Genetic Variability Screening of the *Leptolyngbya Boryana* with Expressed *ChrR* Gene for the Biotransformation of Cr (VI) to Cr (III) Reduction

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Research Article

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Abstract

Chromium is well known pollutant for its mutagenicity, and carcinogenicity in humans. Excessive uses of chromium in leather tanning industries, stainless-steel production, and wood preservatives have resulted as chromium contamination in soil and water. This investigation indicates the effective use of *Leptolyngbya boryana* as an eco-friendly option to overcome Chromium (VI) toxicity in tannery effluents. The main objective of this research was to find out *ChrR* gene and its variability in the context of Cr (VI) stress. This is a novel study in the relation of *Leptolyngbya boryana*. Industrial polluted soil samples were collected and processed according to the standard protocols for *ChrR* variation and 16S rRNA gene. DNA was isolated and amplified through PCR. Amplified DNA was sequenced and aligned with the known sequences. In this study a strong co-relation was established in the nucleotide sequences of *ChrR* and *16S rRNA* genes. MIC was determined for Cr (VI) and pure strains of *Leptolyngbya boryana* were identified and isolated from soil. In the present study presence of *ChrR* gene variability was recorded in *Leptolyngbya boryana* which is a cyanobacterium in the soil of tannery effluent under Cr (VI) stressed condition and its gene variability was confirmed by sequencing. We can conclude that *Leptolyngbya boryana* strain could be eco-friendly option to overcome Chromium (VI) toxicity in tannery effluents.

1. Introduction

Wastes generated from the industries have created big crisis and it is big challenge to convert them in into the eco-friendly compounds by sustained methods. Industrial wastes (effluents) contain various toxic metals, harmful volatile compounds, along with several organic and inorganic compounds. There is a continuous demand of development of new strategies and novel sustained methods to overcome industrial waste management due to increasing urbanization (Evelyne et al., 2014). Long-term exposure of industrial effluents in the atmosphere can cause infectious diseases, neurological disorders, cancer, etc (Megharaj et al., 2003). The liberation of these toxic compounds, there is big losses in all the domains of society (Deepali, 2011).

In the industries effluents many toxic pollutants have been noticed *e.g.* chromium, sulfides, phenolic compounds, magnesium, sodium, potassium etc. One of the important toxic compounds known as chromium is also a necessary micronutrient for the development of many microorganisms (Thacker et al., 2005). But higher amount of chromium is highly toxic in all the environment *e.g.* air, water and soil. In nature, soil may retain Cr concentration ranges from 10 to 50 mg/kg (Pechova et al., 2007). In a survey, Indian tannery industries alone about 2000-3000 tons of chromium emits into the environment with high chromium concentrations from 2000 and 5000 mg/l. Through, it was a safe recommended permissible discharge limits is 2 mg/l (Belay, 2010).

In the nature, some microbes (especially blue green algae) have been reported for biological reduction (Biotransformation) of Cr (VI) into Cr (III). In the several studies it has been tried to identify chromium reductant microorganisms. Some fungi were also studied for their chromium (VI) reducing capacity (Deepali, 2011; Jayalakshmi et al., 2013). The ability of microbes to survive under Cr (VI) metal exposure

and perform detoxification mechanism into Cr (III) is being trying to rectify globally. Every microbe has its own specific metal tolerance ability (capacity) under environmental conditions. In the literature, some mechanisms of Cr (VI) reducing strategies have been mentioned *e.g.* exclusion by permeability barrier, active transport efflux pumps, intra and extra cellular appropriation, enzymatic methods etc (Bruins et al., 2000). One of the bacteria which have been convert toxic Cr (VI) to nontoxic Cr (III) had been mentioned earlier (Jayalakshmi et al., 2013; Qian 2013]. Similarly, one of the fungus which has performed Cr (VI) bio-absorptive property has been explored. During biosorption Cr (VI) gets bound to the functional groups present on the surface of microbes and gets percolated inside (Noorjahan et al., 2014).

In nature vast varieties of cyanobacteria grows on soil surfaces and morphologically phylogenetically might be different (Joo et al., 2007). Well known microbe *Leptolyngbya*, which is a filamentous form of cyanobacterium and have characterized by the thin width of their cylindrical trichomes. *Leptolyngbya* have been isolated from various industrial effluents in the soil. *Leptolyngbya boryana* species is phylogenetically connected to *Leptolyngbya sp* (Anagnostidis et al., 2014).

