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RESEARCH ARTICLE OPEN ACCESS

ChrR Gene Variability in Cr-stressed *Leptolyngbya boryana* for the Biotransformation of Cr (VI) to Cr (III)

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Abstract

Chromium (VI) is a well-known pollutant that is present in industrially polluted soil and water, and has been reported to be mutagenic and carcinogenic. In the present study, we investigated the effective use of *Leptolyngbya boryana* (cyanobacterium) as an eco-friendly option to overcome Cr (VI) toxicity in tannery effluents. The main objective of this study was to identify the Cr reductase (*ChrR*) gene and its variability in the context of Cr (VI) stress. Industrial polluted soil samples were collected and processed according to standard protocols for *ChrR* variation and *16S* rDNA gene analysis. Genomic DNA was isolated from the collected samples and the *ChrR* and *16S* rDNA genes were amplified by PCR. Amplified *16S* rDNA was sequenced and aligned with known sequences. In the present study, a strong correlation was established between the nucleotide sequences of the *ChrR* and 16S rDNA genes. The Minimum Inhibitory Concentration (MIC) was determined for Cr (VI), and pure strains of *L. boryana* were identified and isolated from soil samples. Cr (VI)-stressed conditions and their genetic variability were confirmed by sequencing. In conclusion, the *L. boryana* strain has been identified an eco-friendly option for overcoming Cr (VI) toxicity in tannery effluents.

Keywords: Bioremediation, ChrR gene, Leptolyngbya Boryana, Genetic Variability, Chromium Reductase, Homology

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INTRODUCTION

Waste produced by different industries presents a big crisis, with the main challenge being to convert waste into eco-friendly compounds through sustained approaches. Many industrial wastes (effluents) consist of various toxic materials including metals, harmful volatile compounds, and large organic and inorganic residues. Due to developmental requirements, new strategies and novel sustained approaches are needed to enhance industrial waste management because of increasing urbanization.[1] Several sources can be attribute to the presence of long-term industrial effluents in the atmosphere, and many infectious diseases, neurological disorders, metabolic abnormalities, and cancer have been recorded in recent years.[2] Due to the liberation of these toxic materials, we have faced significant losses and challenges in all domains of society.[3]

Various studies have reported the presence of many toxic pollutants in industrial effluents, such as chromium, sulfides of metals, phenolic compounds, magnesium, sodium, potassium, and mercury.[4] Chromium, an important toxic compound, is also an important micronutrient that is required for the growth of many microorganisms.[5] Chromium in high concentrations is toxic in all ecosystems, i.e., air, water, and soil. Naturally, soil can have a chromium concentration in the range of 10–50 mg/kg.[6] In a study, Indian tannery industries alone produced more than 2000-3000 tons of chromium in the environment with high chromium concentrations of more than 2000-5000 mg/L. Although, it has been found a safe recommended acceptable discharge limit is less than 2 mg/L.[7]

Many microbes, specifically blue-green algae, have been used in biological systems to reduce (biotransformation) Cr (VI) into Cr (III). Moreover, several studies have identified chromium-reducing microorganisms. Additionally, few fungi have also been studied for their Cr (VI) reducing properties.[3,8] These properties of microbes to survive Cr (VI) metal exposure and regulate the detoxification mechanism into Cr (III) are used to rectify globally. Specific microbes have specific metal-tolerance capacities under the optimum environmental conditions. Plasmid-mediated removal of xenobiotics has been described by Bhatt et al.[9] Some mechanisms of Cr (VI) reduction have been reported, e.g. segregation by a permeability barrier, active transport efflux pumps, intra-and extracellular appropriation, enzymatic methods, among this study.[10] A bacterial species that can reduce toxic Cr (VI) to nontoxic Cr (III) has been reported previously.[8,11] Similarly, a fungal species that has Cr (VI) bio-absorptive property has been reported. During biosorption, Cr (VI) is bound to the functional groups present on the surface of microbes and percolated inside.[12] Several studies have reported on the significance of biofilm-mediated bioremediation, which has been used as a powerful tool for the removal of environmental pollutants.[13]

