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Potential use of *Chlorella vulgaris* KCBAL01 from a freshwater stream receiving treated textile effluent in hexavalent chromium [Cr(VI)] removal in extremely acidic conditions

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ABSTRACT

Remediation of hexavalent chromium with conventional chemical and physical methods is a costly process, while replacing some critical steps in physiochemical remediation with self-sustaining bioremediation agents are expected to be cost-effective and environmentally friendly implementation. In this study, a microalga isolated from a freshwater stream receiving treated textile wastewater was identified up to its molecular level and investigated its ability to tolerate and remove hexavalent chromium from extremely acidic conditions under different temperatures. The ability of microalgae to tolerate and remove Cr(VI) was investigated by growing it in BG11 media with different pH (1, 2, 3 & 7), amended with several concentrations of Cr(VI) and incubated under different temperatures for 96 hrs. Microalga was identified as *Chlorella vulgaris* and found that the isolated strain has a higher hexavalent chromium removal potential in extremely acidic conditions than in neutral pH conditions at 25 °C. In contrast, its Cr(VI) removal potential is significantly influenced by the pH and temperature of the growth medium. Furthermore, it exhibited a permanent viability loss at extreme acidic conditions (pH $1-3$) and prolonged exposure to the higher chromium content. The microalga investigated will be a highly useful bioagent in hexavalent chromium remediation in high acidic conditions.

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KEYWORDS
Hexavalent chromium; *Chlorella* sp.; tolerance; removal

Introduction

Cr(VI) is widely used as a raw material in textile manufacturing during the printing and wool dyeing process to improve colorfastness and dye fixation.[1] The effluents containing Cr(VI) should be treated before discharge into the environment owing to their adverse health effects on humans and other living beings.

According to local or international organization guidelines, industrial wastewater containing toxic metals needs to be remediated until toxic metals reach below maximum permissible levels. These permissible discharge levels are introduced to protect humans from potential adverse health effects such as organ dysfunctions, skin and tissue irritations, physical and mental development delays, and carcinogenic and mutagenic effects. Most metals are highly soluble and relatively bioavailable to living organisms, hence bioaccumulating in higher trophic level organisms of food chains and food webs, causing the above adverse effects.[2,3]

Chromium compounds are commonly used in metallurgical productions, catalysts production, pigment production, wood preservation, metal finishing, tanning, printing and textile industries. Chromium has several oxidation states, including metallic chromium (Cr(0)), divalent chromium (Cr(II)), trivalent chromium (Cr(III)), tetravalent chromium (Cr(IV)), pentavalent chromium (Cr(V)), and hexavalent chromium (Cr(VI)) which are stable in a wide range of pH and redox conditions in nature. Among these forms of chromium, Cr (VI) is highly soluble, mobile, and bioavailable, leading to toxic effects on biota.[4] Furthermore, hexavalent chromium-based industry wastewater, including electroplating,[5] and tanning,[6,7] are reported to discharge extremely acidic wastewater (pH $2-4$) into their treatment units.

The toxicity of Cr(VI) toward green plants and algae is evident as such, prolonged exposure to Cr(VI) may inhibit photosynthesis, cause yield reductions, germination reductions, growth reductions, and mortality.[8–12] This may be due to inhibition of nutrient uptake, production of free radicals, and enhancement of chlorophyllase enzyme activity induced by Cr(VI).[13]

Conversely, several algal species can tolerate Cr(VI) under Cr(VI) stressed conditions, including, *Chlorella* sp., *Scenedesmus* sp., *Oscillatoria* sp., *Pandorina* sp., *Zoogloea* sp. etc.[8,14,15] However, it is impossible to define a standard tolerance limit for all algal species as tolerance varies with the algal type, growth medium, environmental conditions etc.[16]

Chlorella is a microalga common in freshwater habitats with a wide tolerance range toward Cr(VI).[17–20] Hence, this organism is considered a promising candidate for

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Table 1. Cr(VI) removal parameters and removal percentages.

Technique	Organism	Removal percentage (%)	Time (hrs.)	pH	Temperature (°C)	Reference
Photoreduction	*Chlorella vulgaris*	87.93	3	3	20	[23]
Biosorption	*Chlorella vulgaris*	49.7	24	2	25	[26]
Biosorption	*Chlorella minutissima*	99.7	12	2	30	[27]
Biosorption	*Chlorella sorokiniana*	95	16	2.5	25	[28]
Biosorption and biosorption	*Chlorella vulgaris*	86.6	180	1.5	25	[29]
Biosorption	*Chlorella sorokiniana*	99.68	72	7	40	[20]
Bioreduction	*Chlorella miniata*	100	150	2	25	[30]

bioremediation of Cr(VI) contaminated sources such as tannery and textile wastewaters.[21,22] Based on this hypothesis, many studies have been done with several strains of live or dead *Chlorella* sp. in Cr(VI) reduction, sorption and or both (Table 1). Deng et al.[23] reported that Cr(VI) remediation through its reduction by *Chlorella vulgaris* is influenced by several environmental factors such as Cr(VI) concentration, initial algal concentration, exposure time and pH. In addition, the light intensity can affect algal growth, including its duration and CO_2 concentration.[24]

Shen at al.[25] reported an adsorption coupled reduction method for Cr(VI) removal and detoxification using freeze-dried *Chlorella vulgaris* which revealed a 51% Cr(VI) removal within 24 hrs. and nearly complete removal after 09 days under extreme acidic conditions (pH 2) of 50 mg/L of initial Cr(VI) content. Furthermore, based on Fourier Transform Infrared Spectrometer analysis (FTIR) analysis, they reported that the carbonyl and amine functional groups of protein complexes of *C. vulgaris* have the potential for Cr(VI) removal through adsorption.