In the present study, research was performed to search novel Cr (VI) reductant bacteria of microbes in tannery effluent soil. The finding of this research may be more suitable, effective, eco-friendly, sustainable and cost-effective biological treatment of leather industry wastewater. In the many studies *Leptolyngbya boryana* has been exposed in the nitrogen fixation and other related genomic analysis (VI) to nontoxic Cr (III) is not mentioned anywhere. So, in the study we have focused on its potential roles for the reduction of Cr (VI) to Cr (III).

2. Materials And Methods

2.1. Chemicals

All the chemicals and reagents were of analytical grade and procured from Merck (USA), Himedia (Mumbai, India), and Qiagen (Germany). The stock solution (1000 mg L^{-1}) of Cr (VI) was prepared utilizing $K_2Cr_2O_7$ in the deionised water.

2.2. Collection of samples

Industrial effluent samples were collected from the nearby industries of Kanpur. Initially three cyanobacterial species had considered, but after the phylogenetically and morphological screening only *Leptolyngbya boryana* has included for the study. Out of 100 Samples from different places, same strains of *Leptolyngbya boryana* were found from 10 places used further in this study. Pathogenic and antibiotic treated *Leptolyngbya boryana* strains were excluded in this study.

2.3. Cyanobacteria and culture conditions

The screened *Leptolyngbya boryana* strains were grown in the Erlenmeyer flasks containing liquid BG-II media with growth conditions: 16:8 light: dark cycle; 30 ± 2°C and the 6 irradiances of 3000-4000 lux

(cool white light). Further, the isolates were routinely cultured and maintained via sub-culturing under its metabolically active state after every 20 days as mentioned previously (Yadav et al., 2021).

2.4. Morphological and Biochemical analysis

The morphology (colony morphology and colour identification) of isolated *Leptolyngbya boryana* strains were performed as described previously (Yadav et al., 2021). The biochemical estimation of Gram's stain, Catalase activity, Sucrose utility and Indole test were also performed in all strains as described (Sundari et al., 2013).

For minimum inhibition concentration (MIC) colony inoculated in large volume (5L) of BG-II medium and will keep at 37 ^{0}C for 10 days at 100 RPM shaker with different concentrations (0-1000 mg) of chromium (VI). *L. boryana* colonies were harvested with centrifugation (1000 RPM at 4 ^{0}C for 30 min.). Harvested colonies were lysed, and reduction of chromium (III) was analyzed from the wild and stressed strain of *L. boryana* (Huang et al., 2017).

2.5. PCR amplification of chromium-responsive genes:

For the genomic study, total genomic DNA was isolated from isolated cells of *L. boryana* by using Qiagen DNA isolation kit (Germany). For PCR, reaction mixture (20 µl volume) contains 10µl master mix (Takara), 1 µl forward and reverse primer each and 30µg DNA template and nuclease free water. The PCR was performed in BIORAD T100 Thermal Cycler using the following conditions initial denaturation at 95°C for 5 min, 35 cycle consisting of 94°C for 30 s, 48°C for 30s, 72°C for 1 min and final extension 72°C for 7 min. Amplified product were visualized by agarose gel electrophoresis (1% agarose in 1X TAE buffer). PCR primers for16S rRNA and *ChrR* gene were listed in Table 1. After the band purification it was submitted for the gene sequencing and following nucleotide sequences were obtained. The exact band size was found 340bp.

Table 1
Primers used in this study for amplification of *16S rRNA* and *ChrR* genes in *L. boryana*

S.N.	Gene name	Sequences	Tm (ºC)	Reference
1.	*16S rRNA* F	5'-AGAGTTTGATCCTGGCTCAG-3'	50	(Noorjahan, 2014)
2.	*16S rRNA* R	5'-TACGGTTACCTTGTTACGACTT-3'	50	
3.	*ChrR* F	5'- TCACGCCGGAATATAACTAC- 3'	53	(Patra et al, 2010)
4.	*ChrR* R	5'- CGTACCCTGATCAATCACTT-3'	54	

Table 2
Morphological, biochemical and molecular characteristics of Cr (VI) reductant strains (*L. boryana*), checked 100 different isolates and found 10 similar useful isolates.

