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Optimizing *Microcystis aeruginosa* inactivation in freshwater using algicidal *Bacillus subtilis* by central composite design

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ABSTRACT

The present study deals with inactivating harmful algae blooms (HABs) in the fresh water samples by using algicidal bacteria (*Bacillus subtilis*). The inactivation process was performed under the direct sunlight and optimized using central composite design (CCD) based on three independent factors included time (1–6 h), bacterial supernatant dosage (1–10 mL 100 mL^{-1}) and pH (5–8). The results revealed that the maximum inactivation of HABs was achieved with 1.61 mL 100 mL^{-1}, within 6 h and pH 8, the reduction was 6 vs. 5.78 log and 90.22 vs. 87.767% of the chl a reduction (observed and predicted, respectively, $R^2 = 0.976$). The inactivation mechanism was explained based on the analysis of untreated and treated HABs cells by field emission scanning electron microscope with energy dispersive X-ray spectroscopy (FESEM-EDX), Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy which revealed a damage in the cell wall structure due to the effect of algicidal substances. Moreover, FTIR analysis showed that the damaging of HABs was due to the adverse effect of algicidal substance, which lead to the damage of protein and carbohydrate structure of the HABs cell wall. These results further demonstrate that the algicidal bacteria can effectively inhibit HABs cells in the freshwater.

Keywords: Harmful algae blooms; Chlorophyll a; Reduction; *Bacillus subtilis*; Mechanism

1. Introduction

Harmful algae blooms (HABs) defined as a natural process occur due to the overgrowth of algae on the surface of natural water systems. HABs have the ability to produce different types of the toxins such as hepatotoxins, and neurotoxins which might be transmitted into the human through the drinking water. The released toxins cause, diarrhoea, vomiting, eye irritation, skin rashes and respiratory symptoms. These symptoms are used as an indication for the infections, since there are no specific diagnostic procedures for these toxins in human blood [1,2]. The occurrence of water toxicity with HABs is more common among the countries which depend on the water desalination such as Saudi Arabia. Fahad dam at Bisha represent one of the natural water system which has HABs due to the contamination of these water with the sewage effluents [3]. The utilization of these waters as drinking water represents a real hazard for the human due to the presence of toxins. The studies have revealed that the reverse osmosis (RO) technology is

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not enough to remove the HABs toxins from the fresh water due to the high solubility and zwitterion properties of these substances, which allow it to diffuse through the RO membrane. Moreover, the HABs cause several operational problems for RO system due to the clogging of the filters [4].

The chemical and physical treatment of HABs recorded more than 90% of the inactivation percentage [5,6]. However both methods are considered as unacceptable techniques due to the toxic by-products which caused a serious secondary contamination and effect negatively on the aquatic organisms in the natural water system [7]. The biological control method appears to be the best alternative for inactivating the HABs in the water systems [8]. The process might be carried out by using algicidal microorganisms which has exhibited high inactivation rate of HABs (>95%) [9,10]. The studies in the literature have revealed that many of the bacterial species which can be used for controlling the algae blooms by producing algicidal agents have the ability to inactivate the algae cells. Algicidal substances are biological substrates produced by bacteria such as *Phaeocystis globosa, Prorocentrum donghaiense, Pseudomonas* sp., *Bacillus* sp., *Aeromonas* sp. and *Heterosigma akashiwo* [11,12]. However, the challenges for the utilization of these organisms in environmental technologies lie in the founding an appropriate strain which have the ability to survive under different environmental conditions. Nonetheless, most algicidal bacteria cleave the algae cells by secreting algicidal substances [13]. Moreover the optimization process based on the main environmental factors might enhance the inactivation process. Response surface methodology (RSM) is one of the best statistical program which might achieve the highest inactivation rate within limited experimental runs [14]. RSM can be effectively implemented when a response or some responses of interest are affected by many variables [15]. Therefore, the aim of the present work is to optimize the inactivation of HABs by algicidal bacteria in the freshwater using RSM. The inactivation mechanism of HABs was investigated using field emission scanning electron microscope with energy dispersive X-ray spectroscopy (FESEM-EDX), Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy.

2. Materials and methods

2.1. Experimental set-up

The experimental set up of the current work consisted of bacterial isolation and subculture, algicidal bioassay, HABs sample preparations and algicidal activity experiments. The Factorial Complete Randomized Design (3 × 1 × 1) in duplicates was used to study the optimal factors affecting the inactivation process with three (3) independent factors, One (1) dependent factors (log reduction of HABs) and 1 control. The inactivation process was conducted with 1,000 mL of the fresh water sample for each run.

2.2. Water samples

The freshwater samples (20 L) with HABs were collected from Fahad Dam in a plastic bottle (20 L) and transferred to the analytical laboratory in an ice box and subjected to analysis within 24 h. The presence of HABs in the freshwater sample was determined using the KDHE procedure as described by the Kansas Department of Health and Environment, USA [16]. The method was performed based on the formation of upper layer on the surface of firewater after the sample was kept in a cold refrigerator overnight [16]. The HABs studied in the present work belongs to *Microcystis aeruginosa* based on the cell morphology of the cells as determined using SEM.

2.3. Algicidal bacterial strain

Bacillus subtilis strain (Fig. 1) was isolated from the secondary effluents sample using direct-plate technique on the nutrient agar (NA) medium according to APHA [18]. The bacterial strain was identified based on the morphological and culture characteristics according to the procedure described by Brown [19]. The bacterial strain was sub-cultured in 1 L of nutrient broth, incubated in the shaker at 30°C, 150 rpm for 24 h. The bacterial culture was centrifuged at 11,000 rpm for 10 min. The supernatant was used for the algicidal activity bioassay and inactivation process. The primary algicidal activities for isolated bacteria were tested against HABs in the LB medium according to Tian et al. [20]. The algicidal activity (%) was determined according to Eq. (1):

$$\text{Algicidal activity}\left(\%\right) = 1 - \frac{D_{t\ \text{treatment}}}{D_{t\ \text{control}}} \times 100 \quad (1)$$

where $D_{t\text{-treatment}}$ (cells/mL) and $D_{t\text{-control}}$ (cells/mL) are the cell densities of treatment and control of HABs with and without bacteria, respectively; t (h) stands for the inoculation time.