Reports on the investigation of the ability of microalgae to remove Cr(VI) under acidic pH, and varying temperatures are rare in the literature. Hence the current study aimed to characterize a microalga previously isolated from tropical freshwater receiving treated textile effluents for its ability to tolerate and remove Cr(VI) in extremely acidic environments by evaluating the influence of temperature on Cr(VI) tolerance and removal.

Methods

Reagents

The stock and working solutions of Cr(VI) were prepared by dissolving $K_2Cr_2O_7$ (AnalaR NORMAPUR, Belgium). The quality control and quality assurance were ensured by analytically verifying the metal solutions using inductively coupled plasma mass spectrometry (ICP-MS) as per method APHA 3120 B: 2017. All glasswares were acid washed before use in order to avoid any binding of the metal.

Characterization of algal isolates

Microalgal culture previously isolated from a freshwater stream receiving treated textile effluent[31] was maintained at the Department of Microbiology, University of Kelaniya, Sri Lanka. The algal culture (approximately 10^6CFU/mL) was reinoculated into BG 11 (pH 7.2) liquid medium in screw-capped flasks and incubated at room temperature (30 °C), for under 24 hrs. continuous light (fluorescence light 36 W) in the environmental shaker (JSSI-202C Series) at 100 rpm for 15 days.

Molecular identification, including DNA extraction, 18S rRNA gene amplification and PCR product sequencing, were carried out at the Genetech Molecular Diagnostics and School of Gene Technology, Sri Lanka.

Almost complete 18S rRNA genes were amplified with the universal eukaryotic primers 5′-GTCAGAGGTGAAA TTCTTGGATTTA-3′ and 5′-AGGGCAGGGACGTAATC AACG-3′.[32] The post molecular identification was followed using the NCBI database and relevant software. 18S rRNA gene fragments of the algal genome were amplified using Forward and Reverse primers by PCR. The edition of chromatogram sequences, the alignment of forward and reverse sequences, and the preparation of the consensus sequence was done using BioEdit Sequence Alignment Editor (Version 7.2.5).

The isolated strain's phylogenetic relationship was revealed by constructing a neighbour-joining phylogenetic tree based on 18S rRNA gene sequences of the strain and other selected species constructed using Mega 10.2.6. The DNA sequences obtained in this study were deposited in the GenBank.

Evaluation of the growth pattern

The growth pattern of microalgae was studied for 21 days by reinoculating the culture into BG 11, providing all required growth conditions. The cell count of the culture was determined in a hemocytometer during every 03 days.

Determination of the Cr (VI) tolerance

The fifteen-day-old microalgal culture (approximately, 10^6CFU/mL) was inoculated into BG 11 liquid medium under different pH conditions (pH = 1, 2, 3 and 7) in screw cap flasks containing different concentrations of Cr(VI)[0.025 − 5.0 mg/L] in deionized water. The total Cr content in each sample was analytically verified by inductively coupled plasma mass spectrometry (ICPMS), following the method APHA 3120 B: 2017.

Dose-response analysis of the microalgae was done at 24 hr intervals for 96 hrs. Media without Cr(VI) but inoculated with algae and medium with Cr(VI) without algae served as biotic and abiotic controls, respectively. Both controls and treatments were incubated at selected temperatures (25 °C, 30 °C, and 40 °C) with continuous shaking under continuous light as described earlier. Growth response of the culture was monitored spectrophotometrically at 430 nm using Thermo Scientific™ Multiskant™ FC Microplate

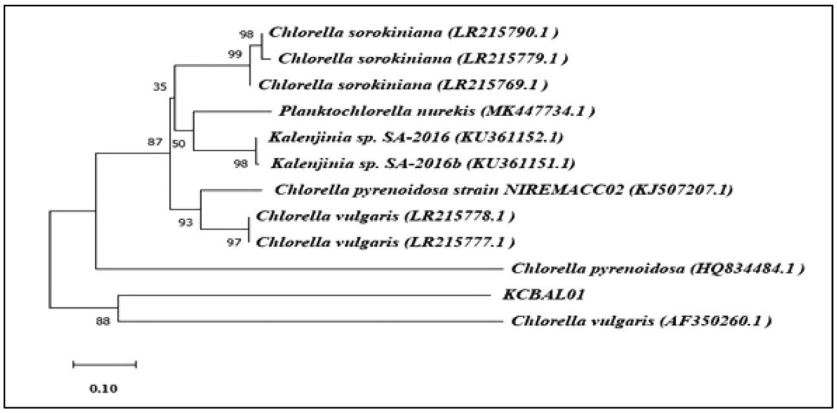

Figure 1. A neighbor-joining phylogenic tree based on 18S rRNA gene sequences showing the phylogenetic relationship among isolated strains and other selected species constructed using Mega 10.2.6. The bootstrap numbers indicate the value of 1000 replicate trees. The NCBI accession numbers are given in parenthesis.