Strain S.N.	Morphological appearance	Motility	Gram's Staining	Catalase Test	Indole Test	Sucrose utility Test	16S rRNA Test
Strain	Thin filamentous, cylindrical trichomes, colourless	Solitary	(+) Ve	(+) Ve	(+) Ve	(+) Ve	(+) Ve

Sequencing was carried out by Chromous Biotech Pvt. Ltd., India. In Brief, sequencing mix composition and PCR conditions were as follows; Total 10µl sequencing reaction mixer was prepared containing big dye terminator (version 3.1) reaction mix 4µl, 1µl primer (10pmol), 1µl template and 3µl sterile water in ABI 3500 Genetic Analyzer. In the DNA sequencing machine 50 cm capillary array column (capillary array; POP-7 polymer) was employed. The analysis protocol was performed using software BDTv3-KB-Denovo-v 5.2 (Seq scape-v 5.2 software. The result was generated in ABI, PDF, and FASTA format and 16S rRNA gene sequence was compared with the most similar sequence using NCBI blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.6. *In silico* analysis

Leptolyngbya boryana strain was confirmed by 16S rRNA based on PCR, the sequences were aligned with Clustal W (https://www.ebi.ac.uk/Tools/msa/clustalo/). Result obtained after gene sequencing were analyzed by for homology analysis by NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.7. Statistical Analysis

For the assessment of MIC and different variables in the study SPSS software version 22 (USA) is used (Corp, 2011). Data represented as mean ±SD after three independent experiments.

3. Results

3.1. Selection and identification of the cyanobacteria based on Morphological and biochemical analysis

The genus *Leptolyngbya* is a simple filamentous cyanobacterial genus with slight morphological variations between species. For the characterization of Cr reductase enzyme present in bacterium *L. boryana*, the optimum pH was recorded 7 and optimum temperature was found 37°C under 200 rpm in a water bath. Same bacterial strain were obtained from 10 places and subjected to optimized level of tolerance 800 mg/L of $K_2Cr_2O_7$ while other isolates were not grown well above 900 mg/L Cr (VI) concentration (Fig. 1).

3.2. Genetic screening of selected strain

The genomic and plasmid DNA were obtained from the morphologically and biochemically confirmed strain *L. boryana*. In this study bacterial genotypical confirmation was performed by gene specific (*16S rRNA*). The obtained *16S rRNA* sequence of *L. boryana* was homology compared with the sequences available in NCBI database. Sequence's homology has showed 98% of identity with known sequences (Fig. 3).

Further, *ChrR* gene was isolated and amplified from *L. boryana*. During the study five isolates were confirmed to have *ChrR* gene (Fig. 2a-c). Four isolates tolerant of Cr (VI) were found in a concentration of 600 mg/L and one *ChrR* gene was detected at the 800 mg/L concentration of Cr (VI).

The band size of *ChrR* gene was found around 340bp (confirmed by using DNA ladder) as shown in the (Fig. 4). The sequenced *ChrR* gene was aligned with known sequences (Fig. 5; Fig. 6).

3.3. Discussion

This study focused on the screening of the in *L. boryana* in the respect of Cr (VI) reductance ability in the soil of tannery effluent. Similar work has been done by the (Ilias et al., 2011; Camargo et al., 2003) and had stated about the Cr (VI) resistant bacteria for Cr (VI) degradation at concentrations between 500-2000 mg/L. The optimum MIC of Cr (VI) has been mentioned is around 740 mg/L, and above this concentration mentioned bacterium had not been found suitable for Cr (VI) reduction. Similar finding has obtained in our study in which optimum MIC is around recorded 800 mg/L (Fig. 1).

Our findings are supported by the results obtained by (Chaturvedi, 2011; Poornima et al. 2010) in which they have been mentioned about the Cr (VI) tolerant in *Pseudomonas putida* and found positive results for catalase, indole, and sucrose utility test positive. Similar results are also obtained in this study and found positive test of catalase, indole and sucrose utility in the *L. boryana*.

