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Research Article

Characterization of the aluminum-resistant microalgae by screening industrial wastewater microorganisms

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Abstract

Aluminum (Al) is a major concern in acidic environments as it can lead to the accumulation of reactive oxygen species (ROS), which induce oxidative stress in the host. Assessment of Al-resistant microorganisms can help scientists to discover their mechanisms and improve bioremediation techniques. The present study aimed to characterize Al-resistant microalgae by screening industrial wastewater microorganisms. The microalgae were treated with 0, 10, and 100 µM Al. Then, H_2O_2, malondialdehyde (MDA), catalase (CAT), and peroxidase (POX) values were measured. In addition, the effects of time (30-300 min), Al concentration (0-370 µM), and pH (4.0-6.5) on Al removal were investigated using the design-expert software. The efficiency of various biosorbents in Al removal was also evaluated in the optimal conditions of the final experiment. According to the results, *Scenedesmus* sp. was the most resistant microalgae and produced more biomass at 100 µM. Moreover, the POX and CAT activities of *Scenedesmus* sp. were increased by the high Al concentrations. In optimum conditions (81.60 µM Al, pH 5.8, 45 minutes), free cells (without modifications) were effective in Al biosorption (93.56%).

Keywords: Aluminum, Antioxidant, Bioremediation, *Scenedesmus*, Oxidative stress

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Introduction

Rapid urbanization and industrialization have led to water resource contamination with significant amounts of heavy metals and chemical synthetics (Kumar *et al.*, 2015b; Nasoudari *et al.*, 2021). Although Al is not a major environmental concern, it is founded in solutions that interferes with other metals' uptake (Lee *et al.*, 2004). Therefore, Al should be considered an important abiotic stress at high concentrations and low pH. Al limits plant growth and production (Huynh *et al.*, 2012) and exerts toxic effects on the central nervous system and the skeletal and hematopoietic systems of humans and animals (Yokel and McNamara, 2001). Food is the primary source of Al, while this element is also found in drinking water. Industrial and medicinal exposure also significantly enhances Al absorption. There are different forms of Al depending on pH values. At low pH, Al causes more toxicity in biological systems through interaction with oxygen donor ligands such as polysaccharides, proteins, and nucleic acids (Mossor-Pietraszewska, 2001). Al^{3+} is the most toxic form of Al, which inhibits cell development and transportation (Mossor-Pietraszewska, 2001) and can be replaced by heme groups (Singh *et al.*, 2009; Auger *et al.*, 2013). Moreover, Al^{3+} attacks to the plasma membrane and binds to its phospholipids, thereby leading to the alteration of permeability and electrochemical potential, interference of membrane transporters, and inhibition of H-ATPase activity (Ma, 2007). Al also induces ROS production (Yamamoto *et al.*, 2002) and membrane lipid peroxidation (Meriga *et al.*, 2004; Ameri *et al.*, 2020a).

Microalgae are the first producers in aquatic environments, which could enrich heavy metal ions, degrade synthetic substances, nutrient, and act as biosorbents in wastewaters (Kumar *et al.*, 2015b; Nasoudari *et al.*, 2021; Bonyadi *et al*., 2022; Sarkheil *et al*., 2022). Dead and fresh algal biomass can absorb heavy metals, while the last one acts quicker within the first few hours (Kumar *et al.*, 2015b). Microalgae have developed passive and active absorption mechanisms to cope with heavy metals and reduce their toxicity. In passive absorption, the functional groups localized on algal cell walls act within the first hours of implementation, while in active transportation, heavy metals accumulate inside the live cells in different cellular compartments for hours (Abdel-Raouf *et al.*, 2012; Suresh Kumar *et al.*, 2015).

Among various plant species, the main mechanism controlling Al stress is described as extracellular metal ion chelations by malate, oxalate, and citrate secretion as intrinsic and major defense mechanisms (Mossor-Pietraszewska, 2001). Obviously, the genotypes of specific organisms have evolved their mechanisms over time, thereby conferring Al resistance. Data are scarce regarding microalgae resistance mechanisms, and the role of antioxidants remains unclear as well. In contact with heavy metals, algal redox homeostasis should remain balanced through the function of several enzymatic and non-

enzymatic antioxidants (Choudhary *et al.*, 2007).

The present study aimed to investigate the enzymatic antioxidant responses and absorption of resistant microalgae in Al exposure.

Materials and methods

Microalgae Sampling and Collection

Al contamination in industrial areas, especially in the regions that are close to tannery factories is important (Haydar and Aziz, 2009). These sites are hazardous to the consumers of local irrigated crop plants. The soil analysis of Charmshahr industrial area in Mashhad, Iran shows a high content of Al (Table 1). In this study, wastewater and soil samples were collected from the Charmshahr (36°13'28.3"N 59°55'00.8"E), and algae purification was performed using plate agar culture with a general algae media including blue-green (BG_{11}) (Allen and Stanier, 1968) and bold basal medium (BBM) (Bold, 1949; Andersen, 2005).