Photometer at 24 hrs. intervals up to 96 hrs.[33] All the tests were done in triplicate for a detailed study.

The isolated microalgal species' Cr(VI) tolerance was measured by calculating the survival percentage (Equation 1) up to 96 h during every 24 hrs. time intervals.

$$\text{Survival percentage} = \frac{100}{\text{OD of control}} \times \text{OD of test} \quad (1)$$

where control represents the optical density of non-Cr (VI) added samples and test represents the optical density of Cr(VI) doped samples.

Cr(VI) removal by microalgae

The microalgal culture (approximately, 10^6CFU/mL, 15 days old) was inoculated into BG 11 medium containing different concentrations of Cr(VI)[0.025 – 5.0 mg/L] and incubated for 96 hrs. at selected different temperatures (25 °C, 30 °C, and 40 °C) and pH ranges (pH = 1, 2, 3 and 7). Samples were withdrawn at 24 hr intervals, and centrifuged (HERMLEZ 206 A) at 6000 rpm for 10 min to obtain cell-free supernatant. Supernatants were analyzed for Cr(VI) following US EPA Method 7169 A with 1,5-diphenylcarbazide (DPC).[34] The experiments were continued for up to 96 hrs., and spectrophotometric measurements were recorded at 24 hr. intervals at 540 nm.

The acidic conditions were maintained using HCl, and temperatures were maintained in 2-Chamber Shaking Incubator (JSSI-202C Series). The initial and final pH values were measured using Hach HQ2100 Portable Multi-Meter.

Cr(VI) removal of the microalgae was calculated as a percentage, using the following mathematical equation 2 based on data collected by DPC colourimetric method described earlier.

Cr (VI) removal percentage = $\frac{Ci - Ce}{Ci} \times 100$(2)where *ci* and *ce* are the initial Cr(VI) concentration and final Cr(VI) concentration after the removal process.[35]

Statistical analysis

The influence of pH and temperature on Cr(VI) removal was statistically analyzed using two-way ANOVA under a 95% confidence ($p < 0.05$) level.

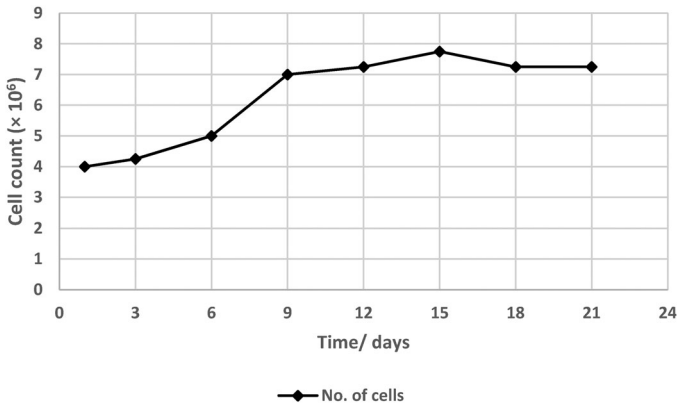

Figure 2. *C. vulgaris* KCBAL01 cell count.
The cell count of the *Chlorella vulgaris* KCBAL01 with time over 21 days is presented in Figure 2. The maximum cell count was achieved on the 15th day of incubation of the *Chlorella vulgaris* KCBAL01 in the B.G. 11 medium under the given growth conditions.

Results and discussion

The 18S rRNA sequence consensus was obtained based on NCBI's BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) query coverage and e-value.[36,37] According to the NCBI database, isolated microalga was identified as related to *Chlorella* sp. MDL7-5 with 99% of query coverage, 0.0 of E value and 99.25% identity. The phylogenetic relationship of the isolates was determined with selected strains from different taxa and nearly identical strains of the isolates by aligning the sequences using the MUSCLE algorithm, and the neighbour-joining phylogenetic tree (Figure 1) was constructed using MEGA 10.2.6. The phylogenetic analysis reveals that isolated *Chlorella* sp. is 88% similar to *Chlorella vulgaris* (AF350260.1) (Figure 1). Therefore, identified microalga in this study was named *Chlorella vulgaris* KCBAL01. The DNA sequences obtained in this study were deposited in the GenBank database under the accession number OK338898.

C. vulgaris KCBAL01 exhibited the highest cell count (7.75×10^6 cells/mL) within fifteen (15) days of inoculating into BG11 medium (Figure 2). Therefore, this culture was used for Cr(VI) tolerance and removal studies and to find optimum conditions such as initial algal density/biomass for efficient Cr(VI) removal, according to Deng et al.[23] and

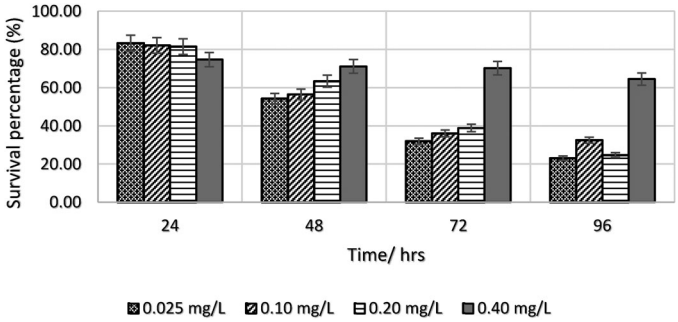

Figure 3. Survival of *C. vulgaris* KCBAL01 cell in lower Cr(VI) concentrations. (Standard error 1.60–5.0 at 95% confidence).

