# Absorption of hexavalent chromium by green micro algae
## *Chlorella sorokiniana*: live planktonic cells

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## Abstract

Hexavalent chromium Cr (VI) is a toxic heavy metal that discharged by many industries into the water streams. It is the most toxic form of chromium compound, which causes significant damage to receiving ecosystems. A microalgal species, *Chlorella* sp., was used as a biosorbent material to remove Cr (VI) from Cr-contaminated effluents. Furthermore, different variables: pH, temperature, contact time, Cr (VI) concentration and algal dose, were optimized in order to determine the optimum conditions that achieve the highest removal efficiency. The optimization process was achieved through two steps: one factor at a time (OFAT) experiments followed by $2^5$ general full factorial. Moreover, molecular identification was performed using 18S rRNA in order to demonstrate the species of *Chlorella*, and it was identified as *Chlorella sorokiniana*. The highest chromium removal efficiency of 99.6793% was achieved at 100 ppm Cr (VI) after three days' contact time. Chlorophyll 'a' estimation as a growth indicator stated that *Chlorella sorokiniana* can tolerate 100 ppm Cr (VI) for three days' exposure. The results suggested that *Chlorella sorokiniana* is a good biosorbent material and it distinguished by its high ability to uptake Cr (VI) from solutions.

Key words: biosorption, chromium removal, *Chlorella* sp, factorial design, microalgae, tolerance

## INTRODUCTION

Water pollution with heavy metals is a severe socio-environmental problem that caused by discharging industrial effluents into water streams. Such effluents are highly contaminated with heavy metals that show many hazardous effects on the aquatic ecosystem (Mahdavi *et al.* 2012; Shanab *et al.* 2012). Hexavalent chromium [Cr (VI)] is a toxic heavy metal that is used in many industries, such as electroplating, leather tanning and metal finishing. It has been reported that Cr (VI) has been found in ground water at concentrations that range from between 0.2 and 180 µg/l. Furthermore, it has been detected in soil and rocks at an average concentration of 2,200 and 2,650 mg/kg, respectively (Chrysochoou *et al.* 2016). Cr (VI) contaminated aquifers have been found in two locations. According to Dermatas *et al.* 2017, the first, Cr (VI) contaminated aquifer was in Northern Greece with a concentration of 64 µg/l and it was considered to be the highest detected geogenic concentration to have been found in regions that have this geological background. The second Cr (VI) contaminated aquifer was at INofyta (Central Greece), with a peak concentration

of 10,000 μg/l as a result of anthropogenic activities (Dermatas *et al.* 2017). Furthermore, Cr (VI) gets to humans through drinking water or food such as fish or plants, where it has many hazardous effects on human health. Therefore, many conventional methods were used for chromium removal: ion exchange, electrodialysis, ultrafiltration, chemical precipitation and reverse osmosis (Nataraj *et al.* 2007; Rajput *et al.* 2016; An *et al.* 2017; Yan *et al.* 2017; Zamri *et al.* 2017). However, these technologies are very expensive, generate toxic non-ecofriendly waste material and are not effective at a low chromium concentration (Singh *et al.* 2014). Alternatively, many researchers were directed to use several non-conventional methods such as biosorption for better and safe removal. Biosorption is the process of binding cations passively by both live and dead biomass (Abdi & Kazemi 2015). Agriculture wastes, plants, fungal, bacterial and algal biomass have been used as potential cost-effective biosorbents (Abioye *et al.* 2018; Almeida & Corso 2014; Fawzy *et al.* 2016; Kavitha *et al.* 2016; Naghipour *et al.* 2016; Thatheyus & Ramya 2016). As algae are photosynthetic organisms, both living and non-living cells are capable of Cr (VI) removal (Sen & Dastidar 2010). Algal strain selection is a key consideration in the chromium removal process. There are many species of algae that can accumulate high concentrations of Cr (VI), such as *Scenedesmus dimorphous, Nanoclropsis, Spirulina, Neochloris oleoabundans, Nannochloroposis, Botrycoccus braunii, Sargassum sp, Dunaliella salina, Chlorella colonials* and *Chroococcus disperses* (Aslan & Kapdan 2006; Chalivendra 2014; Hedayatkhah *et al.* 2018; Kaparapu & Prasad 2018; Jaafari & Yaghmaeian 2019). Although many researchers have investigated *Chlorella* sp. for heavy metal removal, most of them used the dead form only. In this study, a live planktonic form was used in order to investigate the ability of live *Chlorella* sp. to remove Cr (VI) and to estimate its tolerance to chromium toxicity. One factor at a time (OFAT) experiments and general full factorial design were performed to obtain the optimum conditions that maximize the removal of Cr (VI). Chlorophyll A was estimated in order to investigate the ability of *Chlorella* sp. to grow in the presence of Cr (VI). Morphologically identified *Chlorella* sp. was further identified using 18S rRNA. FT-IR analysis of the algal cell was used in order to identify the functional groups that found on the surface of the *Chlorella* sp. and helped in the removal process.

## MATERIALS AND METHOD

### Algal culture conditions

Soil samples were collected from 6th of October industrial zone, Cairo, Egypt. Micro-algal species were isolated and *Chlorella* sp. (microscopically confirmed) was purified from the cultivated sample and sub-cultured using Z-media (see Table 1) under light intensity (3000 lux) with pH 7 and temperature $25 \pm 1\,°C$.

### Chemicals

Different concentrations of chromium were prepared by dissolving an equivalent amount of potassium dichromate ($K_2Cr_2O_7$) in deionized water. Diphenyl carbazide has been used for chromium estimation using (M-ETKAL) 721 spectroscopy (Rice *et al.* 2012).

### Batch one factor at a time (OFAT) experiments

Batch experiments were performed in 250 ml Erlenmeyer flasks containing where Cr (VI) was added to 100 ml of Z-media instead of dist. water at pH 7. The experimental set up was incubated in an algal incubator with a light intensity of 3000 lux, and at room temperature for seven days. Furthermore, the

**Table 1** | Z-media components

| Nutrients | Nutrients | Stock solution (g/100 ml) dist.H₂O | Quantity (ml/l) |
|---|---|---|---|
| Macro-nutrients | $NaNO_3$ | 46.7 | 1 |
| | $Ca(NO_3)_2.4H_2O$ | 5.9 | 1 |
| | $K_2HPO_4$ | 3.1 | 1 |
| | $MgSO_4.7H_2O$ | 2.5 | 1 |
| | $Na_2CO_3$ | 2.1 | 1 |
| | EDTA-$FeCl_3$ solutions | – | 10 |
| Micro-nutrients | $Co(NO_3)_2.6H_2O$ | 14.6 mg | 80 μ/L |
| | $CuSO_4.5H_2O$ | 12.5 mg | |
| | $NiSO_4(NH4)2SO_4.6H_2O$ | 19.8 mg | |
| | $Cr(NO_3)_2.7H_2O$ | 3.7 mg | |
| | $V_2O_4(SO_4)_3.16H_2O$ | 3.5 mg | |
| | $MnSO_4.H_2O$ | 223 mg | |
| | $H_3PO_4$ | 310 mg | |
| | $Na_2SO_4.H_2O$ | 3.3 mg | |
| | KBr | 11.9 mg | |
| | KI | 8.3 mg | |
| | $ZnSO_4.7H_2O$ | 28.7 mg | |
| | $Cd(NO_3)_2.4H_2O$ | 15.4 mg | |
| | $Al_2(SO_4)3 K_2SO_4.24H_2O$ | 47.4 mg | |

sorption process parameters such as contact time in two states (static, shaking), Cr (VI) concentrations, pH values, temperature values and algal doses were optimized. The Cr (VI) was detected using Diphenyl carbazide method at 540 nm by 721 spectroscopy. After that, the removal efficiency (RE) was calculated by the following equation:

$$RE\ (\%) = \frac{C_o - C_e}{C_o} \times 100 \tag{1}$$

where, $C_o$ is the initial metal ion concentration of test solution, mg/l, $C_e$ is the final equilibrium concentration of test solution, mg/l.

The previous OFAT experiments were displayed as preliminary experiments in order to determine the low and the high levels of each factor.