There are limited genetic studies have been mentioned in the relation of Cr (VI) reduction by analyzing *16S rRNA* and *ChrR* genes in the microbes. In this study both 16S rRNA and *ChrR* genes were found out partially and recorded 98% homology identity in 16S rRNA gene and 98% homology identity with *ChrR* gene at the Cr (VI) stress state in the *L. boryana* (Fig. 3; Fig. 6). Similar study had been performed by (Baldiris et al., 2018) which shown the presence of *ChrR* gene in the cyanobacteria and had worked in the reduction of Cr (VI). In another study which has been performed with *S. maltophilia* had demonstrated crucial property for binding of metals like Hg, Co, Zn and Cd in tannery effluents (Rocco et al., 2009). The *16S rRNA* (Fig. 2c) and *ChrR* partial genes sequenced homology has obtained by compared with available sequences in NCBI databases. Similar finding has been obtained by (Rathnayake et al., 2013) for the Cr (VI) bio-transformation in *Phormidesmis molle* with the presence of 16S rRNA and *ChrR* genes. The study had been conducted by (Sundar et al., 2010) also support the presence of *16S rRNA* and *ChrR* genes with 99 % homology in *Bacillus Cereus* strain for Cr (VI) reduction.

In the literature, partial (268 bp) chromate reductase gene had been identified in three Gram positive bacterial isolates from soil of Cr contaminated tannery effluents. In this study we have obtained 340 bp (Fig. 5) partial gene sequences in *L. boryana.* Similarly, (Deng et al., 2015) have been obtained 321 bp

(partial) Cr reductase gene in the Gram positive bacteria. This confirms the presence of chromium reductase gene in the DNA sequences of these two bacteria reaffirming their chromium reducing property. Thus, our findings confirm the presence of the chromate reductase gene in *L. boryana* and strengthen the ability to reduce Cr (VI) to Cr (III) (Tang et al., 2000). This study also has strong co-relation with the finding of (Deshpande et al., 2005) in the relation of reduction of Cr (VI) to Cr (III).

In the Cr (VI) resistant bacteria, Cr reductases gene (*ChrR*) catalyze the reduction of Cr (VI) to Cr (III) with the transfer of electrons from electron donor NADPH to Cr (VI) and resulting the production of reactive oxygen species (ROS) in reactions (Thatoi et al., 2014). Cr reductase gene belongs to the chromate ion transport (*ChrR*) super-family and has been acknowledged vastly in Archaea, Bacteria and Eukarya (Pimentel et al., 2000). In this study (*L. boryana)* the *ChrR* gene was obtained in both plasmid and genomic DNA (Fig. 2a, and Fig. 2b). Similar finding has been mentioned in the literature by some researchers. While some researchers have been mentioned that Cr (VI) resistance gene is deferent from Cr (VI) reduction and location of both genes may be different in the microbes (Juhnke et al., 2002). For getting exact location of these genes there is need of few more studies in the microbes to explore the authentic mechanism Cr (VI) reduction.

4. Conclusion

To our acquaintance, this may be the first study to report on *16S rRNA* and *ChrR* genes co-relation with Cr (VI) reduction in *L. boryana.* The obtained *ChrR* gene in this study has 98% homology with known sequence of NCBI and genetic variability has been observed in stressed *L. boryana*. There is a need of identification and characterization of the enzymes (*ChrR* protein) and to find out location of this gene in the microbes. Study can be enhanced with total genome sequencing methods, comparative genomic approaches to obtain Cr (VI) reductase activities. Additionally, such type of studies is required to know the strength and exact nature of the gene functions to determine the role of Cr (VI) biotransformation in the *L. boryana.*

Declarations

Ethics approval and consent to participate

In this manuscript there was no any ethical clearance required.

Consent for publication

Yes

Availability of data and materials

Data and materials will be available on demand to authorized person

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Ajit Pratap Singh Yadav: Conceived and designed the experiments, Performed the experiments, Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data, Wrote the paper.

Vinay Dwivedi: Conceived and designed the experiments, Analyzed and interpreted the data, Contributed reagents, materials, analysis tools or data.

Satyendra Kumar: Conceived and designed the experiments, Contributed reagents, materials, analysis tools or data.