Table 1: Soil analysis from the industrial area (Charmshahr), Mashhad, Iran

Elements	Al	Pb	As	Mo	Ni	P	Cr
mg/L	5087.81	7.480	3.753	0.226	188.289	792.668	730.440

At the next stage, colonies were selected based on color differences and transferred to solid agar plates. Purification of the isolated colonies was carried out through the inoculation of the colonies on the BG_{11} and BBM, which favor blue-green and green algae. In addition, microscopic observations (100X magnification) were made with oil immersion until a purified culture was obtained. In total, seven isolates were scaled-up in their culture medium with 16:8 (light/dark) photoperiods and continuous aeration to maintain the fresh biomass at the temperature of 25°C (Andersen, 2005). The wastewater samples were collected in November and February 2015 and analyzed by inductively-coupled plasma mass spectrometry (ICP).

Algae selection

Out of seven isolates, one alga was selected for identification based on the most resistant algae to Al in acidic pH (Bose *et al.*, 2013). The chemical speciation of Al shows toxicity in acidic pH, and nutrient availability may affect Al removal (Lee *et al.*, 2004; Ameri *et al.*, 2020a). As such, we designed a new experiment to screen resistant microalgae in toxic and low-nutrient conditions. For this purpose, three factors were considered, including pH (4.0 and 5.0), medium concentration (full and one-third), and Al concentration (0 and 20 µM) (Fig. 1). An equal biomass (2.0 $g.l^{-1}$ fresh weight cells) was added to each treatment. After 48 hours, the most grown algae were selected as the most resistant samples. Following that, 12 well plates were placed under 16:8 fluorescent lighting (20 $\mu mol.m^{-2}\ s^{-1}$) at the temperature of

25°C and shaken at 120 rpm (Ameri *et al.*, 2020). Each treatment was implemented in triplicate.

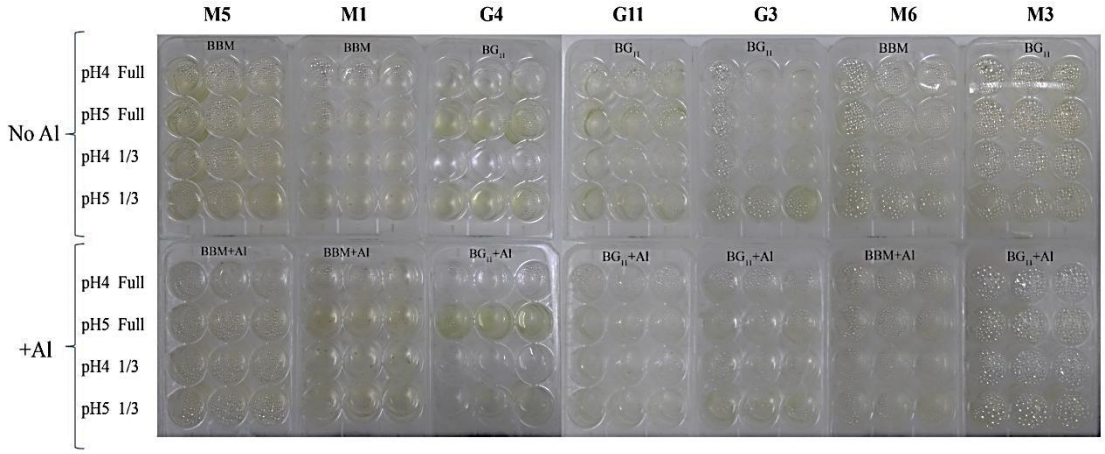

Figure 1: Screening of the microalgae by Al stress (Seven isolates from Charmshahr industrial in Mashhad, Iran; five isolates in BBM and two isolates in BG11 medium compared in different pH [4.0 and 5.0], nutrient availabilities [full and 1/3 medium], and Al concentrations [control and 20 µM]; every three columns indicated one microalga in Al [plate on top] and control condition [plate at bottom] after 48 hours.)

Morphological identification

Al-resistant microalgae were identified based on morphological characteristics such as size, shape, and spines (Prescott, 1962).

Al resistance range

To investigate the physiological responses of Al resistance, the survival threshold of microalgae should be determined initially. For this purpose, the forms of Al species should be examined at different pH (Hu *et al.*, 2006). In addition, the phosphate compounds in media can affect the solubilization of metals, thereby enhancing their uptake by plants (Bolan *et al.*, 2003). Therefore, Al resistance range in different pH and the effects of nutrient availability were investigated in an experiment that was designed based on different Al concentrations (0, 50, 100, and 500 µM), pH (4.0, 5.0, 6.0, and 7.0), and nutrient availability (BBM and BBM/3) for 48 hours.