## 2⁵ General full factorial design experiments

The $2^5$ general full factorial design experiments were performed according to the low and the high levels of the all factors that listed in Table 2. Factorial design matrix and the measured RE were demonstrated in Table 3 with fits and residuals values. The run orders of the experiments were

**Table 2** | Coded factors and its selected levels used in the full factorial optimization model for *Chlorella sorokiniana*

| Factor | Unit | Symbol | Statistical code | Values of coded levels | |
|---|---|---|---|---|---|
| | | | | −1 (Low) | +1 (High) |
| Time | days | Time (d) | A | Sh-1* | St-3** |
| pH | – | – | B | 6 | 8 |
| Temperature | °C | Temp. | C | 24 | 40 |
| Chromium concentration | ppm | Cr (VI) Conc. | D | 100 | 500 |
| Algal dose | ml/100 ml | algal dose | E | 5 | 20 |

*Sh-1 is one day of shaking contact time.
**St-3 is three days of static contact time.

**Table 3** | The design matrix and the $2^5$ full factorial design for *Chlorella sorokiniana*

| Run Order | Time (d) | pH | Temp. | Cr (VI) conc. | Algal dose | RE (%) | FITS1* | RESI1** |
|---|---|---|---|---|---|---|---|---|
| 1 | St-3 | 6 | 29 | 500 | 20 | 62.78771413 | 61.91690912 | 0.870805011 |
| 2 | St-3 | 8 | 40 | 500 | 20 | 78.08583638 | 77.48093371 | 0.604902668 |
| 3 | Sh-1 | 6 | 29 | 100 | 20 | 97.82637147 | 96.95556646 | 0.870805011 |
| 4 | Sh-1 | 8 | 29 | 500 | 20 | 54.25272911 | 53.64782644 | 0.604902668 |
| 5 | Sh-1 | 6 | 29 | 100 | 5 | 57.58835453 | 58.45915954 | 0.870805011 |
| 6 | Sh-1 | 8 | 40 | 100 | 20 | 99.67925634 | 99.07435367 | 0.604902668 |
| 7 | Sh-1 | 8 | 40 | 500 | 5 | 33.49441597 | 32.8895133 | 0.604902668 |
| 8 | St-3 | 6 | 29 | 500 | 5 | 15.86077956 | 16.73158457 | 0.870805011 |
| 9 | Sh-1 | 8 | 29 | 500 | 5 | 35.67302736 | 36.27793003 | 0.604902668 |
| 10 | St-3 | 8 | 40 | 100 | 20 | 98.42262384 | 99.0275265 | 0.604902668 |
| 11 | Sh-1 | 8 | 40 | 100 | 5 | 59.22970304 | 59.83460571 | 0.604902668 |
| 12 | St-3 | 6 | 40 | 100 | 20 | 98.68149952 | 97.8106945 | 0.870805011 |
| 13 | Sh-1 | 8 | 40 | 500 | 20 | 52.41582289 | 53.02072555 | 0.604902668 |
| 14 | St-3 | 6 | 40 | 500 | 20 | 51.60067776 | 52.47148277 | 0.870805011 |
| 15 | St-3 | 6 | 29 | 100 | 20 | 99.21292426 | 100.0837293 | 0.870805011 |
| 16 | St-3 | 8 | 29 | 500 | 20 | 48.27407625 | 48.87897892 | 0.604902668 |
| 17 | Sh-1 | 6 | 40 | 500 | 20 | 55.1353958 | 54.26459079 | 0.870805011 |
| 18 | St-3 | 8 | 40 | 100 | 5 | 34.52859262 | 33.92368996 | 0.604902668 |
| 19 | Sh-1 | 6 | 40 | 500 | 5 | 39.0493818 | 39.92018681 | 0.870805011 |
| 20 | Sh-1 | 6 | 29 | 500 | 5 | 41.14755754 | 40.27675253 | 0.870805011 |
| 21 | Sh-1 | 8 | 29 | 100 | 5 | 69.88588709 | 69.28098442 | 0.604902668 |
| 22 | Sh-1 | 8 | 29 | 100 | 20 | 90.81617814 | 91.42108081 | 0.604902668 |
| 23 | Sh-1 | 6 | 40 | 100 | 20 | 94.63679288 | 95.5075979 | 0.870805011 |
| 24 | St-3 | 6 | 40 | 100 | 5 | 30.18093971 | 31.05174473 | 0.870805011 |
| 25 | Sh-1 | 6 | 40 | 100 | 5 | 71.13638123 | 70.26557622 | 0.870805011 |
| 26 | Sh-1 | 6 | 29 | 500 | 20 | 52.57095878 | 53.44176379 | 0.870805011 |
| 27 | St-3 | 6 | 40 | 500 | 5 | 14.99607798 | 14.12527296 | 0.870805011 |
| 28 | St-3 | 8 | 29 | 500 | 5 | 12.30749964 | 11.70259697 | 0.604902668 |
| 29 | St-3 | 8 | 29 | 100 | 5 | 34.67104568 | 35.27594835 | 0.604902668 |
| 30 | St-3 | 8 | 40 | 500 | 5 | 50.56561324 | 51.17051591 | 0.604902668 |
| 31 | St-3 | 8 | 29 | 100 | 20 | 97.51231598 | 96.90741331 | 0.604902668 |
| 32 | St-3 | 6 | 29 | 100 | 5 | 12.92269182 | 12.0518868 | 0.870805011 |

*Fits are the value of point estimates of the mean CRE for given values of the factors.
**Residuals are the difference between observed value and its corresponding fitted value.

randomized to avoid the systematic errors. The obtained results were analyzed using Minitab 18 software. Furthermore, the main and interaction effects, pareto chart and normal probability were plotted and interpreted.

## Statistical analysis

The OFAT experiments were analyzed using one-way analyses of variance (ANOVA) in order to determine the significance between the different levels at alpha level ($\alpha$) = 0.05. Shapiro-Wilk's test was used to assess the assumptions of normality, while Levene's test was used to determine homogeneity of variances. Data transformation was performed prior to running the ANOVA models. In full-factorial model, analysis of variance (ANOVA) (see Table 4) was performed to determine the significant

**Table 4** | Estimated effects and coefficients for Cr (VI) RE (%)

| Term | Effect | Coef | SE coef | T-value | P-value |
|---|---|---|---|---|---|
| Constant | | 57.661 | 0.530 | 108.76 | 0.000 |
| Time (d) | −10.245 | −5.123 | 0.530 | −9.66 | 0.011 |
| pH | 3.405 | 1.703 | 0.530 | 3.21 | 0.085 |
| Temp. | 4.908 | 2.454 | 0.530 | 4.63 | 0.044 |
| Cr(VI) conc. | −28.045 | −14.022 | 0.530 | −26.45 | 0.001 |
| Ch dose | 38.667 | 19.334 | 0.530 | 36.47 | 0.001 |
| Time (d)*pH | 5.111 | 2.555 | 0.530 | 4.82 | 0.040 |
| Time (d)*Temp. | 4.281 | 2.141 | 0.530 | 4.04 | 0.056 |
| Time (d)*Cr(VI) conc. | 6.588 | 3.294 | 0.530 | 6.21 | 0.025 |
| Time (d)*Ch dose | 14.901 | 7.450 | 0.530 | 14.05 | 0.005 |
| pH*Temp. | 2.971 | 1.485 | 0.530 | 2.80 | 0.107 |
| pH*Cr(VI) conc. | 0.585 | 0.293 | 0.530 | 0.55 | 0.637 |
| pH*Ch dose | −2.529 | −1.265 | 0.530 | −2.39 | 0.140 |
| Temp.*Cr(VI) conc. | 1.651 | 0.825 | 0.530 | 1.56 | 0.260 |
| Temp.*Ch dose | −1.732 | −0.866 | 0.530 | −1.63 | 0.244 |
| Cr(VI) conc.*Ch dose | −12.163 | −6.082 | 0.530 | −11.47 | 0.008 |
| Time (d)*pH*Temp. | 5.050 | 2.525 | 0.530 | 4.76 | 0.041 |
| Time (d)*pH*Cr(VI) conc. | 1.896 | 0.948 | 0.530 | 1.79 | 0.216 |
| Time (d)*pH*Ch dose | −3.483 | −1.742 | 0.530 | −3.29 | 0.081 |
| Time (d)*Temp.*Cr(VI) conc. | 3.165 | 1.582 | 0.530 | 2.98 | 0.096 |
| Time (d)*Temp.*Ch dose | −2.706 | −1.353 | 0.530 | −2.55 | 0.125 |
| Time (d)*Cr(VI) conc.*Ch dose | −4.650 | −2.325 | 0.530 | −4.39 | 0.048 |
| pH*Temp.*Cr(VI) conc. | 6.484 | 3.242 | 0.530 | 6.12 | 0.026 |
| pH*Temp.*Ch dose | 3.291 | 1.645 | 0.530 | 3.10 | 0.090 |
| pH*Cr(VI) conc.*Ch dose | 1.273 | 0.636 | 0.530 | 1.20 | 0.353 |
| Temp.*Cr(VI) conc.*Ch dose | 0.012 | 0.006 | 0.530 | 0.01 | 0.992 |
| Time (d)*pH*Temp.*Cr(VI) conc. | 5.526 | 2.763 | 0.530 | 5.21 | 0.035 |
| Time (d)*pH*Temp.*Ch dose | −0.701 | −0.351 | 0.530 | −0.66 | 0.576 |
| Time (d)*pH*Cr(VI) conc.*Ch dose | −0.271 | −0.136 | 0.530 | −0.26 | 0.822 |
| pH*Temp.*Cr(VI) conc.*Ch dose | −3.597 | −1.798 | 0.530 | −3.39 | 0.077 |