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Figures

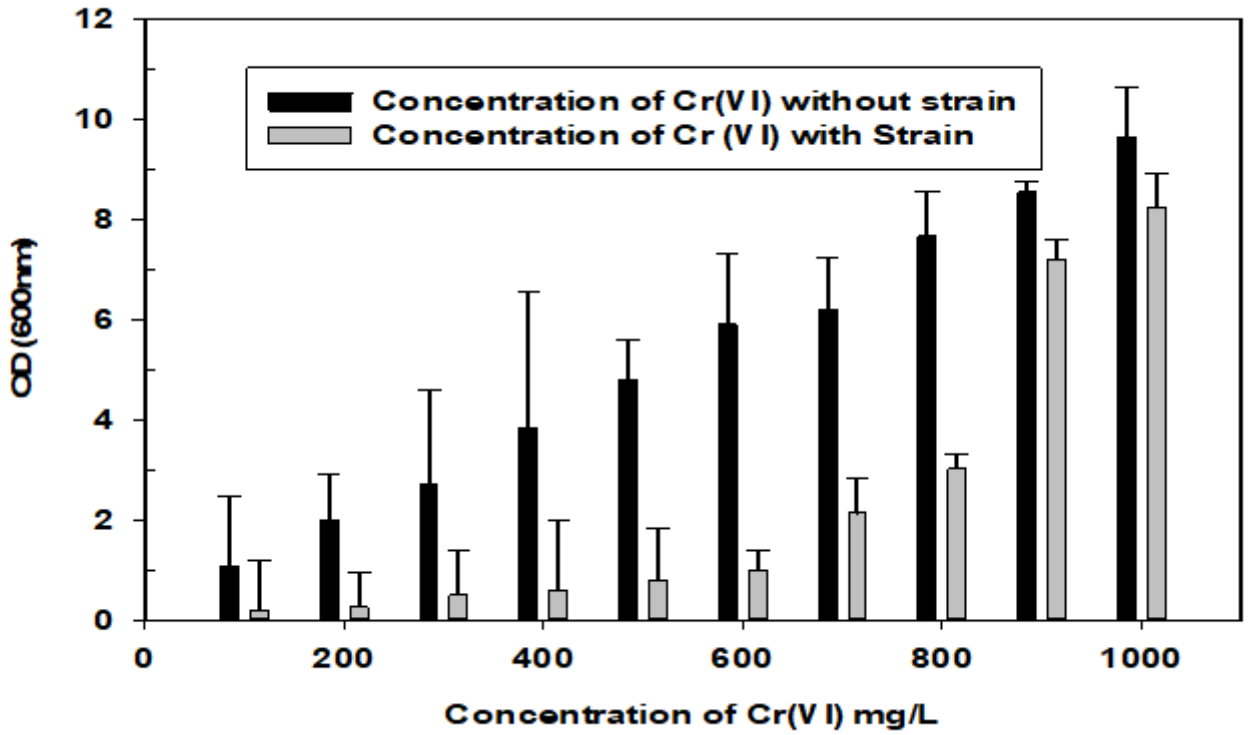

Figure 1

Histogram represents the optimum MIC of Cr (VI) in the growth culture of L. boryana. Values represented as mean ±SD after three independent experiments.