Physiological responses of microalgae to Al

Culture condition

Physiological responses were assessed at different concentrations of Al (0, 10, and 100 µM), at pH 5.0 in BBM/3 (without EDTA). The cells were killed in the presence of Al in acidic pH. Before starting the experiment, the cells from the logarithmic phase were pretreated in BBM/3 at stable pH 5.0 for 24 hours. After 72 hours of Al exposure, the microalgae cells were collected by centrifugation (3,500 ×g; 10 minutes), and a pellet aliquot was used for growth, enzyme activity, and peroxidation analysis.

Growth measurement

After collecting the microalgae cells by centrifugation (3,500×g; 10 minutes), the pellets were weighed to measure the growth (Ameri *et al.*, 2020a). Following that, the sample aliquots were frozen in liquid nitrogen and preserved at -80ºC until antioxidant analysis.

Protein extraction and antioxidant assay

The frozen algae cells were disrupted in liquid nitrogen using a pestle and mortar, and an extraction buffer was also added, which contained 30 mM 3-(N-morpholino) propanesulfonic acid (MOPS) at pH 7.3, 0.6% polyvinylpyrrolidone (PVP), 5 mM sodium-Ethylenediaminetetraacetic acid (Na-EDTA), 10 mM dithiothreitol (DTT), and 1.5 mM phenyl methyl sulfonyl fluoride (PMSF) (Ameri *et al.*, 2020a). The homogenate was centrifuged at 4°C (6,000×g) for 20 minutes. Afterward, the supernatants were considered as the enzyme source, and the protein content was determined using the Bradford protein assay (Bradford, 1976).

To measure the H_2O_2 concentration, fresh algae biomass (100 mg) was extracted with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged (12,000×g, 15 min, 4°C). The supernatant (0.5 mL) was added to 0.5 mL of 100 mM phosphate buffer (pH 7.0) and 1 mL of 1 M KI in water. The absorbance of the mixture was recorded at 390 nm. The H_2O_2 concentration was calculated by comparison with a standard curve and the value was expressed as μmol H_2O_2 g−1 fresh weight (FW) (Velikova *et al.*, 2000). The formation of MDA was started by incubation of a mixture of algae extracts (in 0.1% TCA) and 0.5% thiobarbituric acid (20% TCA) at 90°C. After 30 min, the reactions were cooled at room temperature and centrifuged. Then the absorbance of mixtures was read at 532 and 600 nm and the MDA concentration was expressed as μmol g−1 FW (Stewart and Bewley, 1980). CAT activity was assessed based on measuring the concentration of H_2O_2 in 1 min at 240 nm (Cakmak and Marschner, 1992). POX activity was determined based on the measurement of the changes in absorbance at 470 nm due to guaiacol oxidation by H_2O_2 (Pandolfini *et al.*, 1992).

Al removal by biosorbent treatment

Experiment design

Al removal was optimized using the Box-Behnken design for tree-factor experiments and the response surface methodology (RSM) based on the Al concentration (0-370 μM) at pH 4.0-6.5 for 30-300 minutes. In total, 20 experiments were proposed for Al removal, and the obtained results were analyzed. The optimal value was also predicted by the Design-Expert software version 7.0. Table 2 shows 20 experimental runs of the Box-Behnken design and the actual levels of the variables.

Table 2: Surface method analysis of Al removal designed with design expert software.

Run	pH	Time (min)	Al (µM)	Al removal (%)
1	4.00	200	185	30.17
2	5.25	200	185	80.60
3	4.50	100	75	84.40
4	6.00	300	75	90.62
5	5.25	200	185	71.19
6	5.25	200	0	0.00
7	4.50	100	295	38.37
8	6.00	100	295	55.93
9	5.25	368	185	67.23
10	4.50	300	75	91.56
11	5.25	32	185	53.55
12	5.25	200	185	60.48
13	5.25	200	370	32.24
14	5.25	200	185	61.48
15	4.50	300	295	46.80
16	5.25	200	185	79.10
17	6.00	300	295	56.45
18	6.00	100	75	91.01
19	5.25	200	185	73.19
20	6.51	200	185	77.38

Biosorbent treatments

Six types of free and fresh microalgae were used to remove Al. Moreover, we prepared free cells (FCs), dried cells (DCs), NaOH-treated cells (NCs), HCl-treated cells (HCs), algae beads (ABs), and blank alginate beads (BBs) to be used in batch culture for the comparison of Al removal. Accordingly, biosorbents were also utilized at the predicted points in the Design-Expert results, including the Al concentration of 81.60 µM and at pH 5.8 for 45 min.

