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Molecular Identification and Characterization of Heavy Metal Resistant Bacteria and Their Role in Bioremediation of Chromium

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NJ and MTZ designed the study. Authors MI and MH performed the experiments. Author NMA wrote the first draft of the manuscript and managed literature searches, did the statistical analysis, managed and finalized the final draft.

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ABSTRACT

In this study two bacterial strains *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) were isolated on Cr^{+6} enriched nutrient agar plates at the concentration of 100 µg/ml. Minimum inhibitory concentration of chromium for Cr-S1 was 500 µg/ml while for Cr-S2 was 400 µg/ml. Maximum growth of Cr-S1 and Cr-S2 was noticed at 37ºC and at 8.0 and 7.0 pH, respectively. After careful phenotypic and biochemical characterization, confirmation was done by 16S rRNA gene amplification and sequencing. Partial sequencing results of 16S rDNA of Cr-S1 showed 97% homology with *Bacillus thuringiensis* while Cr-S2 showed 99% homology with *Bacillus pumilus*. Both the bacterial strains, *B. thuringiensis* (Cr-S1) and *B. pumilus* (Cr-S2) were assessed for their bioremediation potential in culture medium containing 100µg/ml of chromium showed 87.04% and 90.1% Cr^{+6} uptake at 37ºC within 24 hours. This research suggests use of *B. thuringiensis* and *B. pumilus* to remove elevated levels of, not only, chromium but also other heavy metals from polluted waters from industry.

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1. INTRODUCTION

Using of the metals by industries such as metal plating and tanning, and also using of these heavy metals as a catalysts, have been eliminated huge quantity of water dissolving effluents containing a large amount of heavy metals such as, chromium, copper, cadmium, mercury, cobalt, manganese, zinc, nickel and silver. In this metal polluted toxic environments having microorganisms that have adjusted to the high toxic level of these heavy metals and turn resistant [1].

It was detected that the concentration of Cr^{+3} is very important for the good working of the living organisms [2] in the breakdown and formation of lipids and glucose such as increased fasting insulin, hypoglycemic symptoms, impaired glucose tolerance and increased cholesterol and triglycerides [3]. Waste water and soil pollution due to heavy metals has also a significant role in environmental problems [4,5]. The main site for the elimination of chromium pollution include from leather tanning effluents, electroplating of chromium, preservation of wood, manufacturing in alloys and also from the nuclear wastes where it is used in nuclear power plants as corrosion inhibitor [6]. In humans and animals chromium effects on bone marrow, blood cells and plasma, adrenals, spleen, kidney, liver and lungs. Occupational exhibited high level of chromium suffers in nasal nuisance and ulcers, puncture of the nasal septum and high sensitivity. “Chrome holes” in skin, respiratory and dermal toxicity of chromium are also reported [7,8].

Formal processes of eliminating these poisonous heavy metals from industrial processing unit wastes water contains ion exchange activity, evaporation recovery, chemical oxidation, reverse osmosis, chemical reduction, electrochemical treatment, chemical precipitation, membrane technologies and filtration [9]. The use of these conventional processes, is often deficient and very costly [10]. Large number of different kind of microorganisms have been known that reduces the soluble toxic chromium (Cr^{+6}) to the low soluble and comparatively low toxic chromium (Cr^{+3}), such as *Actinobacter* and *Ochrobactrum* [11], *Desulfovibrio vulgaris* [12]. *Arthrobacter* [13,14], *Pseudomonas* spp. [15], *Cellulomonas* spp. [16], *Serratia marcescens* [17], *Bacillus* spp. [18], *Ochrobactrum* spp. [19]. The first genus was isolated from soil polluted with Cr^{+6} belongs to *Bacillus* gave proof of chromate reductase [17,20,21].

Escherichia coli has the ability to convert Cr^{+6} higher at 10-45°C in anaerobic condition than at the aerobic condition 10-35°C. A number of genera of microorganisms including, *Bacillus, Pseudomonas, Escherichia, Enterobacter* and also some member of fungi and yeast are very helpful in bioremediation of chromium polluted water, soil and also different metals by bioaccumulation and bio absorption of chromium [22-32,2]. These bacteria has the ability, also restrict 91 percent of the chrome of the medium after 96 hours, and had also reduced chromium wastewater in Lahore to 84% of industrial waste to 144 hours [33,34].