In nature, various species of cyanobacteria live on soil surfaces and might be morphologically and phylogenetically different.[14] *Leptolyngbya*, a filamentous cyanobacterium, is characterized by the width of its cylindrical trichomes. *Leptolyngbya* spp. have been isolated from various industrial effluents in soil. *Leptolyngbya boryana* is phylogenetically coupled to *Leptolyngbya* sp.[15] Although nitrogen fixation by *L. boryana* is well characterized, the ability to reduce Cr (VI) to nontoxic Cr (III) genomic analysis has not been reported previously. Recently, biosurfactants have been found to be powerful tools for bioremediation of heavy metals from contaminated soil.[16]

In our previous study, we reported the successful isolation and characterization of *L. boryana* from tannery soils.[17] In the present study, we aimed to characterize the Cr reductase (ChrR) gene and investigate its variability in the context of Cr (VI) stress. The findings of this study may provide a more suitable, effective, eco-friendly, sustainable, and cost-effective biological treatment of wastewater from the leather industry. Therefore, in this study, we focused on the potential role of *L. boryana* in the biotransformation of Cr (VI) to Cr (III). This study is novel in the context of the bacterial species studied and the methodologies used for the identification and characterization of Cr (VI) toxicity in wastewater/soil.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of analytical grade and procured from Merck

Table 1. Morphological, biochemical and molecular characteristics of Cr (VI) reluctant strains *L. boryana*, checked 100 different isolates and found 10 identical isolates

Strain S.N.	Morphological Appearance	Motility	Gram's Straining	Catalase test	Indole Test	Sucrose Utility Test	16S rDNA test
Strain (10)	Thin filamentous Cylindrical trichomes colourless	Solitary	(+) ve	(+) ve	(+) ve	(+) ve	(+) ve
Strain (90)	Thin filamentous Cylindrical trichomes colourless	Solitary	(+) ve	(+) ve	(-) ve	(+) ve	(-) ve

(Rahway, NJ, USA), HiMedia (Mumbai, India), and Qiagen (Hilden, Germany). A stock solution (1000 mg/L) of Cr (VI) was prepared using $K_2Cr_2O_7$ in deionized water.

Collection of samples

Industrial effluent samples were collected from nearby industries in Kanpur (India) (26°26'59.7228"N 80°19'54.7356"E). Out of 100 samples collected from different places, *L. boryana* (ATCC-27894) was identified in 10 samples. We previously characterized these strains morphologically and biochemically.[17] Pathogenic and antibiotic-treated *L. boryana* strains were excluded from this study.

Cyanobacteria and culture conditions

The screened *L. boryana* strains were grown in Erlenmeyer flasks containing liquid BG-II media under the following growth conditions: 16:8 light:dark cycle, 30 ± 2°C; and 6 irradiances of 3000–4000 lux (cool white light). Furthermore, the isolates were routinely subcultured every 20 days, as mentioned previously.[17]

Morphological and biochemical analysis

The morphology (colony morphology and color identification) of isolated *L. boryana* strains was characterized as described previously.[17] For Cr reductase analysis, isolated and morphologically characterized *L. boryana* strains were inoculated in a large volume (5 L) of BG-II medium and maintained at 37°C for 10 days with constant agitation in at 100 rpm. *L. boryana* colonies were harvested by centrifugation (1000 rpm at 4°C for 30 min). Harvested colonies were lysed, the reduction of Cr (III) by the wild and stressed *L. boryana* strains was analyzed.[18] Cr reductase activity was assessed for up to 14 days in the isolated and characterized strains. For analysis of minimum inhibitory concentration (MIC), cultures were inoculated with different concentrations (0–1000 mg/L) of chromium (VI) and tested at 7 days and 14 days.