A fresh and free cell obtained from the logarithmic phase was separated via centrifugation (1200 ×g; 10 min), and different modifications were applied to FCs. DCs were maintained after 24 hours at 65°C, and treated cells were obtained after 100 mM NaOH or HCl treatment for one hour. Cell immobilization was also performed on 2% Na-alginate and 2% $BaCl_2$ as the cross-linking agents. Equal microalgae concentrations (4 g/l^{-1}) were applied in all the experiments (Ameri *et al.,* 2019; Ameri *et al.,* 2020b).

Statistical analysis

Data analysis was performed in SPSS version 17.0 (SPSS Inc., Chicago, USA), and each treatment was conducted in triplicate. The experiments had a completely randomized and factorial design. Quantitative data were analyzed using one-way ANOVA and Duncan's post-hoc test. In all the statistical analyses, the *P*-value of less than 0.05 was considered significant.

Results

Algae isolation and selection

In total, seven strains were examined, most of which could not survive in the presence of Al. Meanwhile, M3 and M1 showed different responses. In acidic pH (pH 4.0), Al induces more toxicity. As a result, the other strains showed viability at pH 5.0. In addition, S1 needed Al to survive, and this fascinating response made it a proper candidate for investigating its mechanisms of action as resistant microalgae in the presence of Al (Table 3).

Morphological evaluation

The microscopic image of the resistant microalgae indicated that the width of the cells was within the range of 2.5-3.0 micrometers, and the length of the cells was approximately 3.0 micrometers. In addition, the length of the spines was

estimated at 5.0 micrometers. The cenobium consists of two cells with two lateral spines, which have been identified as *Scenedesmus* sp. (Fig. 2) (Prescott, 1962).

Table 3: Screening of resistant microalgae to Al toxicity (Strains codes titled by M and G were isolated in BBM and BG11, respectively).

condition			Strains response (green color and viability)						
Al(Mμ)	Nutrient	pH	M5	M1	G4	G11	G3	M6	M3
0	BBM	4.0	*	-	-	-	-	*	*
0	BBM	5.0	*	-	*	*	-	*	*
0	BBM/3	4.0	*	-	-	-	-	*	*
0	BBM/3	5.0	*	-	*	*	*	*	*
20	BBM	4.0	-	-	-	-	-	-	-
20	BBM	5.0	-	*	*	-	-	-	*
20	BBM/3	4.0	-	-	-	-	-	-	-
20	BBM/3	5.0	-	*	-	-	-	-	*

* The grown microalgae with an inhibition effect

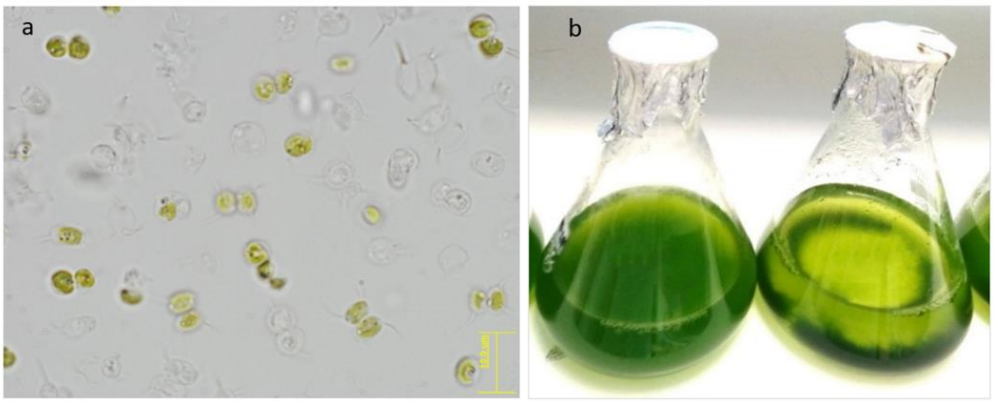

Figure 2: a) The cenobium is two cellular with two lateral spines which are identified as *Scenedesmus* sp. b) *Scenedesmus* sp. culture in presence of Al (right) and control (left) at pH 6.0.

Al resistance range

According to the obtained results, the inhibition of microalgae growth was increased at lower pH and higher Al concentrations. In other words, the cell aggregation occurred at pH 7.0 and 500 µM Al or at pH 4.0 and 100 µM Al (Fig. 3).

Physiological responses of microalgae to Al

Growth measurements

In the present study, cell aggregation occurred immediately after Al exposure, and the growth of *Scenedesmus* sp. was significantly affected by Al concentration. Figure 4 illustrates the induction of biomass production by Al.