Figures 3 and 4 represent the survival of the *Chlorella vulgaris* KCBAL01 when exposed to different concentrations of Cr(VI). It was evident that the survival decreased considerably with time except for 0.40 mg/L.

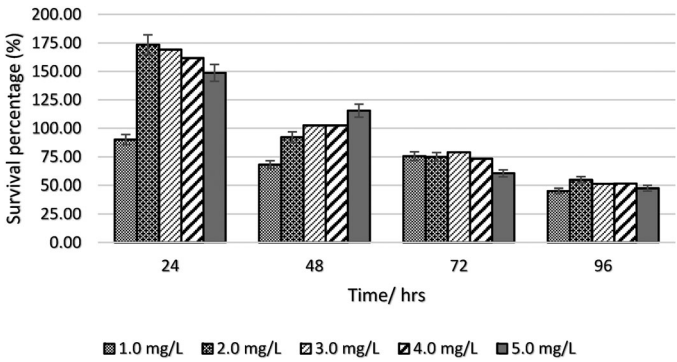

Figure 4. Survival of *C. vulgaris* KCBAL01 exposed to higher Cr(VI) concentrations. (Standard error 2.16–8.43 at 95% confidence).

The tolerance and removal of Cr(VI) by *C. vulgaris* KCBAL01, when grown at different concentrations of Cr(VI) at different pH under the ambient temperature (30 °C), is presented in the Appendix as Figures A1–A4. The highest percentage removal of Cr(VI) was observed when the microalgae were exposed to 0.1 mg/L of Cr(VI) at pH 1,2 and 3 over 96 hrs., however, with decreasing tolerance. At pH 7, though the microalgae could tolerate all the tested concentrations during 96 hrs., the Cr(VI) removal capacity was low.

Gokhale et al.[38] According to the graphical interpretation of the growth curve (Figure 2) a gradual increase of algal cell count was observed while increasing the greenish coloration of the algal culture in BG 11 medium. This gradual increase may be occurred due to the systematic adaptation of *C.vulgaris* KCBAL01 to the newly inoculated medium and favorable growth conditions provided such as agitation, continuous light cycles, and temperature. However, after 15 days, the growth patterns indicated a slight decreasing (7.75×10^6 cells/mL to 7.25×10^6 cells/mL). It is assumed that, *C. vulgaris* KCBAL01 may have reached to its maximum carrying capacity within 15 days.

Lu et al.[39] reported that Cr(VI) at lower concentrations (0.50 – 1.0 mg/L) stimulated growth in *C. vulgaris* while higher concentrations (2.0 mg/L – 5.0 mg/L) inhibited the growth, which is somewhat similar to the observations of the present study. A permanent viability loss observed at lower pH values (pH 1, 2, and 3) at all concentrations tested in this study align with the complete viability loss in higher Cr (VI) contents (8.0 mg/L) after 15 days under pH 7 (data not shown). This complete viability loss was evident from the change of the color of algal cells from greenish to white inoculated in fresh BG 11 medium. This growth depletion can be explained owing to the inhibition of metabolic processes associated with photosynthesis and morphology. Studies by Hörcsik et al.,[40] Laxmi et al.,[41] and Zou et al.[42] revealed that Cr stressed conditions could affect photosystem II (PS II) reaction centers leading to inhibition of photosynthesis in *Chlorella* sp. and *Scenedesmas* sp. Qian et al.[43] has further described this scenario with *Chlorella vulgaris* in terms of damages to thylakoids, disruptions in chloroplast structure, and a decrease in *chlorophyll a* by absorbing chromium into the algal body. Furthermore, there is no clearly defined unique optimal pH value for *Chlorella* sp. as it can vary on the type of strain. This fact is well-evidenced by exhibiting different optimal pH conditions in several previous studies of *Chlorella* strains such as *C. protothecoides* at pH 2.5 and 5,[44] *C. sorokiniana* at pH 6.0 and 6.5,[45] *C. vulgaris* at pH 7 – 9[46] etc. Moreover, it is believed that optimal pH of the selected strain can be laid on pH 7 as it remained viable in neutral growth media (Table 2). Therefore, it can be assumed that, *C. vulgaris* KCBAL01 is highly sensitive for extreme acidic conditions and high concentrations of Cr(VI).

The metal removal studies indicate that *C. vulgaris* KCBAL01 has excellent potential to remediate Cr(VI) through reduction or adsorption. Furthermore, *C. vulgaris* KCBAL01 revealed a higher tolerance under neutral pH conditions at room temperature (30 °C) than extreme acidic conditions (pH 1, 2, and 3) with below and above 30 °C in the presence and absence of Cr(VI). Since the main objective of this study is Cr(VI) removal using *Chlorella* sp. than prolonged cell viability, the current study mainly focused on Cr(VI) removal.