2.4. Optimization of algicidal inactivation of HABs

The inactivation of HABs in the fresh water samples by algicidal bacteria was optimized using the central composite design (CCD) which was selected to create a significant better model compared with other methods of Design-Expert software program. Besides, the CCD requires a smaller number of experiments, where 15 experimental runs were performed in the current work to determine the linear and quadratic effect of bacterial supernatant dosage

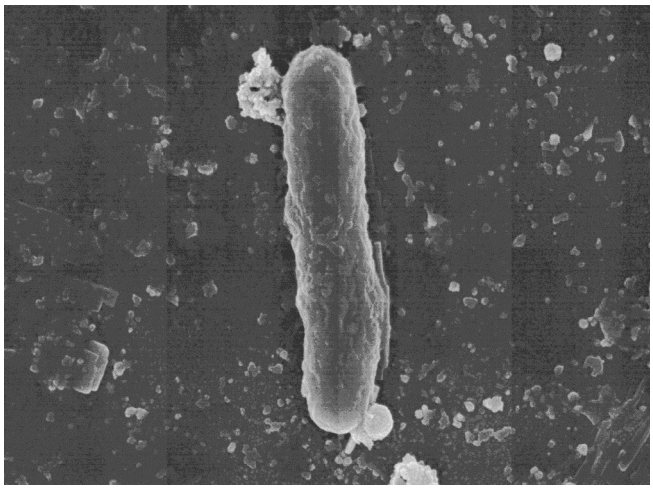

Fig. 1. *B. subtilis* in the freshwater samples (20,000x).

Table 1
Coded and un-coded levels of the independent variables

Factor	Symbol	Level		
		Low (-1)	Middle (0)	High (+1)
Dosage (mL 100 mL^{-1})	x_1	1	5.5	10
Time (h)	x_2	1	3.5	6
pH	x_3	5	6.5	8

(x_1) (1 to 10 mL L^{-1}), time (x_2) (1 to 6 h) and pH (x_3) (pH 5 to 8) as well as the interaction between these factors and their effect on the inactivation of HABs. The ranges of the independent factors (high and low) were represented as minimum (−1), intermediate (0), and maximum (+1) (Table 1).

The quadratic model for the log reduction of HABs as a function of the independent factors is illustrated in Eq. (2):

$$Y = \beta 0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{i<j}^{n} \beta_{ij} x_i x_j \quad (2)$$

while the comparison between the experimental data and the coded variable is represented as Eq. (3).

$$x_i = \frac{\varepsilon_i - \left[\mathrm{HL} + \mathrm{LL}\right]/2}{\left[\mathrm{HL} - \mathrm{LL}\right]/2} \quad (3)$$

where x_i is the coded variable, ε_i is the experimental data, HL is the maximum value of the independent variable, and LL is the minimum value of the independent variable.

The inactivation process using algicidal bacteria was performed in triplicate to obtain the accurate evaluation efficiency of algicidal bacteria.

2.5. Preparation of HABs inoculum

The initial concentrations of HABs in the water samples were enumerated by haemocytometer with microscope as described by Noman et al. [14]. The HABs inoculum was prepared with 10^6 cells mL^{-1} as recommended by STAATT [21].

2.6. Algicidal inactivation processes

Fifteen experimental runs were conducted in order to assess the efficiency of algicidal activity of substances from *B. subtilis* against HABs in the freshwater. Each freshwater sample (1 L) was placed in jar test and inoculated with different dosage of *B. subtilis* supernatant with algicidal substances (1–10 mL per 100 mL of the solution), pH was adjusted using NaOH (0.1 M) and HCl (0.1 N) to be within the range of (pH 4–9) based on the RSM designed in the present study. The freshwater with *B. subtilis* supernatant was mixed at 100 rpm for different time (1–6 h) of HRT and then samples were subjected for low mixing at 30 rpm for 30 min and settlement for another 6 h [21].

The inactivation of HABs was evaluated based on the reduction in the HABs cell numbers as determined by haemocytometer under microscope. The log reduction of HABs was calculated according to Eqs. (4) as recommended by STAATT [20].

$$\log \mathrm{Reduction}\left(\log \mathrm{Kill}\right) = \log \mathrm{IT} - \log \mathrm{RT} \quad (4)$$

where IT is initial numbers of HABs cells (cell mL^{-1} of freshwater sample) before the inactivation process.

RT is the number of HABs cells (cell mL^{-1} of freshwater sample) recovered from a treated samples

Moreover, the chl a reduction was measured based on the determination of the concentrations before and after each treatment process according to Jeffrey and Humphrey [23]. Chl a was extracted from each sample by mixing 1 mL of the water sample with 9 mL of acetone (90%). The absorbance of the mixture was determined using spectrophotometer at λ = 664, 647 and λ = 630 nm. The chl a concentrations were calculated using Eq. (5).

$$\mathrm{Chl}\left(\mathrm{a}\right)\left(\frac{\mu g}{l}\right) = 11.85E_{664} - 1.54E_{647} - 0.08E_{630} \quad (5)$$

where E_{664} = value of absorbance at wavelength 664 nm; E_{647} = value of absorbance at wavelength 647 nm; E_{630} = value of absorbance at wavelength 630 nm.

2.7. Mechanism of algicidal activity against HABs

In order to understand the mechanism of action for algicidal activity against HABs, the HABs cell morphology before and after the inactivation process was imagined using field emission scanning electron microscope with energy dispersive X-ray spectroscopy (FESEM-EDX) (model JEOL JSM-7600F), Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy (X-Plora Plus). For this purpose, 10 mL of freshwater samples before and after the inactivation process were subjected for the centrifugation at 6,000 rpm for 10 min. The pellets of HABs were prepared for FESEM-EDS analysis after coating with gold powder. Moreover, the HABs cells were also subjected for FTIR and Raman spectroscopy analysis to determine the effect of algicidal substances on the functional groups profile of the HABs cell wall as well as the proteins, lipids and carbohydrates profile of the HABs cells.