The objectives of current study include isolation of chromium resistant bacterial strains from the tanneries waste effluent, their biochemical characterization and molecular identification, finding their minimum inhibitory, comparing their growth behavior and optimizing growth conditions. Further evaluation of the strains for the bioremediation of toxic chromium under laboratory conditions.

2. MATERIALS AND METHODS

2.1 Effluent Sampling and Source

For the isolation of chromium resistant bacteria, samples of wastewater were collected in autoclaved screw capped bottles from Sialkot region, Pakistan. The pH and temperature of the samples were measured by pH paper and thermometer respectively.

2.2 Chromium Resistant Bacteria Isolation

Chromium resistant bacteria were isolated by spreading 50 µl of the waste water sample on Petri plates containing nutrient agar with 100 µg/ml of chromium. The colonies developed on these plates after incubation were re-streaked further to facilitate purity. These pure cultures were then preserved as glycerol stocks and used for identification [35].

2.3 Minimum inhibitory Concentration (MIC)

To find out the minimum inhibitory concentration, 20 ml of acetate minimal medium was taken in each flask and various concentrations of chromium were added i.e. 100 mg/L to 600 mg/L. These flasks were inoculated with isolated strains of bacteria and incubated at 37°C in shaking incubator for 24 hours. The OD values of every bacterial isolate was determined at 600 nm wave length by spectrophotometer.

2.4 Identification and Characterization of Isolated Bacteria

According to Bergey's manual of determinative bacteriology and Cowan and Steel's manual bacteria (1979) various biochemical tests were performed for the identification of the isolated bacteria and their colonial and morphological characteristics were noted.

2.5 Determination of Growth Curve

In a conical flask 100 ml LB broth was formulated for obtaining the growth curves and sterilized at 15 lbs per inch square pressure at a temperature of 121°C for 15 minutes. Inoculated the medium with bacterial strains and incubated in a shaking incubator for overnight at a temperature of 37°C. After every 2 hour with micropipette one milliliter bacterial culture drawn and the OD value of each sample was calculated with the help of spectrophotometer at absorbance of 600 nm. By using these values the growth curve were obtained by taking OD value against time in the form of curve.

2.6 Determination of Optimum Growth Conditions

For the determining the optimum temperature, autoclaved LB broth medium was inoculated with bacterial strains and incubated in a shaking incubator for overnight and adjusted the temperature, 15, 20, 25, 30, 35 and 40ºC for each bacterial isolate separately. The optical density (OD) value was recorded at the wavelength of 600 nm with the help of spectrophotometer for each strain at different temperature. The optimum temperature was calculated by plotting the graph against temperature and OD values. For the determining the optimum pH, autoclaved LB broth medium was used and pH ranges from 4 to 10, i.e. 4, 5, 6, 7, 8, 9 and 10 by using of preset quantity of filter sterilized 1 M HCl and 1M NaOH. Then the each flask having media were inoculated with the fresh bacterial culture and incubated in shaking incubator at 37ºC for 24 hours. The OD value was recorded at the wavelength of 600 nm with the help of spectrophotometer. Then optimum pH was obtained by plotting the graph between pH and OD values.

Table 1. Isolation of bacterial strains on agar media

Characteristics	Chromium resistant strains	
	Cr-S1	Cr-S2
Colony color	Creamy off white	Orange yellow
Configuration	Round	Round
Margin	Raised/undulae	Smooth
Elevation	Flat	Raised
Form	Opaque	Opaque
Gram staining	+VE	+VE
Motility	+VE	+VE
Cell shape	Rod shape	Rod shape
Oxygen requirement	Aerobic	Aerobic
Acid fast staining	-VE	+VE
Endospore staining	Spore forming	Spore forming
Catalase test	+VE	+VE
Urease test	-VE	+VE
Gelatin hydrolysis test	+VE	+VE
MR-VP test	+VE	+VE
Citrate test	+VE	-VE
Blood agar test	-VE	+VE
Choclate agar test	+VE	-VE
Mac-Conkey agar test	-VE	-VE
Oxidase test	-VE	-VE
Starch hydrolysis test	+VE	-VE
Nitrate reduction test	+VE	-VE