Previous studies disclose that several *Chlorella* isolates in extreme acidic conditions can efficiently remove Cr(VI) (Table 1). However, this ability was observed in microalgae other than *Chlorella* sp., including *Sargassum* sp.[47] and *Scenedesmus* sp.[48] under pH 1.0, *Chlamydomonas reinhardtii,*[49] *Oedogonium hatei,*[50] and *Dunaliella* sp.[51] under pH 2.

Based on the above previous reports (Table 1), the present study used extreme acidic (pH 1, 2, and 3) and neutral conditions (pH 7) with varying temperatures of 25 °C, 30 °C and 40 °C to determine efficient Cr(VI) removal. The neutral pH conditions were used to compare the viability of cells. As previously described, *C. vulgaris* KCBAL01 exhibited a gradual decrease in their growth after 72 hrs., at all the tested occasions, ie. extreme acidic pHs (pH 1, 2, and 3) and temperatures indicating loss of viability.

Based on the growth response at pH 7 and 30 °C, the strain can survive in Cr(VI) added medium with comparatively lower tolerance than the nonmetal added control. Similar observations were made by Sánchez-Fortún et al.[52] using *Dictyosphaerium chlorelloides* in the presence of Cr(VI) and *Chlorella vulgaris* U.T.E.X. 30 in acidic conditions with Cd(II).[53] Moreover, survival and growth enhancement of *Chlorella* sp. under neutral pH is also evidenced by many growth optimization studies.[46,54,55] Furthermore, it was clearly observed that *C. vulgaris* KCBAL01 could remain viable even at the highest tested

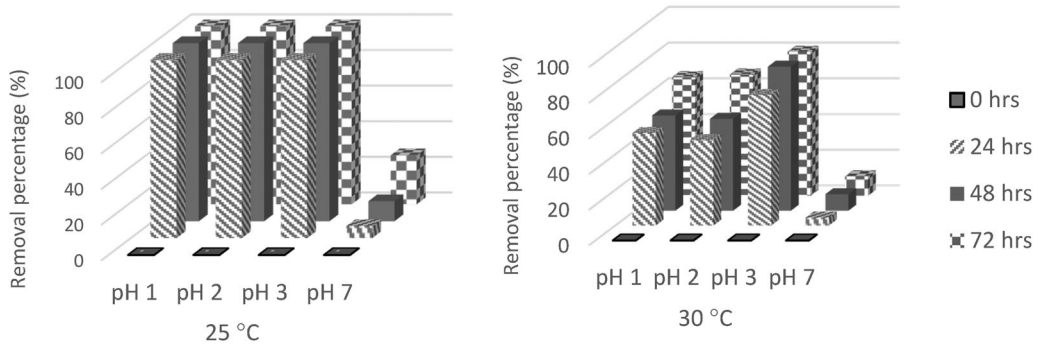

Figure 5. Effect of medium pH and incubation temperature on the efficiency of Cr(VI) removal by *C. vulgaris* KCBAL01 at 0.1 mg/L of Cr(VI).

The effect of medium pH and incubation temperature on the efficiency of Cr(VI) removal by *C. vulgaris* at 0.1 mg/L Cr(VI) is shown in Figure 5. The highest Cr (VI) removal potential was observed at 25 °C. Furthermore, a permanent viability loss was observed after 24 hrs. of incubation when *C. vulgaris* was grown at 40 °C.

Table 2. Survival of *C. vulgaris* KCBAL01 and removal of Cr(VI) after 96 hrs. of incubation.

	pH 1		pH 2		pH 3		pH 7	
Concentration (mg/L)	Survival %	Removal %	Survival %	Removal %	Survival %	Removal %	Survival %	Removal %
0.10	PVL	100.00	PVL	100.00	PVL	78.56	32.47	12.93
0.50	PVL	64.26	PVL	96.31	PVL	60.74	35.65	13.93
1.00	PVL	72.76	PVL	69.04	PVL	58.01	47.54	14.66
2.00	PVL	52.12	PVL	48.90	PVL	57.67	61.75	14.70
3.00	PVL	61.72	PVL	60.69	PVL	45.49	57.36	19.32
4.00	PVL	52.19	PVL	40.71	PVL	41.42	57.35	20.75
5.00	PVL	46.54	PVL	60.38	PVL	29.05	51.93	21.67

PVL-Permanent Viability Loss; Sur. Per (%) - Survival percentage (%), Rem. Per (%) - Removal percentage (%)

The alga grown in the presence of 0.1 mg/L of Cr(VI) showed loss of viability after 96 hrs. of incubation at pH 1,2 and 3 even though the highest percentage Cr(VI) removal occurred at pH 1 and 2. At pH 7, *C. vulgaris* was able to retain its viability at all the concentrations tested until the end of the incubation period, however, with low Cr(VI) removal potential.