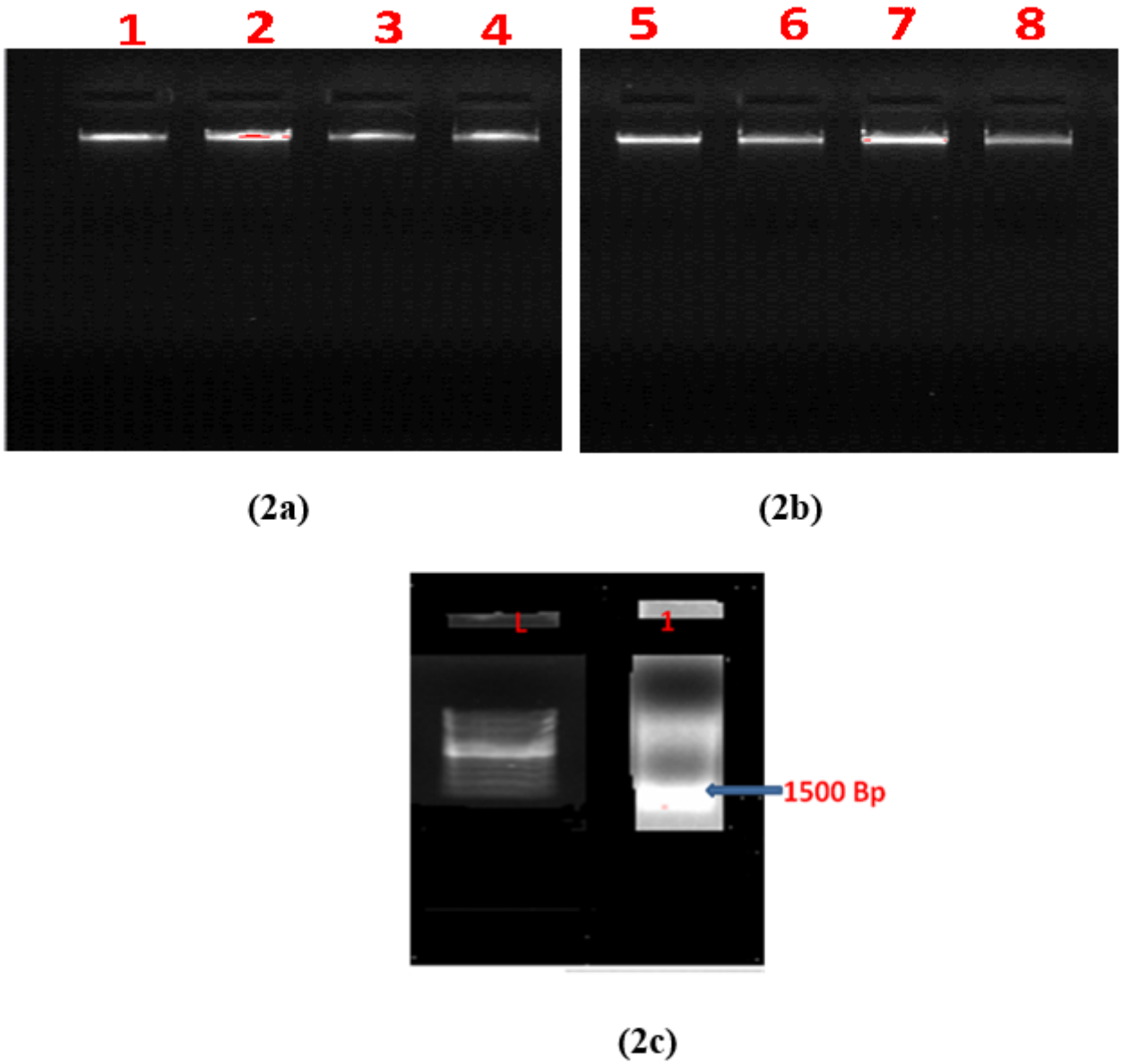

Figure 2

(2a) Lane 1-4 represented genomic DNA of L. boryana, (2b) lane 5-8 represented plasmid DNA of L. boryana, and (2c) L. boryana 16S rRNA amplification represented with 100bp ladder band size approximately 1500bp in 1% agarose gel electrophoresis. L represents Ladder (100bp).