2.7 Isolation of Genomic DNA and PCR

The chromium resistant strains were inoculated and incubated in a shaking incubator at 37ºC overnight. Then the culture was shifted in 50 ml falcon tube and the DNA is isolated by phenol chloroform extraction method. The 16S rDNA of bacteria was amplified with the help 16S F (5'- AGA GTT TGA TCC TGG CTC AG-3') of and 16S R (5'- GGT GTT TGA TTG TTA CGA CTT-3') universal primers.

Fig. 1. Pure culture of Chromium resistant strain *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2)

2.8 Gel Electrophoresis of PCR Products and Gene Clean

For the gel electrophoresis of PCR product one gram agarose dissolved in 100 ml 1X TAE buffer with ethidium bromide. After solidification of the gel by removing the comb, wells are formed the desired product was loaded up and carried out electrophoresis at voltage of 80V for 30-45 minutes and analyzed it in ultraviolet transluminator. The trusted band of gene was cut after electrophoresis of the amplification of the PCR product and measured the weight of agarose gel piece having desired band.

2.9 Ribotyping, Nucleotide Sequence and Phylogeny

The desired gene after cleaning was sent for sequencing to DNA CORE FACILITY Center for Applied Molecular Biology (CAMB) Lahore. The PCR product was sequenced and compared with NCBI database already published the sequence of 16S rDNA. After the comparing the data phylogenetic tree was formulated and equating it with the already known sequences. The 16S rDNA sequences of the bacteria from waste water from tanneries have been uploaded in the GenBank.

2.10 Screening tests for Biosorption of Chromium

Adequate bacterial cells are used to form 100 ml of solution having, 100 µg/ml Cr metal in the form of potassium dichromate. This was shaken at 25°C. The pH was maintained by adding 0.1 M HNO_3 and 0.1 M NaOH just before the experiments. 5 ml sample was separated at regular interval and centrifuged it at 9000 g force for 10 minutes at 10000 rpm. The same process was repeated after every one hour. Hitachi Polarized Zeeman Atomic absorption spectrophotometer (Z8200) was used to evaluate the chromium concentration in each sample.

3. RESULTS

3.1 Isolation of Bacterial Strains

The two strains of bacteria i.e., *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) were isolated on agar plates that containing chromium having varying concentration of Cr^{+6} ions in the form of potassium dichromate salt. The starting concentration of chromium used was 50 µg. Accession No. of strain *Bacillus thuringiensis* (Cr-S1) was DQ286344.1 and that of *Bacillus pumilus* (Cr-S2) was JN037409.1.

3.2 Minimum Inhibitory Concentration (MIC)

Both the chromium resistant bacterial strains were tested to determine the MIC on different concentrations of chromium in acetate minimal medium. First the different concentrations of chromium with acetate minimal medium were prepared and incubated at 37ºC for 24 hours and determined the optical density value of each sample by using the spectrophotometer. The MIC value of two strains *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) were 500 µg/ml and 400 µg/ml respectively.

3.3 Characterization of Chromium Resistant Bacterial Strains

The chromium resistant strains isolated are characterized by colony characteristics, morphologically, physiologically and biochemically.

3.4 Effect of Temperature and pH on Growth of Chromium Resistant Bacteria

The optimum temperature obtained from these both strains i.e. *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) was 37ºC. After 18 hours incubation the growth of chromium resistant strain *Bacillus thuringiensis* (Cr-S1) was maximum at pH 8 while chromium resistant strains *Bacillus pumilus* (Cr-S2) having optimum pH of 7.

3.5 Effect of Chromium on Growth Curve of Chromium Resistant Strains

The curve was obtained by culturing the bacterial strains at a concentration of 100µg/ml in the form of Cr^{+6}. These strains were incubated in shaking incubator at 37ºC for 24 hours and find out the optical density value of interval of every 2 hours and equate the value with the control culture without any stress of metal. The growth curve was plotted against time interval and optical density of both stress and control.

