCV: Defatted *Chlorella vulgaris*, MDCV: Modified defatted *Chlorella vulgaris*, MDCV/Fe\(_3\)O\(_4\): Magnetic modified defatted *Chlorella vulgaris*, Fe\(_3\)O\(_4\): Iron oxide particles, MB: Methylene blue, CCD: Central composite design, RSM: Response surface methodology, \( q_{MDCV} \): MDCV biosorption capacity, \( R_{MDCV} \): Residual MDCV, \( q_{MDCV/Fe_3O_4} \): Biosorption capacity of MDCV/Fe\(_3\)O\(_4\), \( q_t \): Biosorption capacity, \( C_0 \): Initial concentrations of MB, \( C_t \): Final concentrations of MB, ANOVA: Analysis of variance, \( SS_{Total} \): Total sum of the square, \( SS_{Reg} \): Sum of the square of regression, \( SS_{Error} \): Sum of the square of error, \( F_{calc} \): Calculated Fisher distribution, \( F_c \): Critical Fisher distribution, P-value: Probability value, \( R^2_{adj} \): Adjusted determination coefficient, \( q_e \): Equilibrium biosorption capacity, \( C_e \): Equilibrium concentration of MB, \( \Delta G \): Gibbs free energy change, \( \Delta H \): Enthalpy change, \( \Delta S \): Entropy change, XRD: X-ray Diffraction Patterns, FTIR: Fourier Transform Infrared Spectroscopy, SEM: Scanning Electron Microscopy, EDS: Energy-Dispersive X-ray Spectroscopy, BET: Brunauer-Emmett-Teller, VSM: Vibrating Sample Magnetometer, BJH: Barrett-Joyner-Halenda, IUPAC: International Union of Pure and Applied Chemistry.
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Authors' contributions
Naziri S. wrote the main manuscript text and was involved in all tests directly. Salehi Z. was a chemical engineering supervisor of the research. She also edited the manuscript, sponsored the study, and made all the arrangements.

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