3. Results and discussion

3.1. Characteristics of raw freshwater samples

The freshwater sample used in the current work contained Chl a in the average of 250 μg L^{-1} (data not shown). The high concentration is an indication for the presence of

high load of HABs. Moreover, KDHE test showed an upper layer on the surface of the water sample that was tested in the test tube which does mean that the HABs cell concentrations are more than 10^7 cells mL^{-1} (Appendix B). These findings are consistent with the previous study conducted by Hollister and Kreakie [24], who claimed that Chl a is associated with various microcystin health advisory concentrations. However, some of the previous studies claimed that the presence of HABs is associated with 550 μg/L of Chl a [24]. In the current work, the freshwater samples contained 250 μg L^{-1} of Chl a, which is low in comparison with other studies but, the low concentrations of Chl a do not mean the absence of HABs, it indicates that there are HABs but the concentrations are less than 10^7 cells mL^{-1} and might increase with the time to reach 10^7 cells mL^{-1}.

3.2. Optimization of HABs inactivation by algicidal bacteria

The optimization for inactivating HABs in the freshwater samples by using different dosage of algicidal bacteria was studied as a function of three independent variables including bacterial dose (1–10 mL per 100 mL), time (1–6 h) and pH (5–8). The optimization study was carried out in order to determine the best operating parameters at which the maximum inactivation of HABs is achieved. Hence, the CCD was employed with 15 treatment runs (designed by Design-Expert version 10), which performed to cover all possible combination of factor levels (Table 2). Among the treatment runs, four runs was used to represent two-level factorial design, six runs represents star points (axial points), while five runs at the centre. The log reduction (Response variables) was calculated according to Eq. (4). The validity of first and second order model was analysed using ANOVA ($p < 0.05$, with 95% of confident level).

The maximum log reduction of HABs in freshwater within the investigated ranges as displayed in Table 2 was determined with 5.5 mL 100 mL^{-1}, after 3.5 h at pH 6.5 of which the predicted and experimental $\log_{10}$ reductions were the log reduction was 5.7 vs. 4.38 $\log_{10}$ cell mL, respectively. The minimum reduction was noted with 1 mL 100 mL^{-1},1 h and pH 5, where the log reduction was 2.98 vs. 2.17 $\log_{10}$ cell mL of experimental and predicted data, respectively. By contrast, the highest chl a reduction was recorded with 1 mL 100 mL^{-1}, 6 h and pH 8, where the chl a reduction was 87.76% vs. 83.38% of experimental and predicted results, respectively. The lowest reduction was observed with 1 mL 100 mL^{-1}, 1 h and pH 5, where 32.13% vs. 27.75% of chl a was removed. These findings indicated that the algicidal substances from *B. subtilis* have become more effective at moderate pH and after 3.5 h.

The results of ANOVA indicated that a significant model for inactivating HABs (y_1) and removal of chl a (y_2) ($p < 0.0336$ and 0.0072) with the determination coefficients (R^2) equal to 0.9146 and adjusted R^2 was 0.7610 for y_1 reduction and $R^2 = 0.9548$, Adj. $R^2 = 0.8748$ for y_1, indicating the aptness of the model (Table 3). Both x_1 and x_2 factors exhibited positive and significant coefficient ($p < 0.0084$ and 0.0059) with y_1 and y_2, while x_3 has a negative and non-significant coefficient ($p > 0.05$) with both dependent variables (y_1 and y_2) (Table 4). The quadratic analysis for the effect of independent factors on the inactivation process were non-significant ($p > 0.05$), indicating that none of the independent factors has a secondary influence on the inactivation process.

The regression model with the significant coefficients (at 95% confidence level) is given by Eqs. (6) and (7).

Table 2
Log reduction of HABs in freshwater by algicidal substances from *B. subtitles* as a response for independent factors

Run	x_1	x_2	x_3	y_1		y_2	
				Observed	Predicted	Observed	Predicted
1	1.414	0.000	0.000	5.62	6.05	75.77	80.15
2	–1.000	–1.000	–1.000	2.60	2.17	32.13	27.75
3	0.000	0.000	0.000	5.70	4.38	86.45	75.90
4	1.000	1.000	–1.000	3.70	3.28	45.88	41.49
5	0.000	0.000	–1.414	4.80	5.23	72.74	77.12
6	0.000	0.000	0.000	4.54	4.38	77.89	75.90
7	0.000	0.000	0.000	4.31	4.38	72.43	75.90
8	0.000	0.000	1.414	4.70	5.12	77.37	81.75
9	1.000	–1.000	1.000	5.00	4.57	78.98	74.60
10	0.000	0.000	0.000	4.09	4.38	74.66	75.90
11	0.000	–1.414	0.000	0.20	0.63	20.67	25.05
12	–1.414	0.000	0.000	0.50	0.93	25.86	30.25
13	–1.000	1.000	1.000	5.80	5.37	87.76	83.38
14	0.000	0.000	0.000	4.09	4.38	76.82	75.90
15	0.000	1.414	0.000	5.78	6.21	80.32	84.70

x_1 (dosage, mL/L); x_2 (time, min); x_3 (pH), y_1 (Log reduction); y_2 (Chl a reduction).
[a]As determined using Eq. (4).
[b]As determined using Eq. (5).

Table 3
ANOVA analysis of the response surface quadratic model for inactivating HABs in freshwater by algicidal substances from *B. subtitles*

Source	Sum of square	DF	Mean square		*F* value		*p*-value Prob > *F*	
			y_1^*	y_2^{**}	y_1	y_2	y_1	y_2
Model	39.30	9	4.37	756.13	5.80	11.75	0.0336*	0.0072*
x_1	13.11	1	13.11	1,245.34	17.41	19.35	0.0087*	0.0070*
x_2	15.57	1	15.57	1,778.94	20.68	27.64	0.0061*	0.0033*
x_3	5.513E-003	1	5.513E-003	10.70	7.322E-003	0.17	0.9351	0.7003
x_1x_2	2.70	1	2.70	844.48	3.58	13.12	0.1169	0.0152*
x_1x_3	4.48	1	4.48	477.92	5.95	7.43	0.0587*	0.0415*
x_2x_3	6.01	1	6.01	538.19	7.99	8.36	0.0368*	0.0341*
x_1^2	1.53	1	1.53	826.44	2.04	12.84	0.2130	0.0158*
x_2^2	1.78	1	1.78	852.24	2.37	13.24	0.1845	0.0149*
x_3^2	1.22	1	1.22	24.12	1.62	0.37	0.2586	0.5672
Residual	3.76	5	0.75	64.36				
Lack of Fit	1.96	1	1.96	207.40	4.43	7.25	0.1055	0.0545
Pure Error	1.80	4	0.45	28.59				
Cor Total	43.07	14						

*R^2 = 0.9126, Adj. R^2 = 0.7552, **R^2 = 0.9548, Adj. R^2 = 0.8748.