S = 2.998, R-sq = 99.93%, R-sq(adj) = 98.87%.

differences between and within (i.e. low and high levels) factors at $\alpha = 0.05$. Model reduction was displayed to identify all potential terms in the model. Backward elimination method at alpha to remove equal to 0.05 was used to reduce the model. The fitted values and the residuals were calculated for each run in order to calculate the error estimation. Moreover, the response optimizer was used to identify the combination of levels settings from all the factors that maximize the removal efficiency of Cr (VI).

## Chlorophyll 'a' estimation

Effect of chromium on *Chlorella* sp. growth was studied through an estimation of chlorophyll 'a' where different time intervals (1, 2, 3, 4, 6, 7, 9 days) were tested with a fixed pH 7, a chromium dose of 20 mg/100 ml, and a Cr (VI) concentration of 100 ppm. Furthermore, different Cr (VI) concentrations (0, 100, 200, 400, 600 ppm) were investigated when the pH was 7 and the Ch dose 20 mg/100 ml Cr (VI) solution. At the end, the effect of the Ch dose (5, 10, 15, and 20 mg/100 ml

Cr (VI) solution) was studied at 100 ppm Cr (VI), pH 7. All the experiments were undertaken at a room temperature 25 °C $\pm$ 1. Dimethyl sulfoxide (DMSO) method was used for chlorophyll 'a' extraction and estimation as follow:

The chlorophyll 'a' was extracted using 1:1 (v/v) dimethyl sulfoxide (DMSO) and 90% acetone as a solvent. Afterwards, the absorbance was determined at 664, 647 nm.

The following equation has been used for chlorophyll 'a' calculations in μg/l (Jeffrey & Humphrey 1975):

$$\text{Chlorophyll a content} = \frac{11.93 \ E_{664} - 1.93 \ E_{647} \times v}{V \times L} \tag{2}$$

where:

$E_{664}$ = absorbance at 664 nm.
$E_{647}$ = Absorbance at 647 nm.
$v$ = volume of extracting solvent in ml.
V = volume in sample in L.
L = the path-length of cuvette in cm.

## Molecular identification

*Chlorella* sp. that was previously confirmed microscopically was identified using 18S rRNA as follows:

### DNA extraction

The sample was enriched and cultivated on Z-media under a light intensity (3000 lux) with pH 7 at a temperature 25 $\pm$ 1 °C for seven days. After cultivation, the sample was collected and centrifuged at 20,000 rpm for 15 min. The total genomic of micro-algal sample was extracted and purified using a GeneJet Genomic DNA purification Mini Kit (Thermo Scientific) according to manufacturer's protocol. The extracted DNA was diluted to an adequate concentration with sterile water for subsequent PCR reactions to minimize the formation of heteroduplexe molecules and stored at −20 °C until further use.

### Amplification of 18S rRNA of *Chlorella sp*

Primer set, forward (5′-AACCTGGTTGATCCTGCCAG-3′) and Reverse (5′-CACCAGACTTGCCCTCCA-3′) was used to get a fragment of approximately 500 bp. PCR amplification was carried out in a thermal cycler (Creacon, Holland). The reaction mixture (50 μl) contained 1 μl template DNA (50 ng/μl), 25 μl hot start master mix, 1 μl from each primer (5 pmol/μl) and 23 μl biodest water (Hot Start Taq QIAGEN). Thermal cycling was carried out by using an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 1 min. Cycling was completed by a final elongation step of 72 °C for 7 min. Final product for specific amplicon (approx., 869 bp) was photograph and detection using Dig-doc, UVP, INC, England. DNA molecular weight ladder 1 kbp DNA marker (PeqGold 1 Kb, Peqlab, GmH) was used to estimate final amplified product length. The PCR product was loaded on 1.5% (w/v) Agarose gel, stained with ethidium bromide, separated by electrophoresis (75 V, 150 mA) and viewed on UV plate. Gene JET PCR Purification Kit (Thermo Scientific) was used for DNA purification. ABI PRISM® 3100 Genetic Analyzer 3100 (Applied Bio-system) was applied for PCR products and performed by Macrogen Inc. Seal, Korea. Gel documentation system (Geldoc-it,

UVP, and England) was applied for data analysis using Total lab analysis software, ww.totallab.com, (Ver.1.0.1). Aligned sequences were analyzed on the NCBI website (http://www.ncbi.nlm.nih.gov) using BLAST to confirm their identity. The genetic distances and multi-alignments were computed by Pairwise Distance method using Clustalw software analysis (www.ClustalW.com). The sequence was deposited in the Gen Bank with a number of Banklt2088194.

### FT-IR analysis

Algal cells were dried overnight and the dried material encapsulated with KBr discs. Fourier transform infrared spectroscopy (FT-IR) was used in order to detect the functional groups of the algal cell. Spectra were detected in the wavelength range from 400–4,000 $Cm^{-1}$.

## RESULTS AND DISCUSSIONS

### Morphological identification of algal cells

The vegetative *Chlorella* sp. cells, shown in Figure 1, were spherical, 5.5–8.4 μm in diameter, oval after division, with a fluffy, smooth, and colorless membrane. Chloroplast 'a' was a parietal cup with basal pyrenoid and during reproduction, the cell contents divide into (2–4) auto-spores. These are spherical cells 4.5–7.5 μm in diameter.
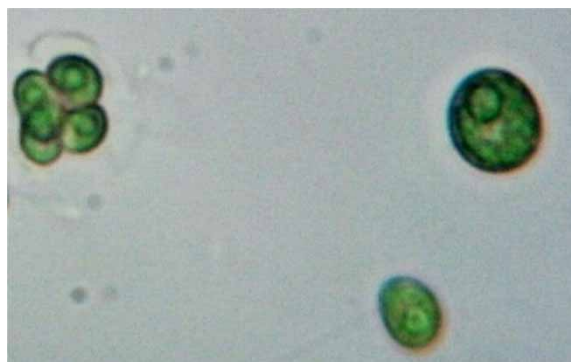


**Figure 1** | Vegetative cells of *Chlorella* sp. and its reproductive stage.

### Full factorial design

The results of a full factorial design experiment were analyzed. Furthermore, the main and the interaction effects, the pareto chart and the normal probability plot were obtained and interpreted as follows:

### Main effects

Plot of the main effects was designed to show the results of the regression analysis. Significant factors of 95% intervals have been displayed by this plot. The main effects were expressed a deviation of the average between the high and the low levels for each factor. The RE was increased as the factor change increased from the low to the high level and this indicated the positive effect of this factor.