Cr(VI) concentration of 5.0 mg/L at these temperature and pH (pH 7, 30 °C) with different survival percentages raging 32 – 61% (Table 2). However, survival had been increased at 0.10 – 2.0 mg/L Cr(VI) while indicating a decrease at 3.0 – 5.0 mg/L Cr(VI) similar to the observations of Lu et al.[39]

Despite the growth response of the isolated strain, higher Cr(VI) removal efficiency with almost 100% at 0.10 mg/L was observed under extremely acidic conditions at 25 °C, than neutral pH and above temperature range (Figure 5). The removal was not efficient at 40 °C due to cell destruction and permanent viability loss. A comparison of metal removal under temperature and pH combination showed a maximum of 0.10 mg/L Cr(VI) removal during the study period of 96 hrs. (Table 2).

It is known that algae can survive with heavy metals, including Cr(VI), via different tolerance mechanisms, including adsorption, absorption and biotransformation through reduction.[29,56,20] The heavy metal adsorption of algal cells is basically functioned by the cell wall, mucilage, and extracellular polymeric components, including carboxyl, hydroxyl, amine, sulfate and other charged functional groups. This phenomenon has been proven by Han et al.,[30] Gokhale et al.,[38] Shen et al.,[25] and Husien et al.[20] with several strains of the genus *Chlorella,* including *C. vulgaris, C.miniata*, and *C. sorokiniana,* with Cr(VI). Furthermore, Fourier Transform Infrared Spectrometer analysis (FTIR), electron dispersive spectroscopy (EDX), and x-ray photoelectron spectroscopy (XPS) analysis of the above studies have clearly shown that functional groups can adsorb Cr (VI).

Moreover, it has been found that *C. vulgaris* can reduce Cr(VI) into Cr(III) through both biological routes of enzymatic activity (enzymatic chromium reductase) and non-biological routes of dead or broken cells.[18,56] Therefore it can be assumed that *C. vulgaris* KCBAL01 may have similar tolerance mechanisms described above since it exhibited Cr(VI) tolerance and removal potentials.

The Cr(VI) removal capability of the strain is influenced by both media temperature and pH. The two-way ANOVA clearly shows, pH value (F-value =616.573, p-value < 0.000), temperature (F-value = 30.678, p-value <0.000) and cumulative effect of both factors (pH × temperature) (F-value = 14.943, p-value < 0.001) affect the Cr(VI) removal significantly. Also, it reveals that both pH value and temperature of the medium have significant interaction.

Conclusion

Isolated *C. vulgaris* KCBAL01 exhibits a higher survival in neutral pH conditions while displaying comparatively lower survival under extremely acidic growth conditions (pH 1 – 3) coupled with a higher potential of Cr(VI) removal at 25 °C. Furthermore, both pH and temperature can significantly influence both survival and Cr(VI) removal potential of *C. vulgaris* KCBAL01. Based on the Cr(VI) removal potentials in extreme acidic conditions, this strain can be suggested to apply as a bioremediation agent for industries generating extremely acidic Cr(VI) contaminated wastes.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Disclosure statement

The authors declare that they have no conflict of interest.