Table 4
Regression coefficient and their significance of the quadratic model for inactivating HABs in freshwater by algicidal substances produced from *B. subtilis*

Source	Coefficient estimate		Standard error		*F* value		*p*-value Prob > *F*	
	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2
Model	4.38	75.90	0.37	3.45	5.80	11.75	0.0336*	0.0072*
x_1	1.81	17.64	0.43	4.01	17.41	19.35	0.0087*	0.0070*
x_2	1.97	21.09	0.43	4.01	20.68	27.64	0.0061*	0.0033*
x_3	–0.037	1.64	0.43	4.01	7.322E-003	0.17	0.9351	0.7003
x_1x_2	–1.16	–20.55	0.61	5.67	3.58	13.12	0.1169	0.0152*
x_1x_3	1.50	15.46	0.61	5.67	5.95	7.43	0.0587*	0.0415*
x_2x_3	1.73	16.40	0.61	5.67	7.99	8.36	0.0368*	0.0341*
x_1^2	–0.45	–10.35	0.31	2.89	2.04	12.84	0.2130	0.0158*
x_2^2	–0.48	–10.51	0.31	2.89	2.37	13.24	0.1845	0.0149*
x_3^2	0.40	1.77	0.31	2.89	1.62	0.37	0.2586	0.5672

$$y_1 = 21.88 - 0.43x_1 - 1.11x_2 - 5.16x_3 - 0.10x_1x_2 + 0.22x_1x_3 + 0.46x_2x_3 - 0.022x_1^2 - 0.08x_2^2 + 0.18x_3^2 \quad (6)$$

$$y_2 = 161.09 + 1.05x_1 + 1.82x_2 - 37.03x_3 - 1.82x_1x_2 + 2.30x_1x_3 + 4.37x_2x_3 - 0.51x_1^2 - 1.68x_2^2 + 0.78x_3^2 \quad (7)$$

Both dosage and time have been reported among the factors which contribute effectivity in inactivating of HABs [25,26]. The results in the present study are in agreement with that reported by Zhang et al. [25], even at different inactivation process. Zhang et al. [25] revealed that the time is a critical factor in inactivating HABs; the study found that at 5 μg mL^{-1} of Prodigiosin, the algae cells showed cytoplasmic hyper vacuolization, and destruction of chloroplast and nucleus rupture, within 2 h, while the cells lost 45.3% of Chl a fluorescence after 24 h. In the current work, the inactivation was conducted for maximum of 6 h; however, 6 log reduction of HABs cells and 87.76% of chl a removal was achieved indicating the effectiveness of algicidal substances from *B. subtitles* for inactivating HABs in freshwater.

The analysis of the interaction between independent factors is presented in Figs. 2 and 3 as well as Table 4. It can be noted that a negative interaction was recorded between x_1 and x_2 indicating that the increasing dosage might minimize the time required for the inactivation time and chl a reduction, but this interaction was not clear and non-significant with y_1 since p value was more than 0.05 (Fig. 2a), while was a significant with y_2 ($p < 0.0152$; Fig. 3a). By contrast, the independent factors x_1 and x_3 as well as x_2 and x_3 occurred positive significant interactions during the inactivation process of