Furthermore, biochemical analysis, including Gram's staining, catalase activity, sucrose utility test, and indole test was performed for all strains as described previously.[19]

PCR amplification of Cr-responsive genes and *In silico* analysis

For genomic analysis, total genomic DNA was isolated from collected *L. boryana* cells using a Qiagen DNA isolation kit (Qiagen India Pvt. Ltd., New Delhi, India). The PCR reaction mixture (20 µL) contained 10 µL of master mix (GoTaq Master Mix, Promega, Madison, WI, USA), 1 µL each of forward and reverse primers, and 30 µg of DNA template and/or nuclease-free water. PCR was performed in a BioRad T100 Thermal Cycler (BioRad, Hercules, CA, USA) using the following cycling conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 1 min; and final extension at 72°C for 7 min. The amplified product was visualized by 1% agarose gel electrophoresis.

Further validation of the cyanobacterial isolate was performed using *16S* rDNA sequencing. PCR analysis was performed on freshly grown culture as described by Yadav et al.[17] Sequencing was performed by Chromous Biotech Pvt. Ltd. (Bengaluru, India). Briefly, the sequencing reaction

(10 µL) contained 4 µL of the BigDye Terminator mix (version 3.1), 1 µL of primer (10 pmol) (Table 2), 1 µL of DNA template, and 3 µL of sterile water. Sequencing was carried out using an ABI 3500 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). A 50 cm capillary array column (capillary array; POP-7 polymer) was employed in the DNA sequencer. The analysis was performed using the protocol BDTv3-KB-Denovo-v 5.2 (Seq scape-v 5.2 software, Thermo Fisher Scientific, Waltham, MA, USA). The results were generated in ABI, PDF (Figure S4), and FASTA format, the *16S* rDNA gene sequence was subjected to NCBI Blast to identify the similar sequences, and the phylogenetic tree was constructed using the MEGA5 software.[20]

To check the expression of *ChrR*, RNA was isolated using an RNA isolation kit (Qiagen India Pvt. Ltd.), and the concentration was determined using a Nanodrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of the isolated RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Relative gene expression was quantified using the qPCR SYBR Green PCR Master Mix (Applied Biosystems). Relative expression was calculated by a 2-ΔCt method[21] using *16S* rDNA as an endogenous control. The amplified product was visualized by 2% agarose gel electrophoresis. After purification, the band was subjected to gene sequencing. Primers used for *16S* rDNA and *ChrR* genes are listed in Table 1. *L. boryana* strain was confirmed by *16S* rDNA based on PCR results, and the sequences were aligned using Clustal W (https://www.ebi.ac.uk/Tools/msa/clustalo/). The results obtained after gene sequencing were subjected to homology analysis using the NCBI

Table 2. Primers used in this study for amplification of 16S rDNA and ChrR genes in *L. boryana*

S.N.	Gene name	Sequences 5'→3'	Tm (°C)	Amplicon size	Reference
1.	16S *rDNA* R	AGAGTTTGATCCTGGCTCAG	50	1.5kb	12
2.	16S *rDNA* R	TACGGTTACCTTGTTACGACTT	50		
3.	ChrR F	TCACGCCGGAATATAACTAC	53	340bp	38
4.	ChrR R	CGTACCCTGATCAATCACTT	54	Used in qRT-PCR	
5.	16S rDNA F	CACACTGGGACTGAGACAC	56	190bp	39
6.	16S *rDNA* R	CTGCTGGCACGGAGTTAG	56	Used in qRT-PCR	