Antioxidant assays

Al showed no significant difference in terms of the malondialdehyde (MDA) content in the *Scenedesmus* sp. cells, while H_2O_2 significantly increased with the exposure of the cells to Al. However, no significant increase was observed in H_2O_2 production at higher Al concentrations (Fig. 5).

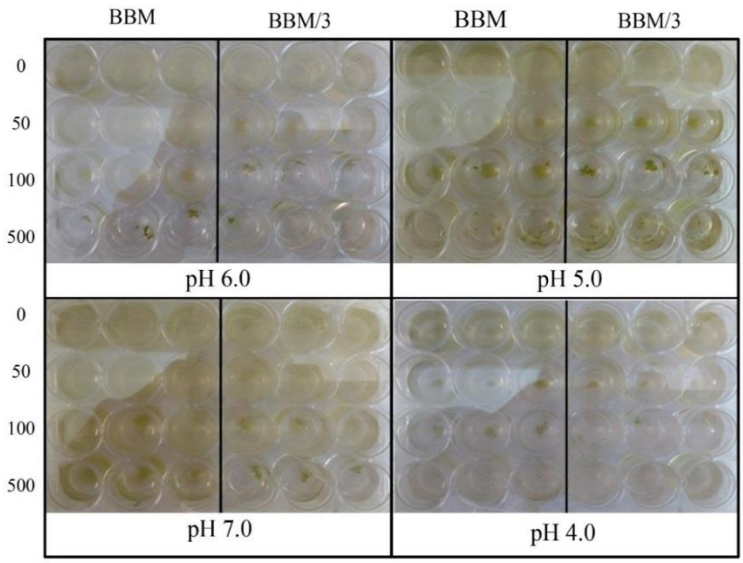

Figure 3: Al concentrations (0- 500 µM) in different media (BBM and BBM/3) at different pH (4.0-7.0).

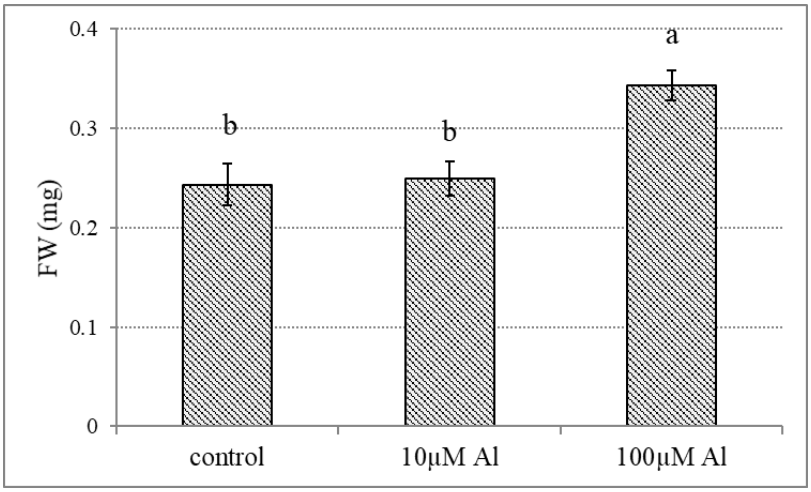

Figure 4: Growth of *Scenedesmus* sp. under Al stress. Different letters denote significant differences between treatments (p<0.05).

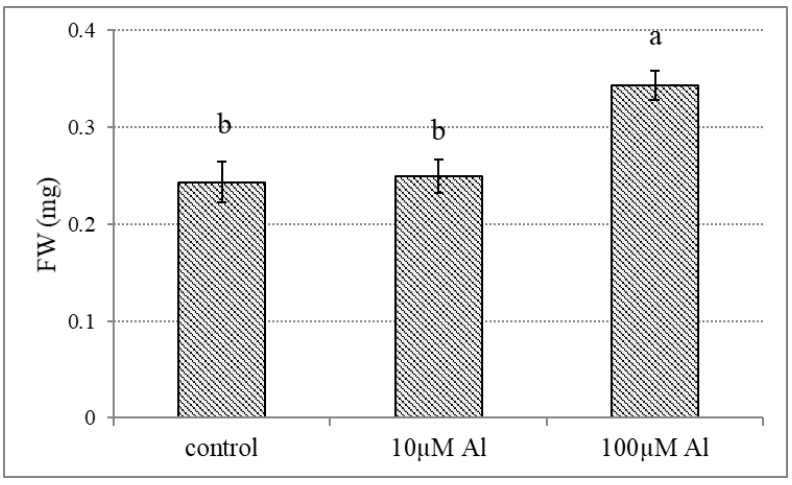

Figure 5: H_2O_2 content under Al stress in *Scenedesmus* sp. Different letters denote significant differences between treatments (p<0.05).