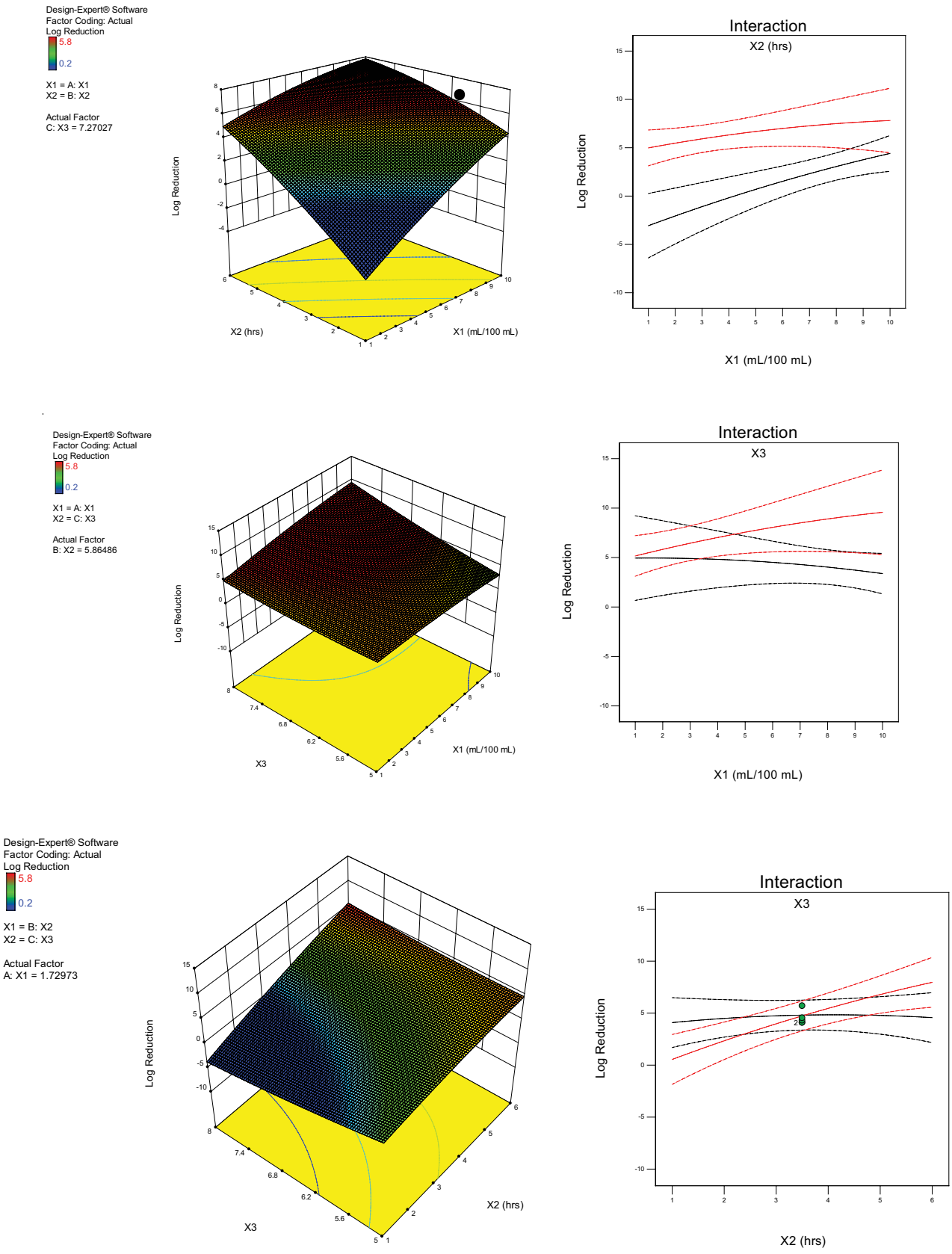

Fig. 2. Three-dimensional response surface plot for log reduction of HABs in freshwater by algicidal substances produced from *B. subtilis* as a response of interaction between independent factors. x_1 (dosage mL 100 mL^{-1}); x_2 (time, h); x_3 (pH).

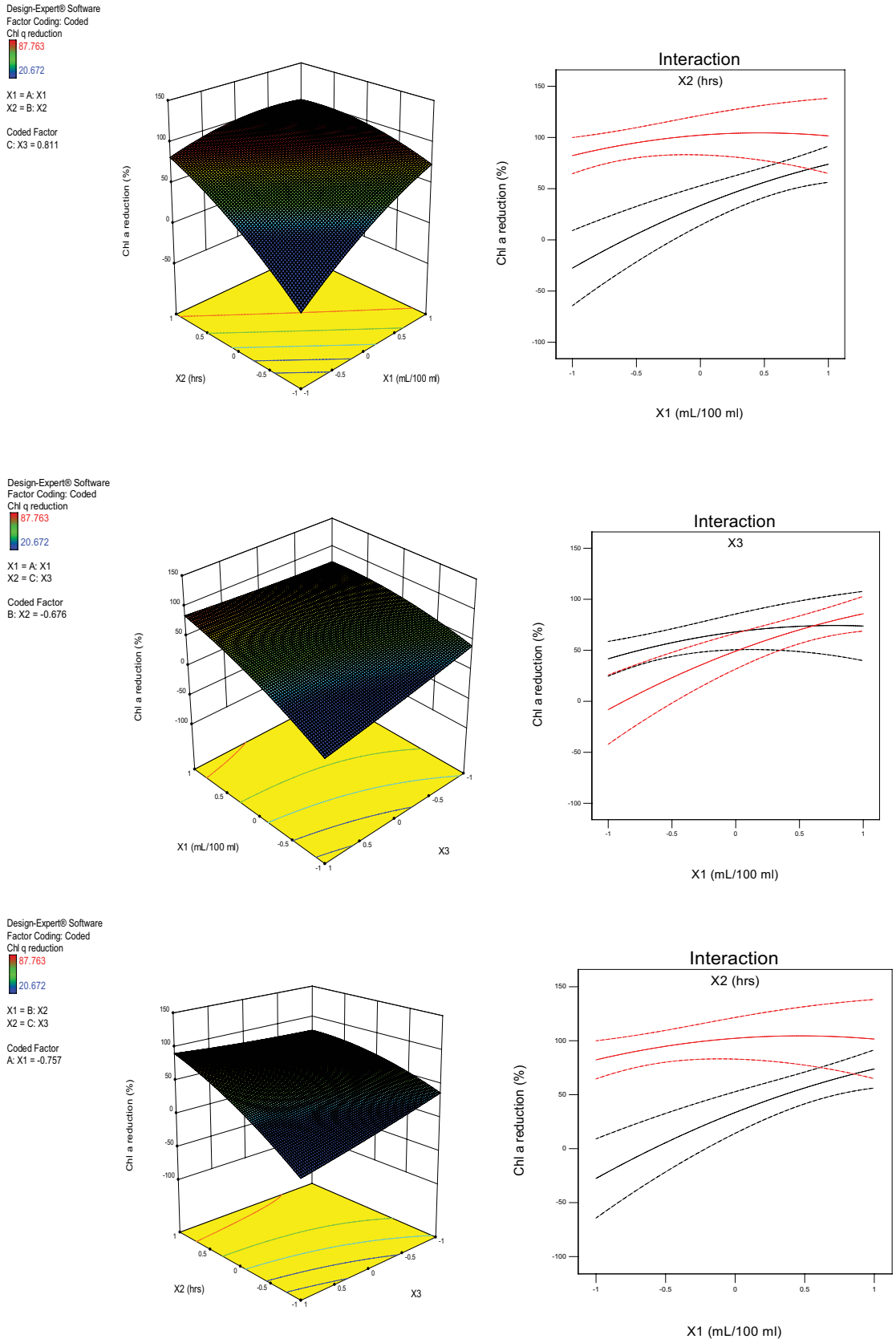

Fig. 3. Three-dimensional response surface plot for Chl a of HABs in freshwater by algicidal substances produced from *B. subtilis* as a response of interaction between independent factors. x_1 (dosage mL 100 mL^{-1}); x_2 (time, h); x_3 (pH).

HABs in the freshwater. The ANOVA analysis revealed that the increasing bacterial dosage becomes more influential at high pH at a constant time (Figs. 1b and 2b), while a short time is required to achieve high log reduction in the HABs at constant bacterial dosage (Figs. 2c and 3c). It can be concluded that, although, the x_1 and x_2 has no significant interaction, both factors have more influences at constant pH (7.5) than that recorded as a result of interaction between x_1 and x_3 at constant time (1.6 h) and between x_2 and x_3 at constant dosage (1.1 mL per 100 mL).