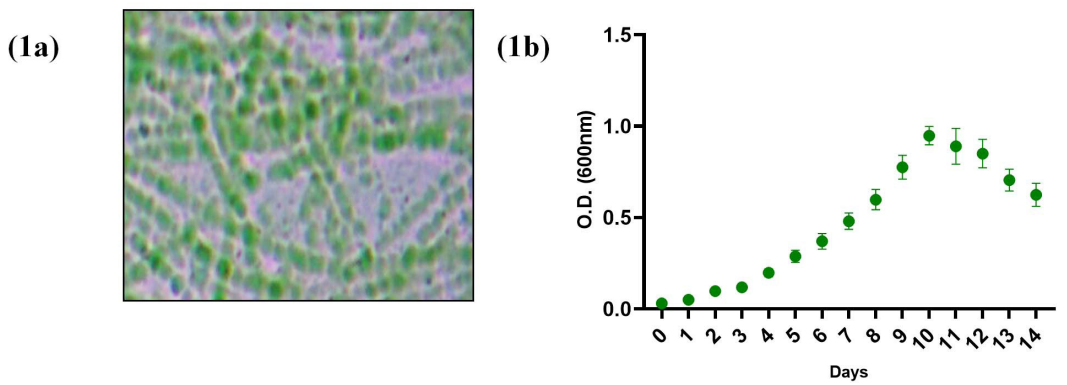

Figure 1. (1a) Representative micrograph depicts morphological characteristics of L. boryana (10X). (1b) Graph represents the growth curve of isolated culture observed up to 14 days. Each dot represents the mean value of O.D. at 600 nm of three independent culture sets

nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE, BlastSearch).

Statistical Analysis

In the present study, MIC and dissimilar variables were assessed using the SPSS software version 22 (IBM, Armonk, NY, USA).[22] Data are presented as mean ± standard deviation of three independent experiments.

RESULTS

Selection and identification of the cyanobacteria based on morphological and biochemical analysis

In the present study, 10 out of 100 samples collected from different locations contained only *L. boryana* and were subjected to biochemical, morphological, and genetic analysis. The genus *Leptolyngbya* is a simple filamentous cyanobacteria that exhibits slight morphological