3.6 Isolation of Genomic DNA

The genomic DNA was isolated. About 5 µl isolated DNA of the two strains *Bacillus thuringiensis* (CR-S1) and *Bacillus pumilus* (Cr-S2) was mixed with 2 µl loading dye and loaded for gel electrophoresis. A molecular marker of 10kb also loaded for the comparison. After 30-40 min sharp bands were observed above than 10 kb.

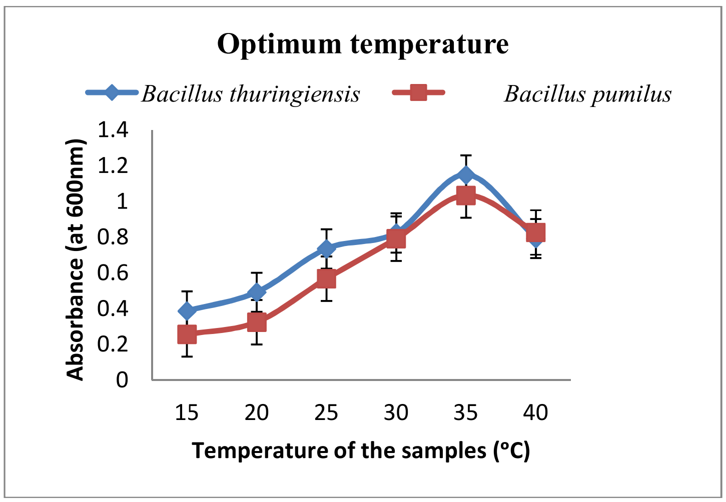

Fig. 2. Minimum inhibitory concentration

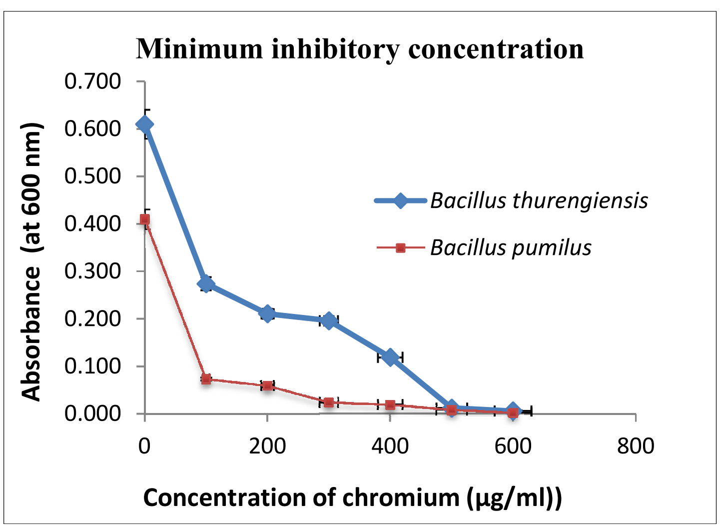

Fig. 3. Optimum temperature of bacterial isolates

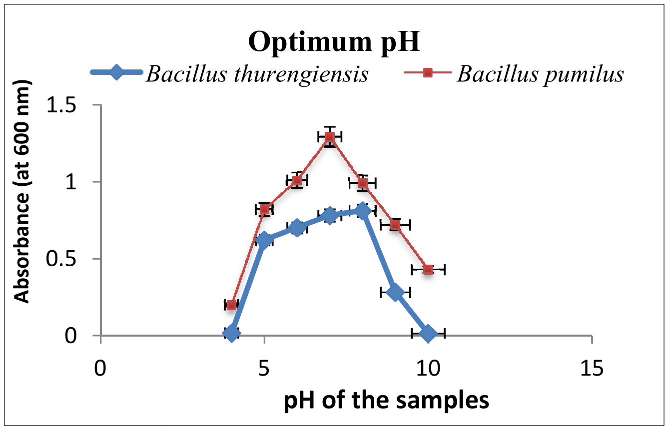

Fig. 4. Optimum pH of bacterial isolates

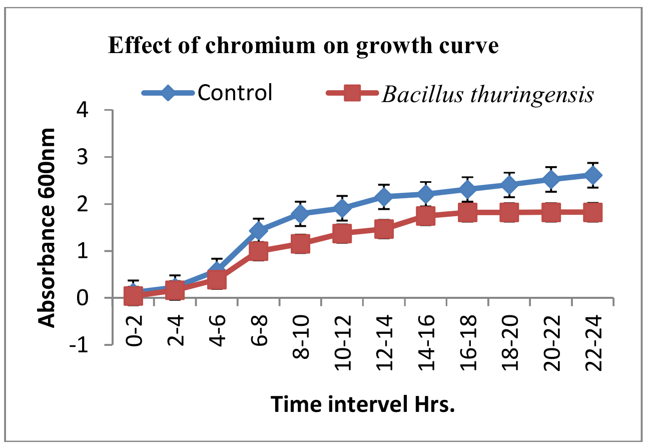

Fig. 5. Effect of chromium on bacterial strains Cr-S1 for growth curve

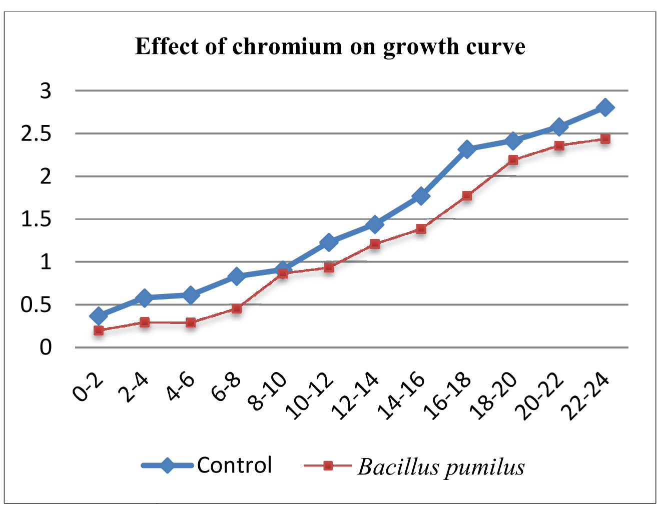

Fig. 6. Effect of chromium on bacterial strains Cr-S2 for growth curve

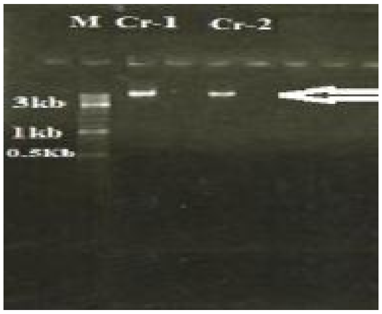

Fig. 7. 1% agarose gel for *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) shown the genomic DNA isolated from chromium resistant bacteria. M represents marker of 10kb molecular weight

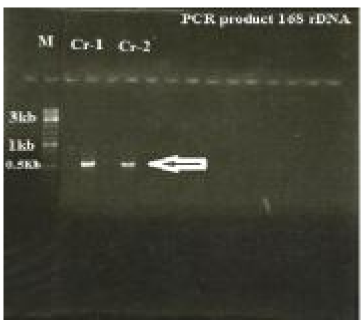

Fig. 8. 1% agarose gel of 16S rDNA PCR product by using universal primers 16S forward and 16S reverse. *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) represent the PCR product while M represents the molecular marker having 10kb weight

3.7 Ribotyping of Chromium Resistant Bacteria (PCR of 16S rDNA)

From the isolated genomic DNA, by using the universal forward and reverse primers the conserved region of 16S rDNA gene was amplified. The PCR product was observed by using UV transluminator and documented using Gel Documenter. Size of the PCR product was equal 0.5 kb as using ladder mix with 1% agarose gel.

3.8 Sequencing of the Gene from PCR Product and Phylogeny

The PCR product bands were cut of the both strains *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) from the gel. The gene clean was performed with the help of DNA recovery kit (Vivantis) and sent to (CAMB) for the sequencing. The partial sequence of 16S rDNA gene of Cr-S1 showed 97% homology with *Bacillus thuringiensis*. While partial sequence of 16S rDNA sequence of Cr-S2 showed 99% homology with *Bacillus pumilus*. The phylogeny tree of both strains also shows their close relationship to other strains.