3.3. Validation of the optimal parameters

In order to validate the best operating parameters suggested by the Design-Expert, the experiment conditions suggested by the software were tested in the laboratory. The results for the validation of the optimal parameters are presented in Table 5. The percentage error was investigated for the optimization of experiments, the errors between model and experimental values were calculated according to the formula in Eq. (8)

$$\text{Error} = \frac{\text{Model error} - \text{Exp error}}{\text{Model Error}} \times 100 \quad (8)$$

The best operating parameters for inactivating HABs in freshwater as a response for algicidal substances from *B. subtitles* were tested with two suggestions to achieve high inactivation. The inactivation of HABs in the freshwater samples with low dosage concentrations 3.47 mL 100 mL^{-1} within 2.62 h and at pH 5, which might achieve 4.42 log reduction and 69.54 % of chl a reduction (R^2 = 0.703). The second suggestion might be carried out with 1.606 mL 100 mL^{-1}, within 6 h and pH 8, the reduction was 6 log and 90.22% of the chl a reduction (R^2 = 0.976).

A few studies have been carried out on the optimization of HABs inactivation by RSM. However, these studies indicated that pH and dosage concentrations were among the factors which contribute effectively in the chl a reduction. In the previous study, Hamed et al. [22] revealed that a significant interaction was recorded between fungal dosage and pH for removing Chl a. In comparison with other bacterial strains, it was noted that *B. subtilis* strain in the current study exhibited high efficiency in inactivating HABs, but the dosage used was more than that recorded with other bacterial strains such as *Bacillamide* which showed excellent algicidal efficiency against many of the HABs included (*Skeletonema costatum* and *Gymnodinium catenatum*) with concentrations ranged from EC50 value of 0.011 to 0.58 mg/L [27]. However, the differences might be due to the use of raw algicidal substances in this work as well as the inactivation factors which are different.

3.4. Inactivation mechanism of HABs cells

The results of SEM imaging showed that HABs in the fresh water samples (before the inactivation process) have spherical shape with smooth surface, where there was no critical influence on the algal cells under the normal environmental conductions were observed (Fig. 4a). In contrast, the results in Fig. 4b revealed changes in the surface of HABs cells which might be related to the effect of algicidal secreted by *B. subtilis* [28]. According to Eckersley et al. [30] most of HABs cells have a spherical shape, therefore, any changes on the shape or surface of the HABs is used as indication

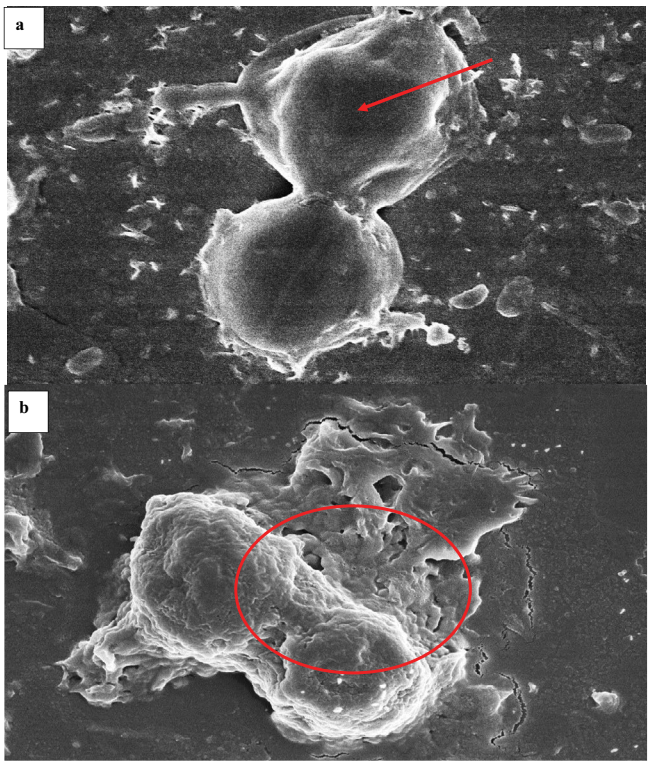

Fig. 4. FESEM image shows destruction of HABs cells (10,000X) in freshwater using algicidal substances produced from *B. subtilis*, (a) before the inactivation (control); (b) after the inactivation (X = 10 k).

Table 5
Operating parameters for achieving highest inactivation of HABs in freshwater by algicidal substances produced from *B. subtilis* and as a response for independent factors

Run	x_1 (mL 100 L^{-1})	x_2 (h)	x_3	y_1		y_2		Desirability
				Observed	Predicted	Observed	Predicted	
1	3.466	2.615	5	4.423	4.249	69.542	66.665	0.703
2	1.606	6	8	6	5.784	90.221	87.767	0.976

y_1 (Log reduction); y_2 (Chl a reduction).

for inactivating or deforming HABs cells. Moreover, in the present study, the EDS analysis found that the percentage of nitrogen has reduced in the HABs after the inactivation process, while the elements such as P, S were increased as a result of their release from the cytoplasm into the surface of the HABs (Fig. 5). The FTIR analysis for the functional groups on the HABs cell wall was carried out to confirm the presence or absence of the adverse effect as a result of algicidal substances (Fig. 6). The results revealed a reduction in the main functional groups which included OH, –C–H, non-conjugated groups, C–OH; these groups are available

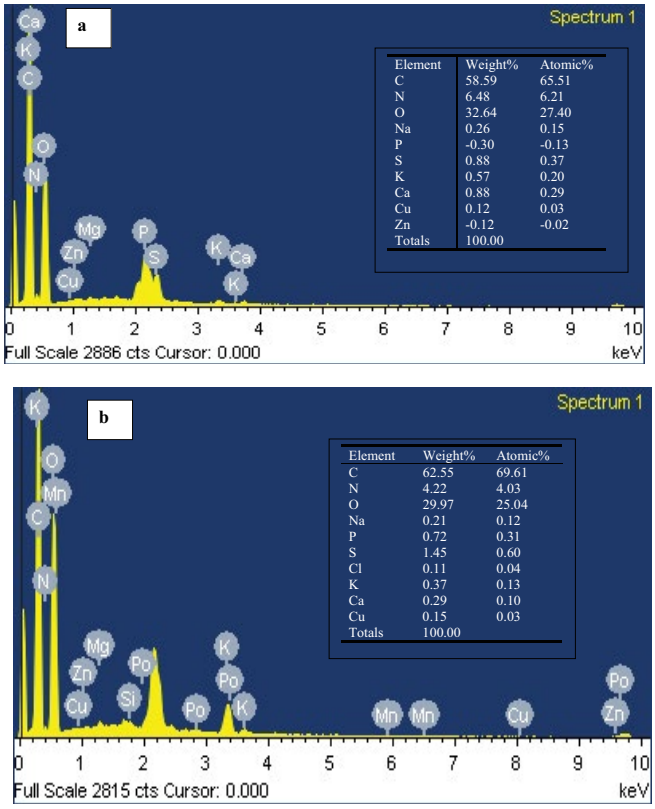

Fig. 5. EDS analysis shows the element contents in the HABs before (a) and after (b) the inactivation in freshwater using algicidal substances produced from *B. subtilis*.