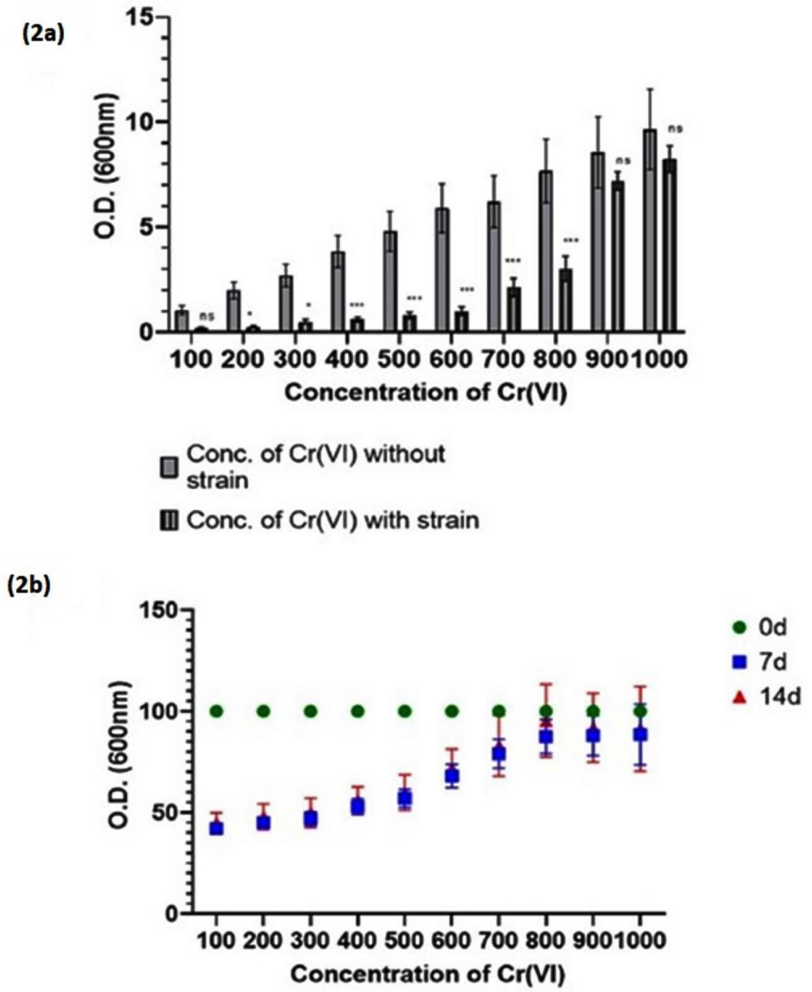

Figure 2. (2a) Histogram represents the optimum MIC of Cr (VI) mg/L in the growth culture of *L. boryana*. (2b) Histogram represents the Cr (VI) mg/L stress in the growth culture of L. boryana observed at 0 day, 7 days and 14 days. Values represented as mean ±SD after three independent experiments. ***p<0.001, *p<0.05, and ns=not significant

differences between species. The same bacterial strain was isolated from 10 locations (Figure 1a). The isolated *L. boryana* strains were observed for up to 14 days. Culture growth increased from the 6th day to the 10th day of culture and start decreasing from the 11th day of culture compared with that on the 1st day of culture (Figure 1b). Biochemical tests characterized the cultures as thin filamentous bacteria with cylindrical trichomes, gram-positive, catalase-positive, indole test positive, and sucrose test positive (Table 1).

Genetic screening of the selected strain

Genomic DNA was isolated from the morphologically and biochemically characterized *L. boryana* (Figure S1a). In the present study, bacterial genotypic confirmation was performed using *16S* rDNA sequencing (Figure S1b). The obtained *16S* rDNA sequence of *L. boryana* strain was homologous to sequences available in the NCBI database (Figure S1f). The strain exhibited 98% sequence identity with known sequences (Figure S1c–e).