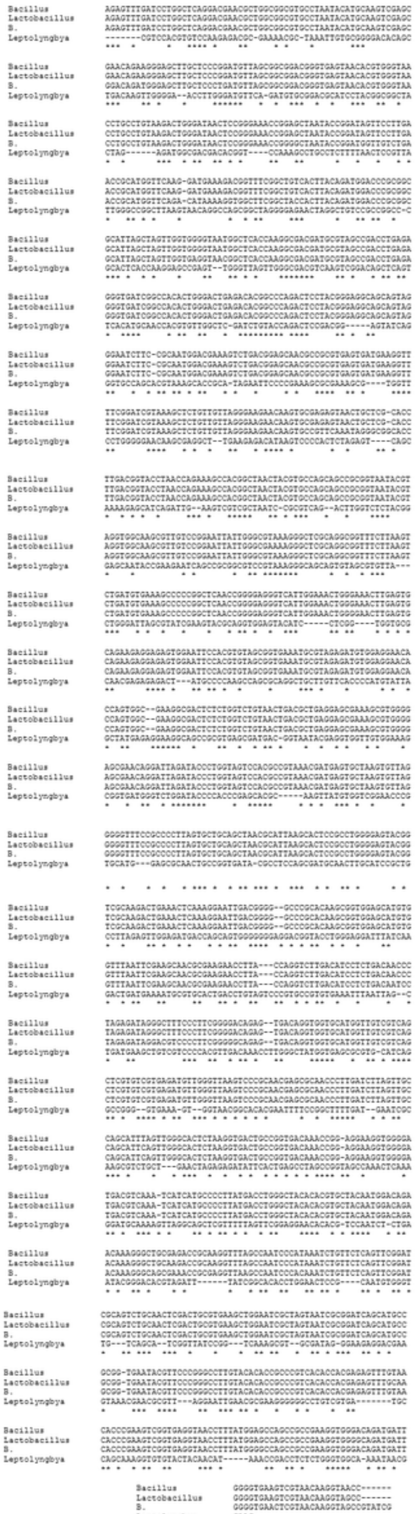

Figure 3

Multiple sequence alignment (MSA) of bacterial 16S rRNA by ClustalW after confirmation of isolated strain by 16S rRNA.

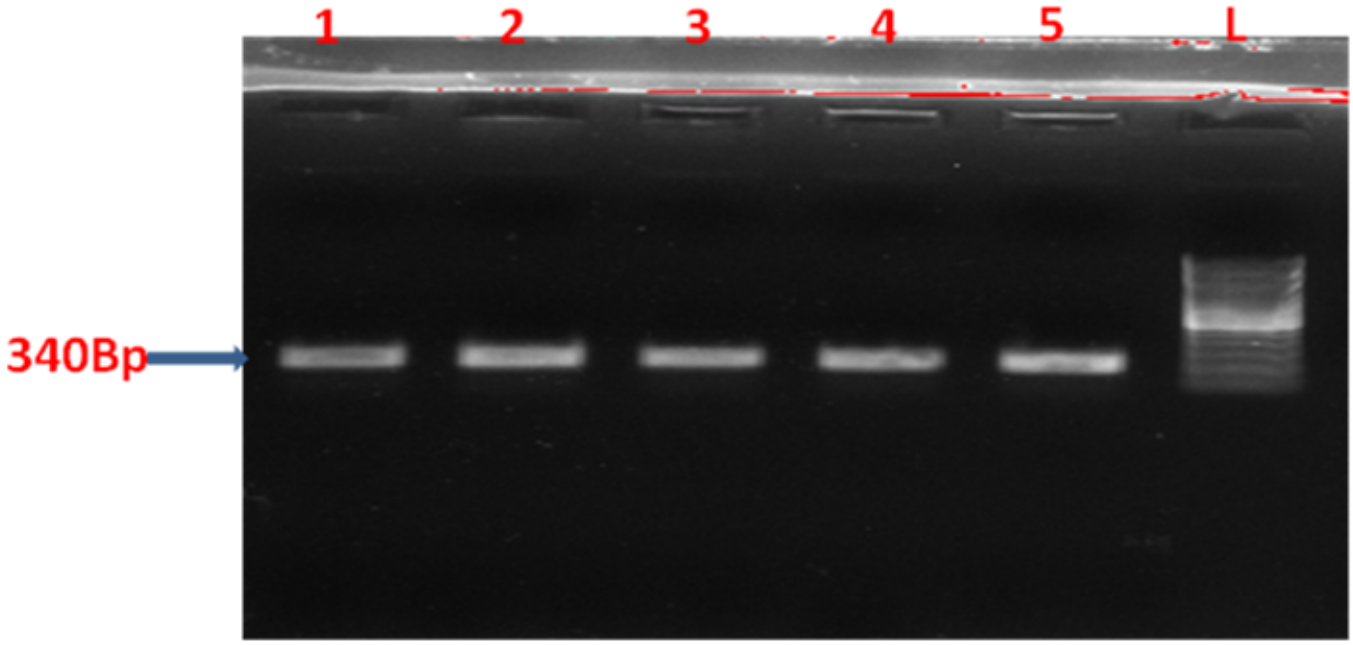

Figure 4

PCR product of Chromium Reductase Gene (ChrR gene) (approximately band size 340bp) on 1% agarose gel from isolated strain showing Chromium reductase activity. In this photo lane 1-3 were obtained from the amplification of genomic DNA and lanes 4-5 were obtained from the amplification of Plasmid DNA. L represents Ladder (100bp).