In contrast to the negative effect, the RE was decreased as a result of reduction that occured from the low to the high level of the factor (Ponnusami *et al.* 2007). Furthermore, the larger the vertical line between the two levels, the larger the effect on the RE. This result was attributed to the length of the vertical line that was directly proportional to the statistical significant of a factor (Palanikumar & Davim 2009). Table 4 indicated that time, temperature, Cr (VI) concentration and algae dose (Ch dose) were significant factors, which aligns with previous studies (Saadat & Karimi-Jashni 2011). Furthermore, the main effects plot (Figure 2) indicates that Cr (VI) concentration and algae dose (Ch dose) were the most significant factors. However, Ch dose had a positive effect on the Cr (VI) RE. Nevertheless, Cr (VI) concentration had a negative effect. Furthermore, the plot interpretation suggests that the low level of Cr (VI) concentration factor, the low level of time and the high level of algal dose achieved the highest Cr (VI) RE.
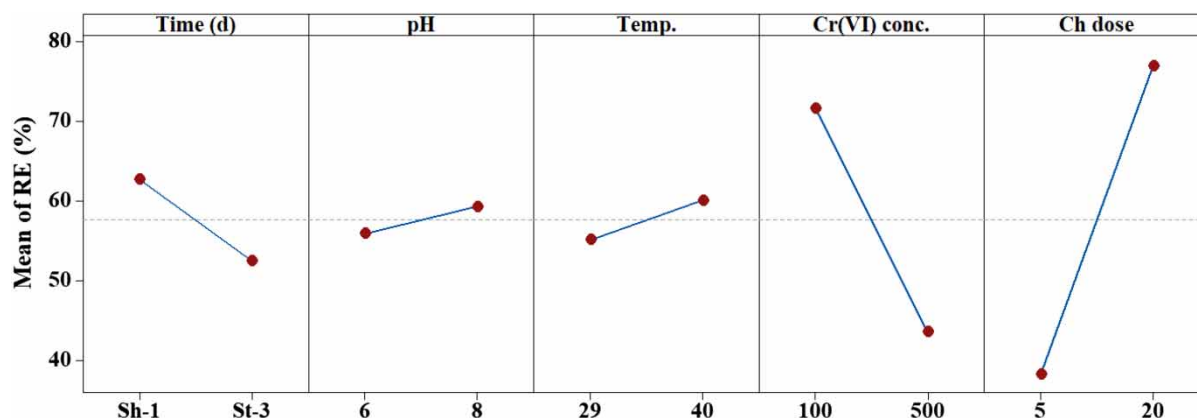


**Figure 2** | Main effects plot for the chromium removal effeciency (RE) at the low and the high levels of each factor by *Chlorella* sp.
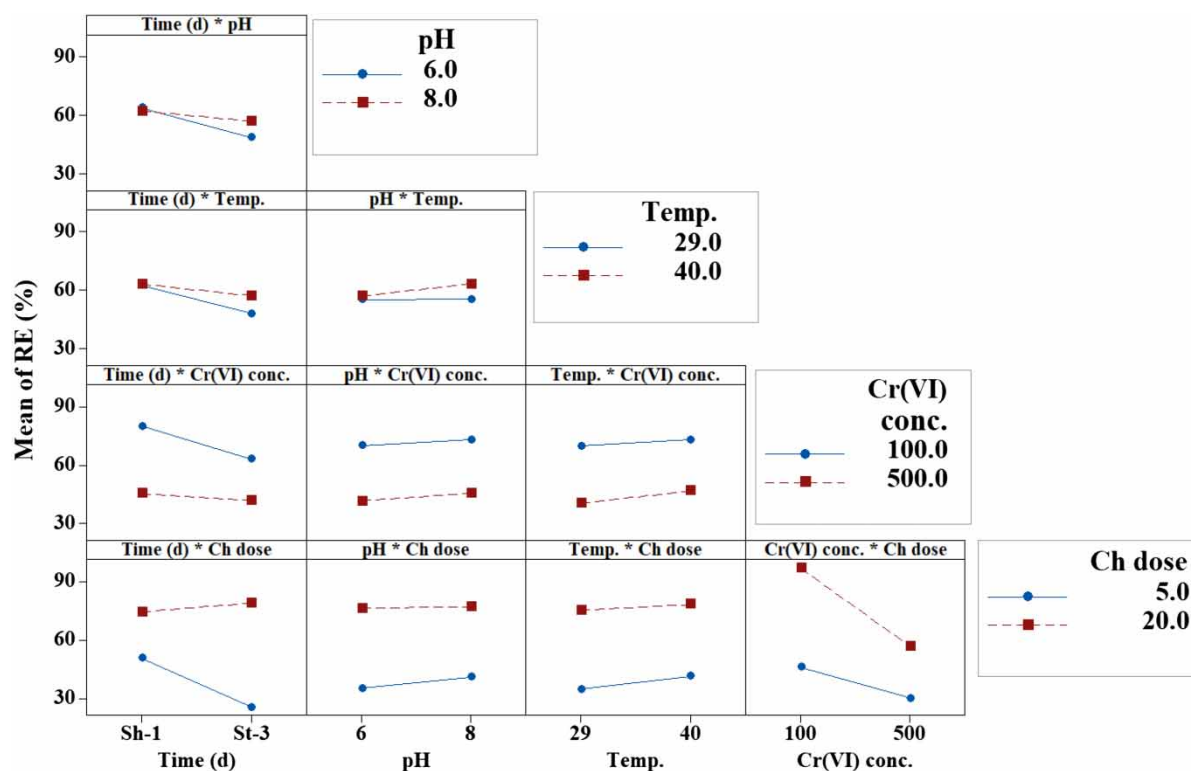
## Interaction effects

It is well known that effective interaction is achieved when there are changes from the low to the high levels of a factor, dependent on the levels of other factors. For instance, when lines of the levels of two factors don't run in parallel (Mathialagan & Viraraghavan 2005). Table 4 states that the two-way-interactions between 'Time and pH', 'Time and Cr (VI) conc.', 'Time and Ch dose', and 'Cr (VI) and Ch dose' were statistically significant, which means it affected the Cr (VI) RE. In addition, Figure 3 exhibites that the interactions between 'Time and pH' had a positive effect on the Cr (VI) RE due to the non-parallel lines that occurred between the levels of the two factors. Furthermore, Table 4 demonstrates that the three-way interactions 'Time, pH, and temperature', 'Time, Cr (VI) concentration, and Ch dose', 'pH, temperature, and Cr (VI) concentration', and the four-way interactions 'Time, pH, temperature, and Cr (VI) concentration affected the Cr (VI) RE.
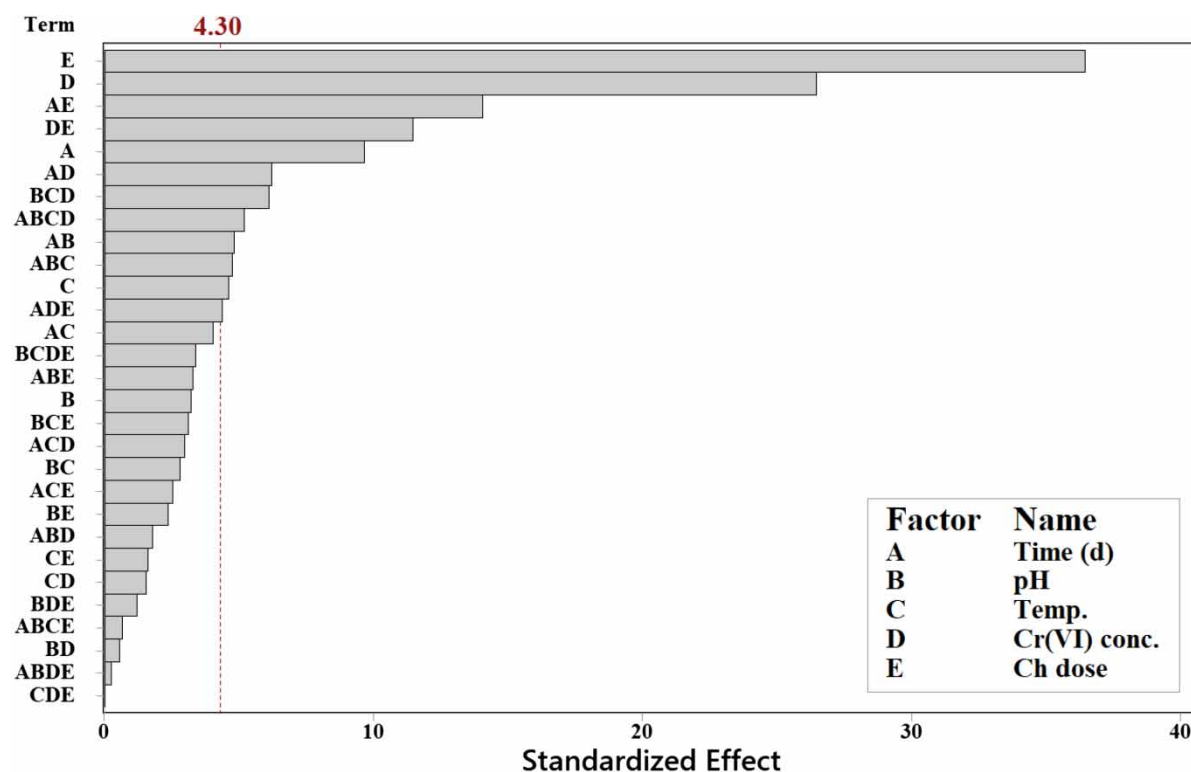
## Pareto chart