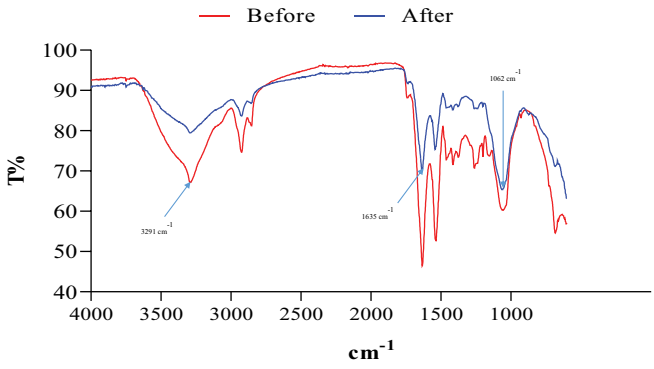

Fig. 6. FTIR analysis of HABs before and after the inactivation in reshwater using algicidal substances produced from *B. subtilis.*

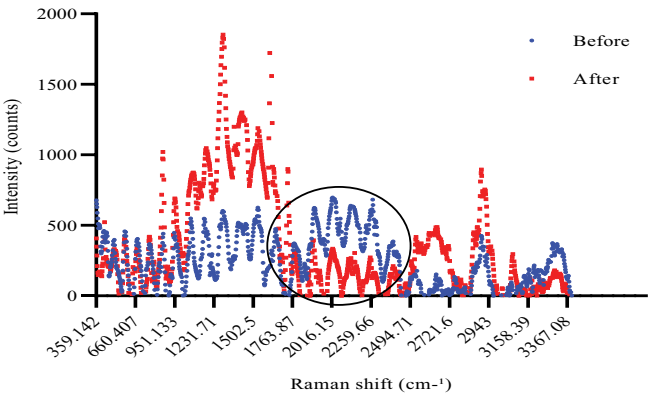

Fig. 7. Raman spectroscopy analysis of HABs before and after the inactivation in freshwater using algicidal substances produced from *B subtilis.*

in the protein and carbohydrate compounds (amine, pectin, cellulose). Therefore, the reduction in these functional groups is indication for the effect of algicidal substances on the protein and carbohydrate structures on the cell wall of HABs cells. The advance analysis using Raman spectroscopy confirmed the reduction in the amino groups due to the destruction caused by algicidal substances (Fig. 7). Indeed, the inactivation mechanism of algicidal activity against HABs based on the chemical analysis of the protein and carbohydrates structure of the HABs cell wall have not been reported before. Therefore, the current work has contributed effectively to understand more the inactivation mechanism of HABs by using algicidal substances.

4. Conclusion

The current work optimized the algicidal activity of HABs by using algicidal substances from *B. subtilis*. The optimal inactivation rate as determined based on the reduction of log cells and Chl a was recorded with 1.606 mL 100 mL^{-1}, within 6 h and pH 8, the reduction was 6 log and 90.22% of the chl a reduction (R^2 = 0.976). The SEM analysis confirmed the damaging of HABs due to the adverse effect of algicidal substance, which lead to the damage of protein and carbohydrate structure of the HABs cell wall. It can be concluded that algicidal substances from *B. subtilis* is suitable for controlling and preventing the distribution of HABS in the freshwater systems.

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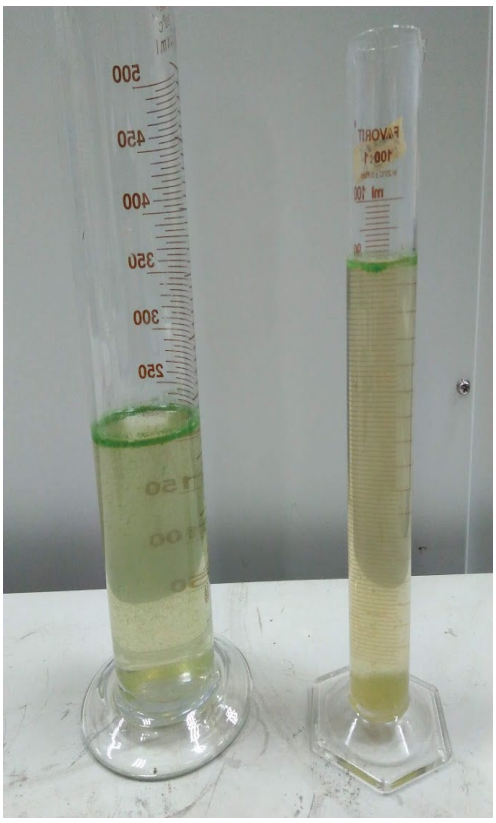
References

[1] L. Villacorte, S. Tabatabai, N. Dhakal, G. Amy, J. Schippers, M. Kennedy, Algal blooms: an emerging threat to seawater reverse osmosis desalination, Desal. Wat. Treat., 55 (2015) 2601–2611.
[2] L. Grattan, S. Holobaugh, J. Morris Jr, Harmful algal blooms and public health, Harmful Algae, 57 (2016) 2–8.