>sequence 1

CGTTGTACAACGAGGACGTCGAGGCCGAAGCGCCGCCGGAGGCGTGGAAGCGGTTTCGCGA

GGAGATCCGTCGCAGTGATGCGGTGTTGTTCGTCACGCCAGAATACAACCGATCAGTGCCGG

GCTGCCTGAAAAATGCCATCGATGTGGGCTCGCGGCCGTACGGGCAAAGTGCCTGGAGTGG

CAAGCCGACGGCGGTGGTGAGTGTATCGCCCGGGGCCATTGGCGGCTTTGGCGCCAACCAT

GCCGTGCGCCAGTCGCTGGTGTTTCTGGACATGCCGTGCATGCAGATGCCCGAAGCCTACAT

TGGCGGTGCCGCAAGCCTGTTCGACGATTCGGG

Figure 5

Partial gene sequence of ChrR gene in L. boryana after PCR, gel electrophoresis and gene sequencing.

6a

```
Query  1    CGTTGTACAACGAGGACGTCGAGGCCGAAGCGCCGCCGGAGGCGTGGAAGCGGTTTCGCG  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  CGTTGTACAACGAGGACGTCGAGGCCGAAGCGCCGCCGGAGGCGTGGAAGCGGTTTCGCG  297

Query  61   AGGAGATCCGTCGCAGTGATGCGGTGTTGTTCGTCACGCCAGAATACAACCGATCAGTGC  120
           |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  298  AGGAGATCCGTCGCAGTGATGCGGTGTTGTTCGTCACGCCAGAACACAACCGATCAGTGC  357

Query  121  CGGGCTGCCTGAAAAATGCCATCGATGTGGGCTCGCGGCCGTACGGGCAAAGTGCCTGGA  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  CGGGCTGCCTGAAAAATGCCATCGATGTGGGCTCGCGGCCGTACGGGCAAAGTGCCTGGA  417

Query  181  GTGGCAAGCCGACGGCGGTGGTGAGTGTATCGCCCGGGGCCATTGGCGGCTTTGGCGCCA  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  GTGGCAAGCCGACGGCGGTGGTGAGTGTATCGCCCGGGGCCATTGGCGGCTTTGGCGCCA  477

Query  241  ACCATGCCGTGCGCCAGTCGCTGGTGTTTCTGGACATGCCGTGCATGCAGATGCCCGAAG  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  ACCATGCCGTGCGCCAGTCGCTGGTGTTTCTGGACATGCCGTGCATGCAGATGCCCGAAG  537

Query  301  CCTACATTGGCGGTGCCGCAAGCCTGTTCGACGATTCGGG  340
           ||||||||||||||||||||||||||||||||||||||||
Sbjct  538  CCTACATTGGCGGTGCCGCAAGCCTGTTCGACGATTCGGG  577
```

6b

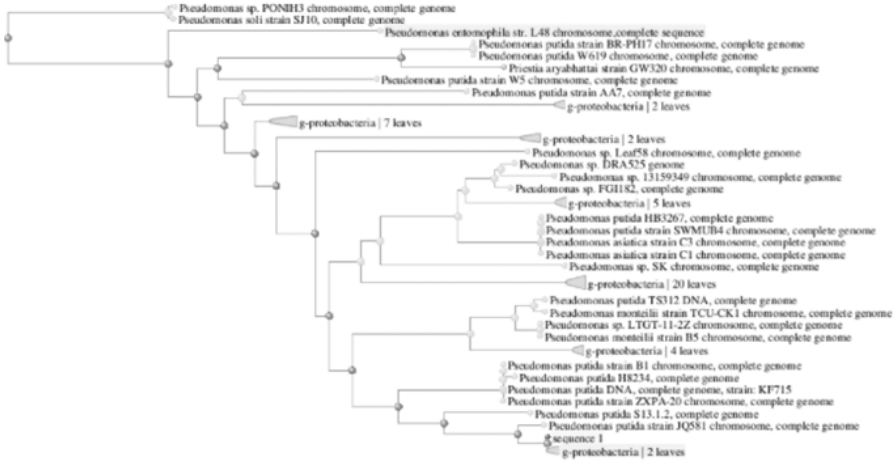

Figure 6

(6a) Homology and (6b) phylogenetic analysis of obtained partial gene sequence of ChrR gene in L. boryana.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.jpg