CAT activity decreased due to Al exposure, while POX activity significantly increased (Fig. 6).

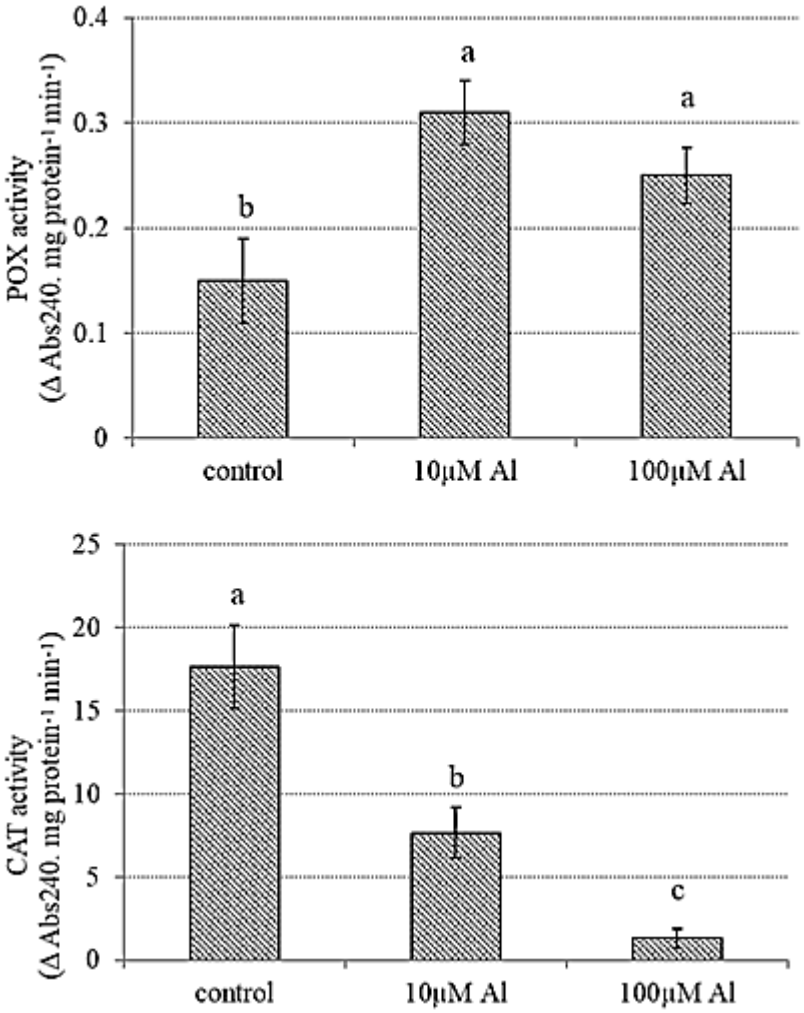

Figure 6: POX and CAT activities under Al stress in *Scenedesmus* sp. Different letters denote significant differences between treatments (p<0.05).

Al removal by biosorbent treatments

In this study, Al removal was carried out in 20 runs (Table 4). According to the information in Table 4, a significant model and a non-significant lack of fit validated the regression model. Furthermore, the findings indicated that Al biosorption is a time-independent reaction, while pH had a significant direct correlation with Al biosorption. Al concentration also had a negative significant effect on biosorption (Fig. 7).

In the present study, maximum removal (95.35%) could be predicted at a higher pH (pH 6.0) and lower Al concentration (75 µM), independent of time by *Scenedesmus* sp. In addition, higher pH (6.0) had the most significant impact on Al removal.

The analysis of the data obtained from the Box-Behnken design in the

Design-Expert software predicted that the microalgae could remove Al completely, and the experimental data indicated 95.3% removal.

Table 4: ANOVA for Response Surface Reduced Linear Model Analysis of variance table (Partial sum of squares- Type III) using the Design-Expert software.

Source	Sum of Squares	Df	Mean Square	*F* -value	*p*-value	
Model (quadratic)	6414.66	2	3207.33	36.60	<0.0001	significant
A-pH	917.42	1	917.42	10.47	0.0049	
C-Al	5497.24	1	5497.24	62.73	<0.0001	
Residual	1489.72	17	87.63			
Lack of Fit	1125.82	12	93.82	1.29	0.4145	not significant
Pure Error	363.90	5	72.78			
Cor Total	7904.38	19				