A Student's t-test was performed to estimate if the calculated effects were significantly different from zero or not (Ponnusami *et al.* 2007). For the 95% of confidence intervals and 16 degrees of freedom, the t-value reference line (Figure 4) was 4.30. It is well known that values that exceed the reference line are significant values, while values that do not exceed the reference line are non-significant values. Based on the previous rule, it was observed that algal dose (E), Cr (VI) concentration (D), time & Ch dose (AE), time (A), time & Cr (VI) concentration (AD), pH & temperature & Cr (VI) concentration (BCD), time & pH & temperature & Cr (VI) concentration (ABCD), time & pH (AB), time

**Figure 3** | Interaction effects plot for for the chromium removal effeciency (RE) by *Chlorella* sp.



**Figure 4** | Pareto chart of the standardized main and interaction effects of *Chlorella* sp.

& pH & temperature (ABC), temperature (C) and time & Cr (VI) concentration & algal dose (ADE) were significant values. The (E) and (D) were higher significantly than the other values that exhibited their positive effect on the Cr (VI) RE (Mathialagan & Viraraghavan 2005). These results were in a

good a agreement with previous studies where the concentration of pollutants was decreased from the low to the high levels of the factor (Boubakri *et al.* 2014).

### Normal probability plot

In the statistical data analysis, it's necessary to know if the data comes from normal distribution or not (Ruiz Espejo 2006). Data plotted in Figure 5 shows the normal probability distribution of the data. The points that fall close enough to the straight are normally distributed. Moreover, appearance of far points of algal dose (E) and Cr (VI) concentration (D) shows that they are real factors that had a great influence on the Cr (VI) RE (Palanikumar & Davim 2009; Brasil *et al.* 2006). This result confirmed the previous result obtained from the pareto chart.

From the all of previous results, it can be observed that the Cr (VI) RE was increased on increasing the Ch dose and decreased on increasing the Cr (VI) concentration. This may be attributed to the large number of the functional groups, which occurred on the cell wall surface of *Chlorella*, resulting from the increasing dose. Many researchers have reported that increasing doses caused an increase of pollutants' RE due to the availability of functional groups on the cell wall of *Chlorella*, such as carboxylic, amino, hydroxyl, amines, sulphonic etc. that increased the RE (Volesky 2007; Gupta & Rastogi 2008; Jaafari & Yaghmaeian 2019). On the other hand, the RE was decreased by increasing the Cr (VI) concentration due to the damage inflicted on the *Chlorella* cells by the higher concentrations of Cr (VI), as previously stated (Singh *et al.* 2016).
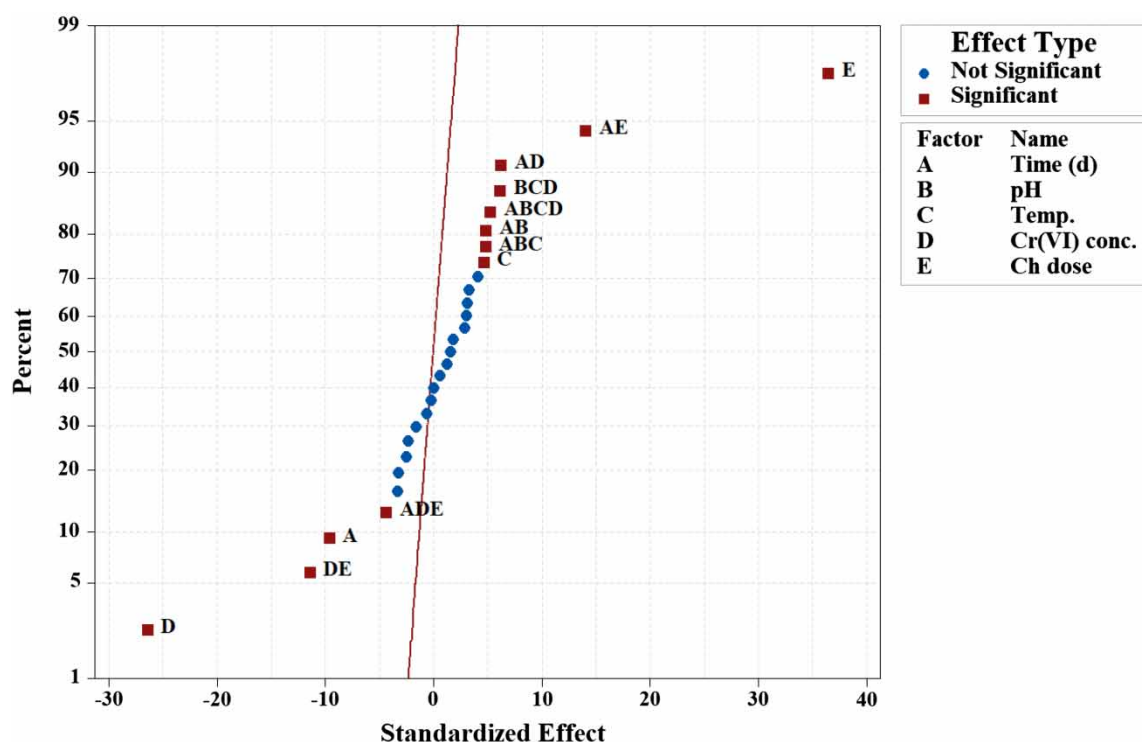


**Figure 5** | Normal probability plot of the standardized main and interaction effects.

### Response optimizer

Response optimizer is a tool that is used to determine the optimum conditions that achieve the highest RE. In the current work, the optimum combined conditions were a time of three days in the static state, at pH 6.2, temperature 29 °C, Cr (VI) concentration 100 mg/l and algal dose 20 mg/100 ml Cr (VI)-solution for a predicted RE of 99.67%, with a desirability score of 1 (see Figure 6).
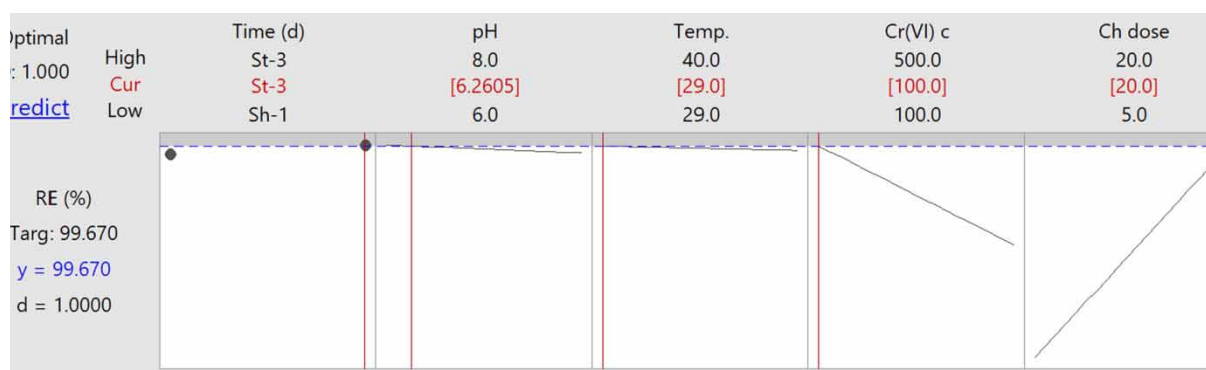
**Figure 6** | The optimization plot of the fitted values for the predictor settings to maximize the chromium removal effeciency (RE).