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APPENDICES

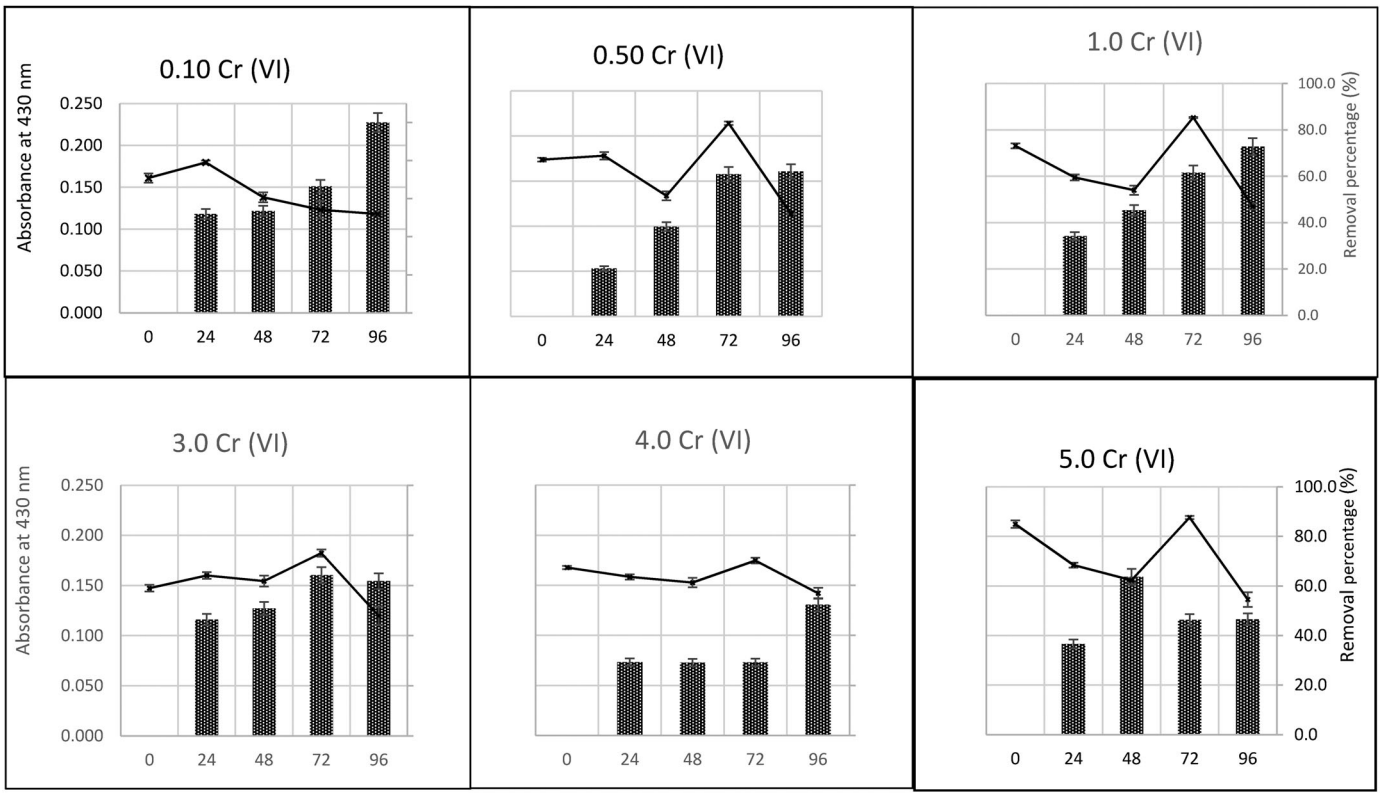

Figure A1. *C. vulgaris* KCBAL01 growth and Cr(VI) removal in pH 1.

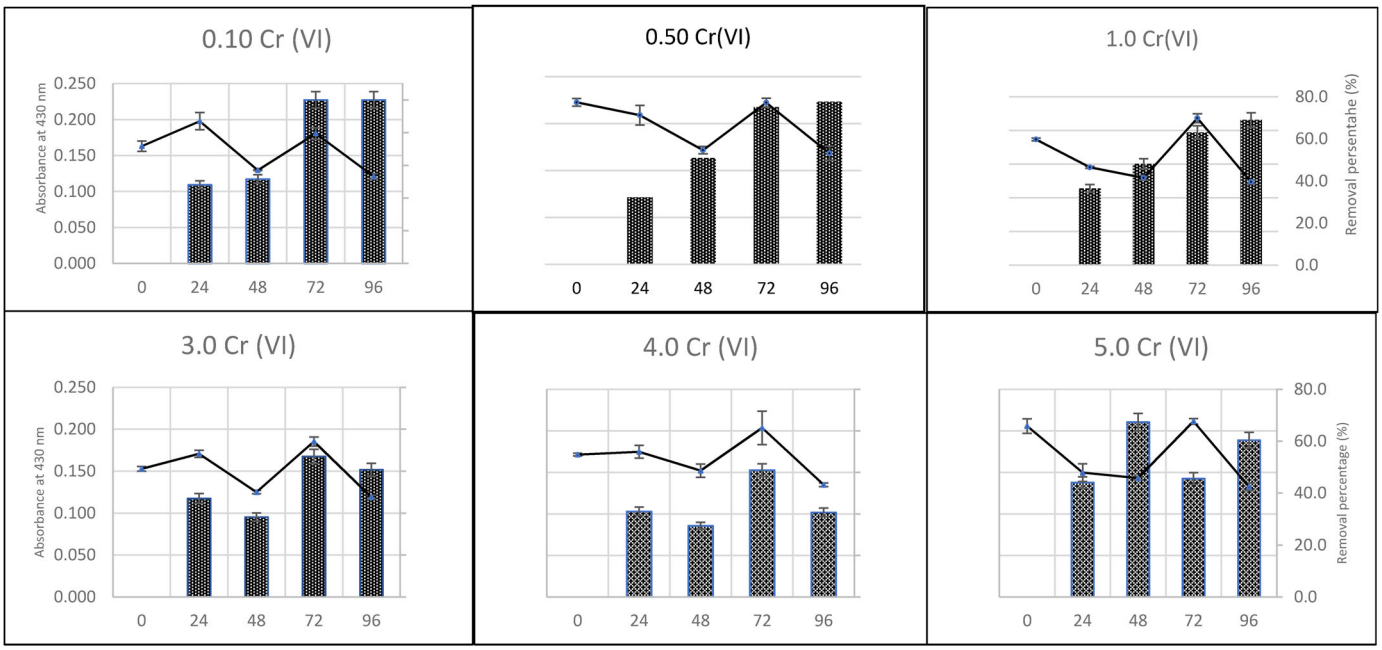

Figure A2. *C. vulgaris* KCBAL01 growth and Cr(VI) removal in pH 2.