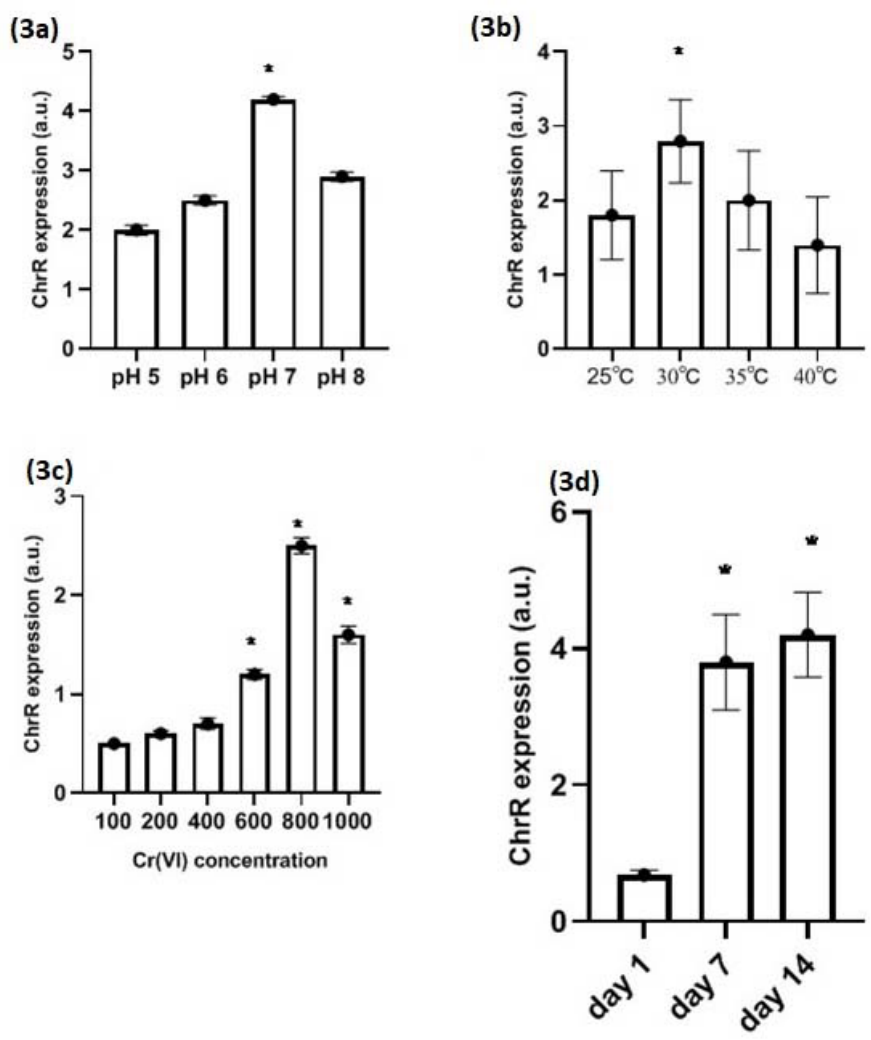

Figure 3. Expression of ChrR genes in different physiological conditions of L. boryana culture, (3a) ChrR genes expression at pH 5-8, (3b) ChrR genes expression at temp 25-40 °C (3c) ChrR genes expression at Cr (VI) concentration 100-1000mg/L, (3d) ChrR gene expression observed at 0 day, 7 days and 14 days in L. boryana. Values represented as mean ±SD after three independent experiments *p<0.05

Expression of *ChrR* gene in the selected strain under Cr (VI) stress

The optimized level of tolerance for Cr (VI) of the selected strain was found to be 800 mg/L of $K_2Cr_2O_7$ while other isolates did not grow well at Cr (VI) concentrations above 800 mg/L (Figure 2a). Cr (VI) stress tolerance was also assessed for up to 14 days. No significant differences were observed between 7 and 14 days (Figure 2b). Furthermore, *ChrR* gene was isolated and amplified from *L. boryana*. In the present study, five isolates were confirmed to express the *ChrR* gene (Figure S2a) when cultured with 800 mg/L of Cr (VI), and the band size of *ChrR* gene was found to be approximately 340 bp (confirmed with a DNA ladder) (Figure 3a). The sequenced *ChrR* gene was aligned with known sequences (Figure S2b, c).

High *ChrR* expression was observed in *L. boryana* at an optimum pH of 7 and at an optimum temperature of 37°C (Figure 3a, b). High *ChrR* expression was also observed in *L. boryana* strains when cultured with 800 mg/L of Cr (VI) ($p < 0.05$) and after 7 days of culture ($p < 0.05$); however, no significant difference was observed between 7 and 14 days (Figure 3c, d) and (Figure S3 a–d).

DISCUSSION

The present study focused on assessing the genetic variability and expression of *ChrR* gene in *L. boryana* isolated from tannery soils with respect to Cr (VI) reductase activity. Similar work has been done by Ilias et al.[23] and Camargo et al.[24] who reported that Cr (VI)-resistant bacteria degrade for Cr (VI) at concentrations between 500–2000 mg/L. The optimum MIC of Cr (VI) was reported to be approximately 740 mg/L, and above this concentration, the bacterium was not found to be suitable for Cr (VI) reduction. A similar finding was obtained in our study; the optimum MIC was found to be approximately 800 mg/L (Figure 2a). Our findings are supported by the findings of Chaturvedi[25] and Poornima et al.[26] who reported Cr (VI) tolerance in *Pseudomonas putida* and found positive results for in catalase, indole, and sucrose utility tests. Similar results were also obtained in the present study; *L. boryana* exhibited positive tests for catalase, indole, and sucrose utility (Table 1).