[3] O. DeNicola, S. Aburizaiza, A. Siddique, H. Khwaja, D. Carpenter, Climate change and water scarcity: the case of Saudi Arabia, Ann. Global Health, 81 (2015) 342–353.
[4] M. Darwish, H. Abdulrahim, S. Hassan, B. Shomar, Reverse osmosis desalination system and algal blooms Part I: harmful algal blooms (HABs) species and toxicity, Desal. Wat, Treat., 57 (2016) 25859–25880.
[5] N. Noyma, L. de Magalhães, L. Furtado, M. Mucci, F. van Oosterhout, V. Huszar, Controlling cyanobacterial blooms through effective flocculation and sedimentation with combined use of flocculants and phosphorus adsorbing natural soil and modified clay, Water Res., 97 (2016) 26–38.
[6] J. Naceradska, M. Pivokonsky, L. Pivokonska, M. Baresova, R. Henderson, A. Zamyadi, V. Janda, The impact of pre-oxidation with potassium permanganate on cyanobacterial organic matter removal by coagulation, Water Res., 114 (2017) 42–49.
[7] J. Su, X. Yang, Y. Zhou, T. Zheng, Marine bacteria antagonistic to the harmful algal bloom species *Alexandrium tamarense* (Dinophyceae), Biol. Control, 56 (2011) 132–138.
[8] C. Miao, Y. Tang, H. Zhang, Z. Wu, X. Wang, Harmful algae blooms removal from fresh water with modified vermiculite, Environ. Technol., 35 (2013) 340–346.
[9] R. Eaton, P. Sandusky, Biotransformations of (+/−)-geosmin by terpene-degrading bacteria, Biodegradation, 21 (2010) 71–79.
[10] B. Zhou, R. Yuan, C. Shi, L. Yu, J. Gu, C. Zhang, Biodegradation of geosmin in drinking water by novel bacteria isolated from biologically active carbon, J. Environ. Sci., 23 (2011) 816–823.
[11] J. Luo, Y. Wang, S. Tang, J. Liang, W. Lin, L. Luo, Isolation and identification of algicidal compound from Streptomyces and algicidal mechanism to *Microcystis aeruginosa*, PloS One, 8 (2013) e76444.
[12] H. Zhang, Y. Peng, S. Zhang, G. Cai, Y. Li, X. Yang, T. Zheng, Algicidal effects of Prodigiosin on the harmful algae *Phaeocystis globosa*, Front. Microbiol., 7 (2016), doi: 10.3389/fmicb.2016.00602.
[13] X. Yang, X. Li, Y. Zhou, W. Zheng, C. Yu, T. Zheng, Novel insights into the algicidal bacterium DH77-1 killing the toxic dinoflagellate *Alexandrium tamarense*, Sci. Environ., 482 (2014) 116–124.
[14] E. Noman, N. Rahman, M. Shahadat, H. Nagao, A. Al-Karkhi, A. Al-Gheethi, A. Omar, Supercritical fluid co2 technique for destruction of pathogenic fungal spores in solid clinical wastes, Clean Soil Air Water, 44 (2016) 1700–1708.
[15] E. Noman, A. Al-Gheethi, B. Talip, R. Mohamed, A. Kassim, Inactivating pathogenic bacteria in greywater by biosynthesized Cu/Zn nanoparticles from secondary metabolite of *Aspergillus iizukae*; optimization, mechanism and techno economic analysis, PloS One, 14 (2019) e0221522.
[16] M. Bezerra, R. Santelli, E. Oliveira, L. Villar, L. Escaleira Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta, 76 (2008) 965–977.
[17] KDHE (2015), Harmful Algae Blooms: A Tool kit for health departments. Kansas department of health and environment bureau of epidemiology and public health informatics, 1000 SW Jackson Street, Suite 330. Topeka, KS. 66612–1365. www.kdheks.gov/algae-illness/index.htm.
[18] APHA, (2012) Standard Methods for the Examination of Water and Waste Water. 22nd Edition, American Public Health Association, American Water Works Association, Water Environment Federation, 2012.
[19] A. Brown, Benson's Microbiological Applications: Laboratory Manual in General Microbiology, P.E. Reidy, J.S. Fornango, Eds., McGraw-Hill, Inc., New York, 2005.
[20] C. Tian, X. Liu, J. Tan, S. Lin, D. Li, H. Yang, Isolation, identification and characterization of an algicidal bacterium from Lake Taihu and preliminary studies on its algicidal compounds, J. Environ. Sci., 24 (2012) 1823–1831.
[21] STAATT (2005) Technical Assistance Manual: State Regulatory Oversight of Medical Waste Treatment Technology, Report of the State and Territorial Association on Alternative Treatment Technologies (STAATT).
[22] A. Hamed, A. Noman, A. Al-Gheethi, R. Mohamed, B. Talip, A. Ab. Aziz, M Amir Hashim, Statistical Optimization of Optimization of algicidal activity of indigenous strain Schizophyllum commune 104UTHM against *Microcystis aeruginosa* in freshwater using response surface methodology, Desal. Wat. Treat., (2019) 356–368.
[23] S. Jeffrey, G. Humphrey, New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton, Biochemie Physiol. Pflanz., 167 (1976) 191–194.
[24] B. Hollister, Kreakie, Associations between chlorophyll a and various microcystin health advisory concentrations, F1000 Res., 5 (2016), doi: 10.12688/f1000research.7955.2.
[25] H. Zhang, H. Wang, W. Zheng, Z. Yao, Y. Peng, S. Zhang, S. Hu, Z. Tao, Z. Zheng, Toxic effects of prodigiosin secreted by *Hahella* sp. KA22 on Harmful Alga *Phaeocystis globosa*, Front. Microbiol., 8 (2017) 999.
[26] J. Shao, Y. Jiang, Z. Wang, L. Peng, S. Luo, J. Gu, R. Li, Interactions between algicidal bacteria and the cyanobacterium *Microcystis aeruginosa*: lytic characteristics and physiological responses in the cyanobacteria, Int. J. Environ. Sci. Technol., 11 (2013) 469–476.
[27] T. Shimizu, T. Oda, H. Ito, I. Imai, I. Isolation and characterization of algicidal bacteria and its effect on a musty odor-producing cyanobacterium *Dolichospermum crassum* in a reservoir, Water Sci. Technol. Water Supply, 17 (2016) 792–798.
[28] B. Wang, Y. Tao, Q. Liu, N. Liu, Z. Jin, X. Xu, Algicidal activity of Bacillamide alkaloids and their analogues against marine and freshwater harmful algae, Marine Drugs, 7; 15 (2017) pii: E247, doi: 10.3390/md15080247.
[29] P. Uronen, P. Kuuppo, C. Legrand, T. Tamminen, Allelopathic effects of toxic haptophyte *Prymnesium parvum* lead to release of dissolved organic carbon and increase in bacterial biomass, Microbial Ecol., 54 (2007) 183–193.

Appendix

Appendix A show the algae bloom in Fahad dam at Bisha.


500
450
400
350
300
250
100 ml