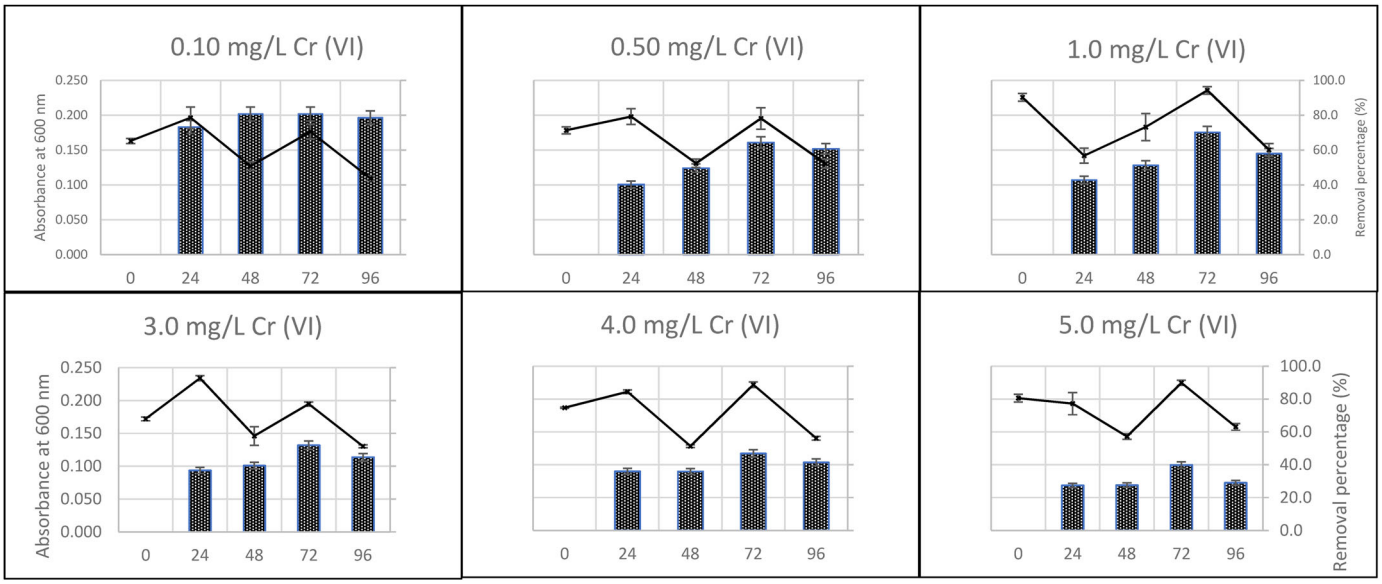

Figure A3. *C. vulgaris* **KCBAL01 growth and Cr(VI) removal in pH 3.**

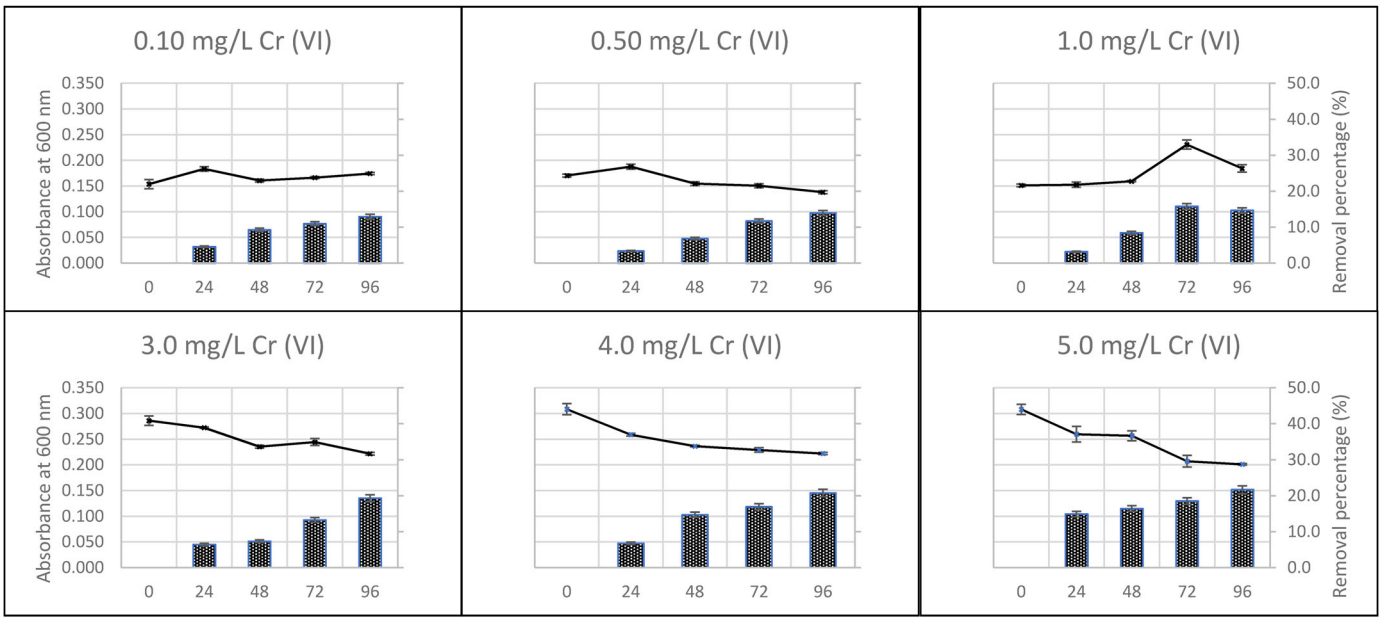

Figure A4. *C. vulgaris* **KCBAL01 growth and Cr(VI) removal in pH 7.**