## Molecular identification

The phylogenetic analysis of the band sequence revealed that the sample was related to *Chlorella sorokiniana* strain KU-1019 18S ribosomal RNA gene, partial sequence with accession number of KF444207.1 with Query coverage of 97% and Identity of 86%.

## *Chlorella sorokiniana* tolerance to hexavalent chromium

In order to investigate the ability of *Chlorella sorokiniana* to tolerate Cr (VI) stress, chlorophyll 'a' (growth indicator) was estimated. For instance, Figure 7 showed a comparison of chlorophyll 'a' estimation between the Cr (VI)-treated and the non-treated (control) *Chlorella sorokiniana*. It was observed that the *Chlorella sorokiniana* can tolerate Cr (VI) until the third day where the growth in the third day was nearly 1,890.177 μg/l in comparison to the control 6,680.79 μg/l. After that, a slight decrease was observed from the fourth day where the chlorophyll 'a' result recorded 163.023 μg/l. Furthermore, Figure 8 demonstrated the effect of increasing algal dose with a correlation to its growth in Cr (VI)-treated medium when Cr (VI) concentration was 100 ppm, and pH 7.
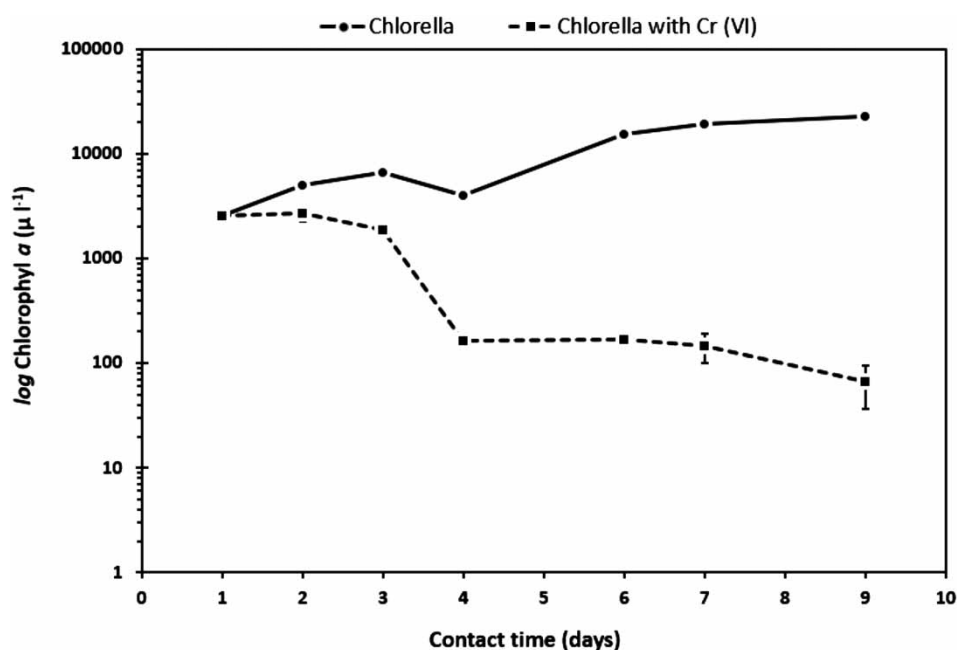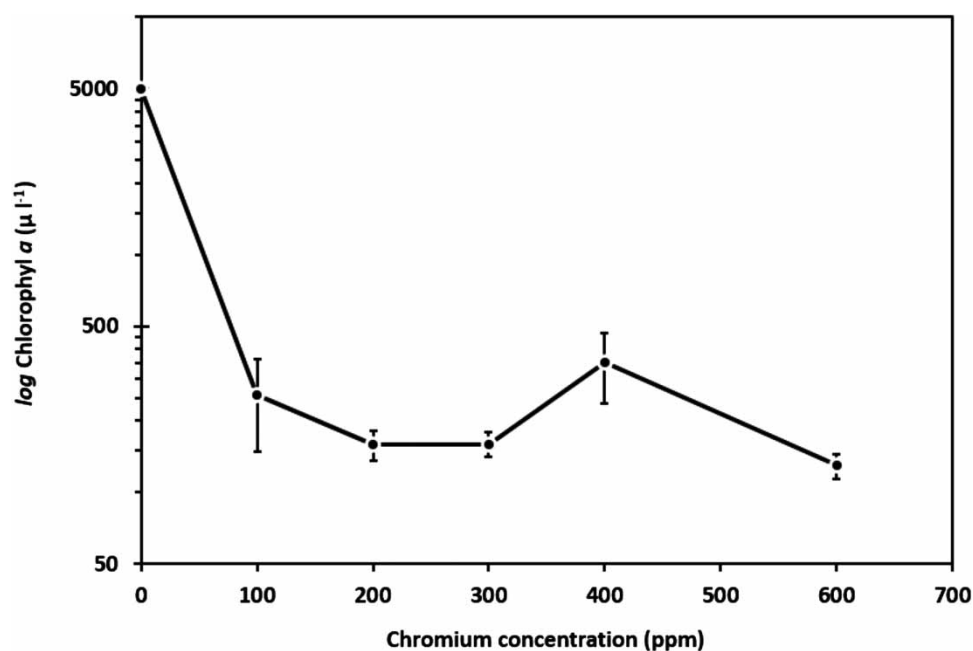


**Figure 7** | Chlorophyll a at different contact time.
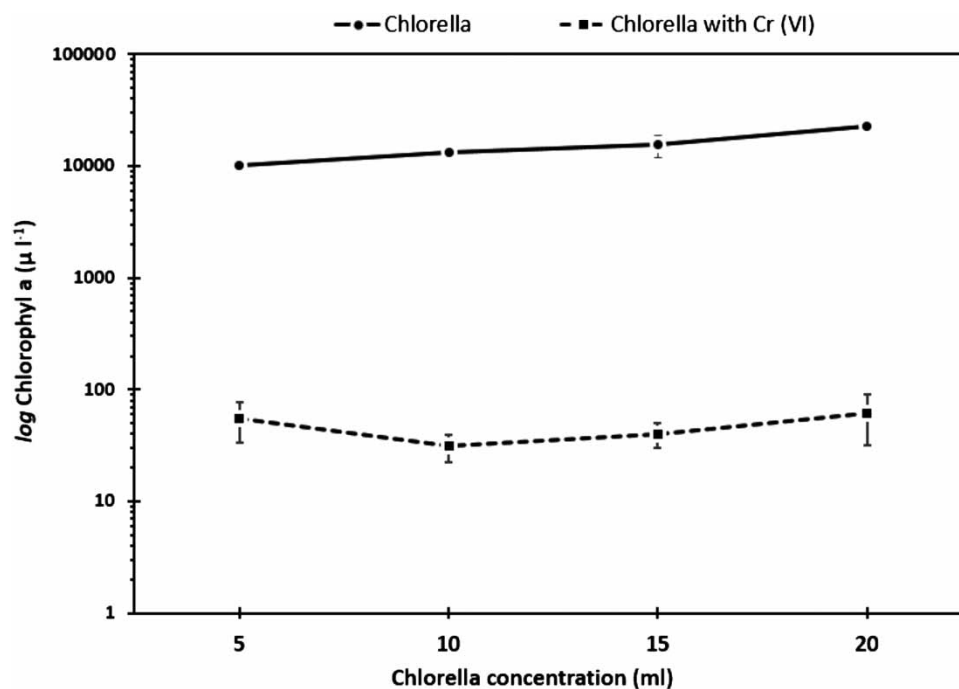
**Figure 8** | Chlorophyll a at different doses of *Chlorella* sp.