There are limited genetic studies that have analyzed *16S* rDNA and *ChrR* genes in microbes with relation to Cr (VI) reduction. In the present study, both *16S* rDNA and *ChrR* genes were found out and showed 98% homology identity with *16S* rRNA gene and 98% homology identity with *ChrR* gene in *L. boryana* under Cr (VI) stress state (Figure S1 and S2). A similar study was performed by Baldiris et al.,[27] who showed the presence of *ChrR* gene in cyanobacteria and reduction of Cr (VI). In another study by Rocco et al., which used *S. maltophilia*, a crucial property for the binding of metals, such as Hg, Co, Zn, and Cd in tannery effluents was demonstrated.[28] The *16S* rDNA (Figure S1) and *ChrR* partial gene sequence homology was obtained by comparison with available sequences in NCBI databases (Figure S2). A similar finding was obtained by Rathnayake et al.,[29] for Cr (VI) biotransformation in *Phormidesmis molle* in the presence of *16S* rDNA and *ChrR* genes. Additionally, the findings of Sundar et al.,[30] also support the presence of *16S* rDNA and *ChrR* genes with 99% homology in *Bacillus Cereus* strains for Cr (VI) reduction.

In the recent past, Cr (VI)-reducing bacteria on biosolids from wastewater were isolated by Velez et al.,[31] and the data generated supported the findings of our study. In the literature, a partial (268 bp) chromate reductase gene has been identified in three gram-positive bacterial isolates from soils contaminated with Cr from tannery effluents. In the present study, we obtained a 340 bp (Figure S2) partial gene sequence for *L. boryana*. Similarly, Denget al.,[32] obtained a 321 bp (partial) Cr reductase gene in gram-positive bacteria. This confirmed the presence of the *ChrR* gene in the DNA of these two bacteria, reaffirming their chromium-reducing property. Thus, our findings confirm the presence of the ChR gene in *L. boryana* and strengthen the ability to reduce Cr (VI) to Cr (III).[33] The present study is also consistent with the findings of Deshpande et al.,[34] in relation to the reduction of Cr (VI) to Cr (III). In the literature, biofilm formation by xenobiotic-degrading microorganisms was reported by Bhatt et al.,[35] which supported by the

findings of our study in terms of Cr (VI) reduction to Cr (III).

In Cr (VI)-resistant bacteria, *ChrR* gene catalyzes the reduction of Cr (VI) to Cr (III) with the transfer of electrons from the electron donor NADPH to Cr (VI), resulting in the production of reactive oxygen species.[36] The *ChrR* gene belongs to the chromate ion transport (*ChrR*) super-family and has been widely acknowledged in Archaea, bacteria, and Eukarya.[37] The data obtained in the present study was very similar to the findings of Mishra et al.,[16] in the context of Cr (VI) reduction. In the present study, the *ChrR* gene was obtained from genomic DNA of *L. boryana* (Figure S1). Similar findings have been previously reported in the literature. Some researchers have reported that the Cr (VI) resistance gene is different from that associated with Cr (VI) reduction, and the location of both genes may be different in different microbes.[27] To determine the exact location of these genes, there is a need for more studies on such microbes to explore the mechanisms of Cr (VI) reduction.

CONCLUSION

This is the first study to report on *16S* rDNA and *ChrR* genes in relation with Cr (VI) reduction in *L. boryana*. The *ChrR* gene obtained in this study exhibited 98% homology with known sequences in the NCBI database, and genetic variability has been experimental in stressed *L. boryana*. There is a need for the identification and characterization of the enzymes (protein encoded by ChR gene) and to determine the location of this gene in microbes. The findings of this study can be further verified with total genome sequencing methods and comparative genomic approaches to elucidate Cr (VI) reductase activity. Additionally, such studies are required to determine the strength and exact nature of gene functions to establish the role of Cr (VI) biotransformation in *L. boryana*. The data generated from this study may be helpful for young researchers to plan their research and help society obtain safe drinking water and ecosystems.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.17.1.36

Additional file: Additional Figure S1-S4.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AK, SK, VD and APSY conceived and designed the experiments. APSY performed the experiments. APSY, AK, VD and SK contributed reagents, materials, analysis tools or data. VD and APSY analyzed and interpreted the data. APSY wrote the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

Not applicable.

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