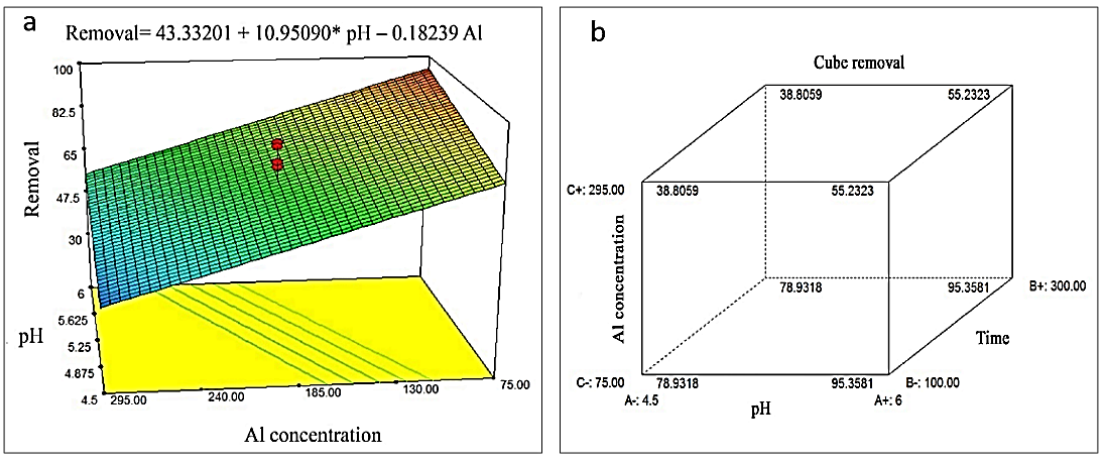

Figure 7: Influential factors in Al Removal in *Scenedesmus* sp. A: effects of pH and Al concentration were more significant than time; Al removal decreased at lower pH and higher Al concentration, B: interactive effects of pH, Al concentration, and time on Al removal).

On the other hand, a significant difference was observed in Al removal between various microalgae. *Scenedesmus* sp. free and live cells did not require any modification to biosorbent Al. Notably, Al mostly binds to the cell surface as passive transportation, which is recognized as DCs (81.86%). Interestingly, immobilization in alginate included BBs and ABs showed the lowest Al removal (Fig. 8).

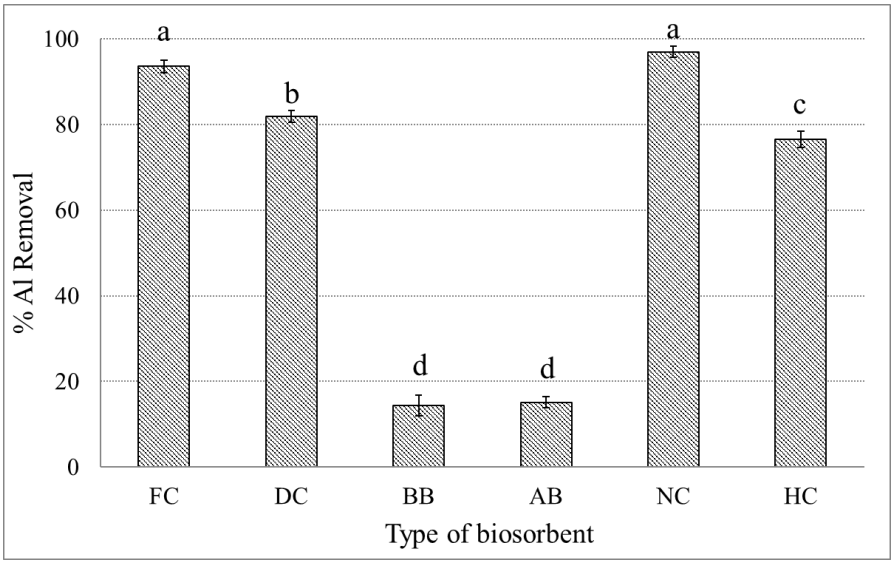

Figure 8: Effect of different biosorbents on Al removal. Free and fresh microalgae cells (FC), dry cells (DC), NaOH- treated cells (NC), HCl- treated cells (HC), algae beads (AB), and blank alginate beads (BB). Different letters denote significant differences between treatments (p<0.05).

Discussion

In an acidic paddy field, Al induces toxicity in N_2-fixing cyanobacteria, thereby decreasing the growth of cyanobacteria and the yield of the paddy field. Al^{3+} is the most toxic form of Al at pH 4.5 (or below) (Pettersson *et al.*, 1985) with increased pH, other mononuclear Al species may also appear, such as $Al(OH)^{2+}$ and $Al(OH)^{2+}$. Two major mechanisms involve plants in acidic areas that are polluted by Al. First, the resistance mechanism excludes Al from the root, while after the symplast entry of Al, the tolerance mechanism prevents Al absorption. The resistance mechanism of Al is associated with organic acid biosynthesis and transportation. Organic acids of Al complexes could be used for internal and external Al detoxification. According to the results of the present study, *Scenedesmus* sp. could be a resistant microorganism to produce more biomass in the case of Al exposure (Fig. 6).