Furthermore, it was noticed that a slight increase in chlorophyll 'a' that resulted from increasing the Ch dose (see Figure 8). Nevertheless, increasing in the Cr (VI) concentration has displayed a negative effect on the *Chlorella sorokiniana* growth where it exhibited 256.55 μg/l at 100 ppm when the algal dose was 20 mg/100 ml, and the pH of 7. After increasing the Cr (VI) concentration to 100 ppm, the chlorophyll 'a' content was reduced respectively to the half of the value. Figure 9 illustrates the decreasing in the detected chlorophyll 'a' by increasing the concentration of Cr (VI) from (0–600). Error bars illustrated the standard deviation of each values.



**Figure 9** | Chlorophyll 'a' at different chromium concentrations.

### FT-IR analysis

The FT-IR spectrum of the *Chlorella sorokiniana* (Figure 10) displays a number of absorption peaks that indicate the complex nature of the examined sample. The appearance of different characteristic peaks refer to the heterogeneity of *Chlorella sorokiniana* as a biosorbent material. The IR absorption of each peak indicated a certain functional group: a peak of 3,422 showed the appearance of a strong, broad –OH group. The 2,953 band formation suggested a stretch –CH group and the peak 1,653 was stretch –C=O. Moreover, 1,544, 1,454, and 1,404 indicate the formation of stretch –C=C. $SO_3^{2-}$ and $PO_4^{3-}$ were formed at 1,244.46 and 1,072.74, respectively, and the peak 669 showed the appearance of halogens. Copious functional groups appeared on the cell wall of *Chlorella sorokiniana*, indicating its affinity for metal ions removal.

### Biosorption mechanism

Live planktonic cells of *Chlorella sorokiniana* have successfully removed Cr (VI) from polluted waste-water through a process called biosorption. Biosorption as a word consists of two parts: sorption, that means attachment of one substance on the surface of another substance via physico-chemical process, and bio 'prefix', that means by using of biological material. Furthermore, this method has the ability to remove heavy metals by two mechanisms (Gadd 2009). The first mechanism is a slow metabolic dependent that occurs in living cells only where metal ion uptake into the cell occurs through the cell membrane and cytoplasm of the cell. This type of mechanism was known as intracellular uptake or active uptake and bioaccumulation (Vymazal 1990; Herrero *et al.* 2008). Accordingly, death of *Chlorella* cells after three days' exposure to 100 ppm Cr (VI) suggested that the amount of absorbed Cr (VI) was toxic to the cells and caused its death. Furthermore, the second mechanism, which is regarded as a rapid metabolic independent and known as adsorption or passive uptake, was observed. In this mechanism, metal ion binds to the surface of the cell wall and extracellular material and is common between both live and dead cells. This return to multiple chemical groups carboxyl, sulphonate, hydroxyl and amino groups' availability on the surface of the cell wall of the algal cell that was confirmed by the FT-IR spectrum (Davis 2003). Another concerning point, heavy metals binding on the surface of the algal cells are dependent on many factors, such as the availability of active sites, their accessibility and the affinity between site and metal (Lacher & Smith 2002). Additionally, the role of shaking in the adsorption mechanism can't be denied where 99.07% RE was observed after 24 h shaking and this result was nearly the same obtained after three days' static time:
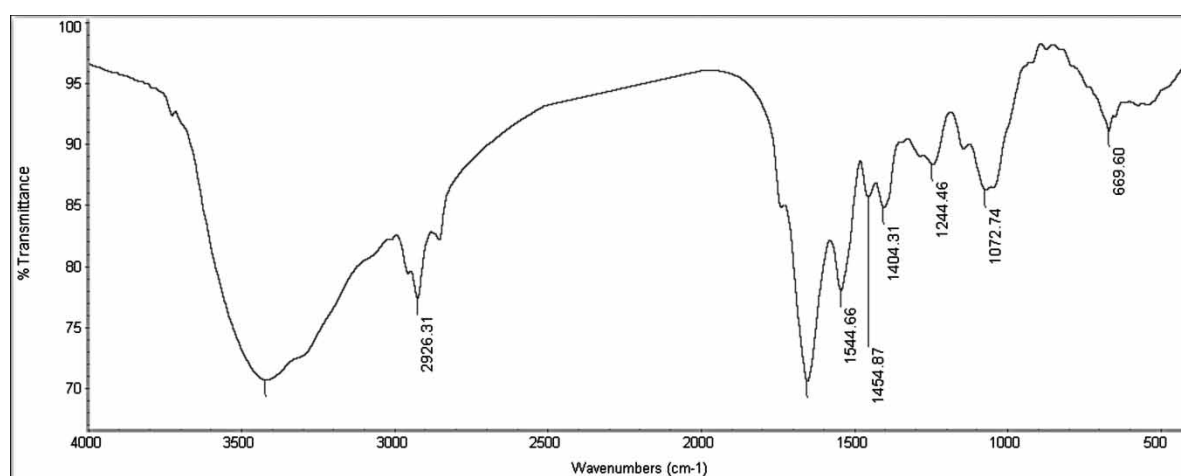


**Figure 10** | FT-IR spectra of *Chlorella* sp.

99.02%. This may be attributed to passive uptake discussed previously and as stated in previous research (Friis & Myers-Keith 1986; Al-Rub *et al.* 2006; Murphy *et al.* 2008).

## CONCLUSION

*Chlorella* sp. was successfully isolated, purified and applied in the Cr (VI) removal. The highest Cr (VI) RE of 99.6793% was achieved after 24 h contact time, pH 7, temperature 40 °C, shaking model and Cr (VI) concentration 100 ppm. Based on this result, the live planktonic form of *Chlorella* sp. was stated as an efficient biomass for Cr (VI) removal. Additionally, the chlorophyll 'a' result was nearly 1,890 μg/l after three days' exposure to 100 ppm of Cr (VI); after that growth declined. This suggested the ability of *Chlorella* sp. to tolerate Cr (VI) toxicity. Additionally, *Chlorella* sp. was successfully identified using 18Sr RNA as *Chlorella sorokiniana*. However, further studies are still needed to investigate a new method that facilitates harvesting and dewatering of algae cells after treatment processes such as biofilm formation from micro-algae.

## ACKNOWLEDGEMENTS

## REFERENCES

Abdi, O. & Kazemi, M. 2015 A review study of biosorption of heavy metals and comparison between different biosorbents. *Journal of Materials and Environmental Science* **6**(5), 1386–1399.

Abioye, O., Oyewole, O., Oyeleke, S., Adeyemi, M. & Orukotan, A. 2018 Biosorption of lead, chromium and cadmium in tannery effluent using indigenous microorganisms. *Brazilian Journal of Biological Sciences* **5**(9), 25–32.

Almeida, E. & Corso, C. 2014 Comparative study of toxicity of azo dye Procion Red MX-5B following biosorption and biodegradation treatments with the fungi *Aspergillus niger* and *Aspergillus terreus*. *Chemosphere* **112**, 317–322.

Al-Rub, F. A., El-Naas, M., Ashour, I. & Al-Marzouqi, M. 2006 Biosorption of copper on *Chlorella vulgaris* from single, binary and ternary metal aqueous solutions. *Process Biochemistry* **41**(2), 457–464.

An, A. K., Guo, J., Lee, E.-J., Jeong, S., Zhao, Y., Wang, Z. & Leikness, T. 2017 PDMS/PVDF hybrid electrospun membrane with superhydrophobic property and drop impact dynamics for dyeing wastewater treatment using membrane distillation. *Journal of Membrane Science* **525**, 57–67.

Aslan, S. & Kapdan, I. K. 2006 Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. *Ecological Engineering* **28**(1), 64–70.