Al has the potential to connect to proteins or lipids and replace Ca^{2+} to disrupt plasma membrane flexibility (Akeson *et al.,* 1989). This connection also results in plasma membrane depolarization (Kinraide *et al.,* 1992) and the reduction of H^{+}-ATPase (Sivaguru *et al*., 2002). Al^{3+} can interact with photosystem II (Yang *et al.*, 2012; Hasni *et al.,* 2015) and induce oxidative stress due to the excessive generation of ROS. Excess ROS damages the rigidification of the plasma membrane (Hasni *et al.*, 2015; Kumar *et al.*, 2015a; Silva *et al.,* 2018), and the polyunsaturated fatty acid content of lipids is the initial target of ROS damage. Therefore, lipid peroxidation occurs due to Al stress and leads to the generation of oxygen free radicals (Yamamoto *et al.,* 2003; Gupta *et al.,* 2013). The results of the present study

indicated H_2O_2 generation during Al exposure, while no significant difference was observed in the MDA content (Fig. 7).

Due to Al stress, plant cells produce ROS by activating various peroxidases (pox) and oxidases, and the scavenging system (antioxidants) will be further activated to protect cells against the destructive effects of ROS (Kumar *et al.,* 2015a; Sun *et al.,* 2018). As such, algal redox homeostasis should remain balanced through the function of several enzymatic and non-enzymatic antioxidants (Pinto *et al.,* 2003). Antioxidants may overlap in ROS elimination, such as the scavenging activity of ascorbate peroxidase (APX), glutathione peroxidase (GPX), POX, and CAT (Fridovich, 1997; Trchounian *et al.,* 2016). Wang *et al*. (2010) introduced POX and APX as major defense tools in H_2O_2 elimination rather than CAT in Pb-treated plants. Our findings in this regard showed the reduction of CAT activity at the higher concentrations of Al, while POX activity was observed to increase (Fig. 8).

As mentioned earlier, Al^{3+} is the most toxic form of Al, which inhibits cell development and transportation (Mossor-Pietraszewska, 2001) and could be replaced by heme groups. In addition, CAT contains metal cofactors like Fe, and it has been suggested that Fe could be replaced by other metals, like Al (Singh *et al.,* 2009; Auger *et al.,* 2013). The replacement of Al by other metals like Fe) could inhibit enzyme activity. In other research, the cells treated with 15 mM Al showed a reduction in the Krebs cycle and iron-dependent isoforms (Singh *et al.,* 2009).

Al can bind to an oxygen ligand with a greater affinity than other elements. These donor ligands are found in the phosphate or carboxylate groups of cell walls and the outer surface of the plasma membrane, respectively (Yamamato *et al.,* 2001; Gupta *et al.,* 2013). The cell wall is the primary target of Al and plays a key role in the perception and biosorption of Al (Yang *et al.,* 2011; Singh *et al.,* 2017). For instance, cells of the algae *Chara coralline* showed an Al removal rate of 99.99% (Taylor *et al.,* 2000). Al accumulation in the cell wall is not entirely pH-dependent, while the transfer of Al^{3+} across membranes is more dependent on pH and often occurs at an acidic pH. (Taylor *et al.,* 2000; Herburger *et al.,* 2016). In the composition of some algae and land plant cell walls, a polymer of α-D-1,4-galacturonan is found, which provides the binding of free carboxylic groups to Al after pectin methyl esterase activity (Schmohl *et al.,* 2000; Popper *et al.,* 2011; Domozych *et al.,* 2014).

According to the results of the present study, Al removal was mostly accomplished by the cell wall (81.87% by DCs) within the first minutes, which indicated passive transportation. Passive transportation involves cell walls and plasma membranes, while active transportation (11.70% in live cells) requires more energy and time to cross a membrane and reach the intracellular targets. Immobilized microalgae showed good ability in nutrients (Sarkheil *et al.,* 2022) and heavy metal biosorption

(Ameri *et al.*, 2020b) while they are not good in Al removal. However, the carboxyl groups on algal cells are considered to be greatly responsible for Al binding (Kumar *et al.*, 2015b). Al stress also triggers a binding to the oxygen donor ligands located in intracellular compartments such as proteins, polysaccharides, and nucleic acids. These events increase ROS production and lead to oxidative stress (Mossor-Pietraszewska, 2001).

According to the results, other antioxidant mechanisms may be responsible for the control of cell activity in the case of Al stress. Furthermore, *Scenedesmus* sp. was observed to be a resistant microalga with a high ability to remove Al through the cell walls without any modification. Therefore, it is suggested to use this microalgal species for the phytoremediation of Al-contaminated wastewater as it can be effective within minutes.

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