Boubakri, A., Helali, N., Tlili, M. & Amor, M. B. 2014 Fluoride removal from diluted solutions by Donnan dialysis using full factorial design. *Korean Journal of Chemical Engineering* **31**(3), 461–466.

Brasil, J. L., Ev, R. R., Milcharek, C. D., Martins, L. C., Pavan, F. A., dos Santos Jr, A. A., Dias, S. L., Dupont, J., Norena, C. P. Z. & Lima, E. C. 2006 Statistical design of experiments as a tool for optimizing the batch conditions to Cr (VI) biosorption on *Araucaria angustifolia* wastes. *Journal of Hazardous Materials* **133**(1–3), 143–153.

Chalivendra, S. 2014 *Bioremediation of Wastewater Using Microalgae*. University of Dayton, Dayton, OH.

Chrysochoou, M., Theologou, E., Bompoti, N., Dermatas, D. & Panagiotakis, I. 2016 Occurrence, origin and transformation processes of geogenic chromium in soils and sediments. *Current Pollution Reports* **2**(4), 224–235.

Davis, T. 2003 A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research Water Research* **37**(18), 4311–4330.

Dermatas, D., Panagiotakis, I., Mpouras, T. & Tettas, K. 2017 The origin of hexavalent chromium as a critical parameter for remediation of contaminated aquifers. *Bulletin of Environmental Contamination and Toxicology* **98**(3), 331–337.

Fawzy, M., Nasr, M., Abdel-Gaber, A. & Fadly, S. 2016 Biosorption of Cr (VI) from aqueous solution using agricultural wastes, with artificial intelligence approach. *Separation Science and Technology* **51**(3), 416–426.

Friis, N. & Myers-Keith, P. 1986 Biosorption of uranium and lead by *Streptomyces longwoodensis*. *Biotechnology and Bioengineering* **28**(1), 21–28.

Gadd, G. M. 2009 Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. *Journal of Chemical Technology and Biotechnology* **84**(1), 13–28.

Gupta, V. & Rastogi, A. 2008 Equilibrium and kinetic modelling of cadmium (II) biosorption by nonliving algal biomass *Oedogonium* sp. from aqueous phase. *Journal of Hazardous Materials* **153**(1–2), 759–766.

Hedayatkhah, A., Cretoiu, M. S., Emtiazi, G., Stal, L. J. & Bolhuis, H. 2018 Bioremediation of chromium contaminated water by diatoms with concomitant lipid accumulation for biofuel production. *Journal of Environmental Management* **227**, 313–320.

Herrero, R., Lodeiro, P., Rojo, R., Ciorba, A., Rodríguez, P. & de Vicente, M. E. S. 2008 The efficiency of the red alga *Mastocarpus stellatus* for remediation of cadmium pollution. *Bioresource Technology* **99**(10), 4138–4146.

Jaafari, J. & Yaghmaeian, K. 2019 Optimization of heavy metal biosorption onto freshwater algae (*Chlorella coloniales*) using response surface methodology (RSM). *Chemosphere* **217**, 447–455.

Jeffrey, S. t. & Humphrey, G. 1975 New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochemie und Physiologie der Pflanzen* **167**(2), 191–194.

Kaparapu, J. & Prasad, M. K. 2018 Equilibrium, kinetics and thermodynamic studies of cadmium (II) biosorption on *Nannochloropsis oculata*. *Applied Water Science* **8**(6), 179.

Kavitha, G., Sridevi, V., Venkateswarlu, P. & Babu, N. C. 2016 Biosorption of chromium from aqueous solution by *Gracilaria corticata* (red algae) and its statistical analysis using response surface methodology. *Open Access Library Journal* **3**(09), 1.

Lacher, C. & Smith, R. W. 2002 Sorption of Hg(II) by *Potamogeton natans* dead biomass. *Minerals Engineering* **15**(3), 187–191.

Mahdavi, S., Jalali, M. & Afkhami, A. 2012 Removal of heavy metals from aqueous solutions using Fe 3 O 4, ZnO, and CuO nanoparticles. *Journal of Nanoparticle Research* **14**(8), 846.

Mathialagan, T. & Viraraghavan, T. 2005 Biosorption of pentachlorophenol by fungal biomass from aqueous solutions: a factorial design analysis. *Environmental Technology* **26**(5), 571–580.

Murphy, V., Hughes, H. & McLoughlin, P. 2008 Comparative study of chromium biosorption by red, green and brown seaweed biomass. *Chemosphere* **70**(6), 1128–1134.

Naghipour, D., Taghavi, K., Jaafari, J., Mahdavi, Y., Ghanbari Ghozikali, M., Ameri, R., Jamshidi, A. & Hossein Mahvi, A. 2016 Statistical modeling and optimization of the phosphorus biosorption by modified *Lemna minor* from aqueous solution using response surface methodology (RSM). *Desalination and Water Treatment* **57**(41), 19431–19442.

Nataraj, S., Hosamani, K. & Aminabhavi, T. 2007 Potential application of an electrodialysis pilot plant containing ion-exchange membranes in chromium removal. *Desalination* **217**(1–3), 181–190.

Palanikumar, K. & Davim, J. P. 2009 Assessment of some factors influencing tool wear on the machining of glass fibre-reinforced plastics by coated cemented carbide tools. *Journal of Materials Processing Technology* **209**(1), 511–519.

Ponnusami, V., Krithika, V., Madhuram, R. & Srivastava, S. 2007 Biosorption of reactive dye using acid-treated rice husk: factorial design analysis. *Journal of Hazardous Materials* **142**(1–2), 397–403.

Rajput, S., Pittman Jr., C. U. & Mohan, D. 2016 Magnetic magnetite (Fe3O4) nanoparticle synthesis and applications for lead (Pb2+) and chromium (Cr6+) removal from water. *Journal of Colloid and Interface Science* **468**, 334–346.

Rice, E. W.American Public Health, A., American Water Works, A. and Water Environment, F. 2012 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, D.C.

Ruiz Espejo, M. 2006 Design of Experiments for Engineers and Scientists. *Technometrics* **48**(2), 304–305.

Saadat, S. & Karimi-Jashni, A. 2011 Optimization of Pb (II) adsorption onto modified walnut shells using factorial design and simplex methodologies. *Chemical Engineering Journal* **173**(3), 743–749.

Sen, M. & Dastidar, M. G. 2010 Chromium removal using various biosorbents. *Iranian Journal of Environmental Health Science and Engineering* **7**(3), 182–190.

Shanab, S., Essa, A. & Shalaby, E. 2012 Bioremoval capacity of three heavy metals by some microalgae species (Egyptian isolates). *Plant Signaling & Behavior* **7**(3), 392–399.

Singh, S. K., Dixit, K. & Sundaram, S. 2014 Effect of acidic and basic pretreatment of wild algal biomass on Cr (VI) biosorption. *IOSR Journal of Environmental Science, Toxicology and Food Technology* **8**, 38–41.

Singh, V., Tiwari, A. & Das, M. 2016 Phyco-remediation of industrial waste-water and flue gases with algal-diesel engenderment from micro-algae: a review. *Fuel* **173**, 90–97.

Thatheyus, A. J. & Ramya, D. 2016 Biosorption of chromium using bacteria: an overview. *Science International* **4**, 74–79.

Volesky, B. 2007 Biosorption and me. *Water Research* **41**(18), 4017–4029.

Vymazal, J. 1990 Uptake of lead, chromium, cadmium and cobalt by *Cladophora glomerata*. *Bulletin of Environmental Contamination and Toxicology* **44**(3), 468–472.

Yan, X., Chai, L., Li, Q., Ye, L., Yang, B. & Wang, Q. 2017 Abiological granular sludge formation benefit for heavy metal wastewater treatment using sulfide precipitation. *CLEAN–Soil, Air, Water* **45**(4), 1500730.

Zamri, M. F. M. A., Kamaruddin, M. A., Yusoff, M. S., Aziz, H. A. & Foo, K. Y. 2017 Semi-aerobic stabilized landfill leachate treatment by ion exchange resin: isotherm and kinetic study. *Applied Water Science* **7**(2), 581–590.