3.9 Bioremediation of Chromium (VI) through Bacterial Isolates

Both the bacterial strains *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) were capable to bioremediation of 100µg/ml of chromium from the medium 87.04% and 90.1% respectively within 24 hours. It was observed that *Bacillus pumilus* showed similar ability to reduce Cr^{+6} to the *Bacillus thuringiensis.*

4. DISCUSSION

The present study represents the chromium contamination in the tanneries waste water from District Sialkot [36]. Chromium (Cr) is also a dominant species of heavy metal in aquatic as well as terrestrial environment which is found mostly in two form Cr^{+6} and Cr^{+3} [37-39,6,3]. The two vital parameters which affects on concentration and detection of chromium in waste water sample are the pH and temperature. The tanneries waste water samples temperature and pH ranges were 25ºC to 32ºC and 6 to 8 respectively. It is also observed from waste water of tanneries District Sialkot. In the current study the optimum pH for growth of *Bacillus thuringiensis* and *Bacillus pumilus* were observed 8 and 7 respectively which is supported by the results of [38,40]. The study shows that the temperature at which the growth of bacterial strains, *Bacillus thuringiensis* and *bacillus pumilus* is maximum at 37°C have same results [41].

The *Bacillus thuringiensis* and *Bacillus pumilus* strains have been isolated and checked for the resistant to chromium salt ($K_2 Cr_2 O_2$). Similarly, chromium resistant bacteria were isolated from tanneries waste water [42,18,13,43,44]. The bacterial strains having highest tolerance capability for chromium were 500 µg/ml and 400 µg/ml. Other studies showed maximum tolerance concentration value ranges from 100 µg/ml to 500 µg/ml [45]. It was also reported that a number of bacterial strains that was resistant to hexavalent chromium isolated from the effluents of leather processing units are capable to

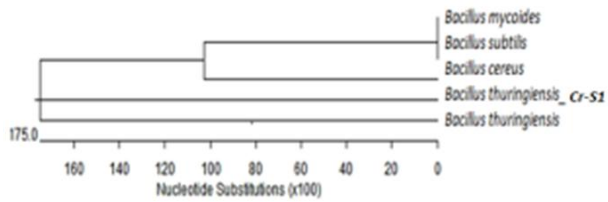

Fig. 9. Phylogeny tree of *Bacillus thuringiensis*_Cr-S1

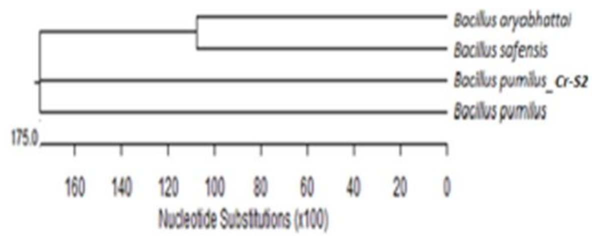

Fig. 10. Phylogeny tree of *Bacillus pumilus*_Cr-S2

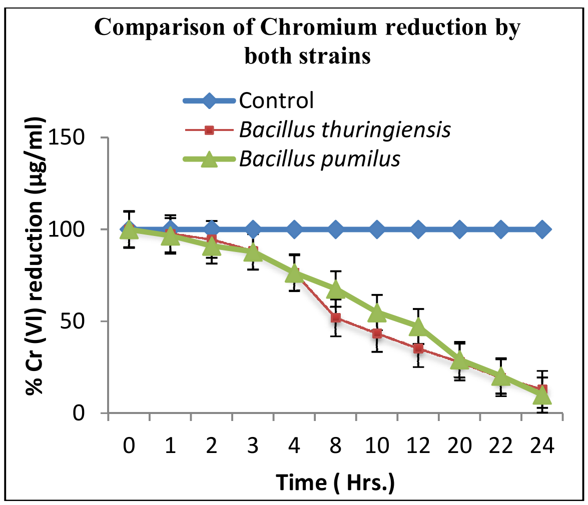

Fig. 11. Comparison of bioremediation between *Bacillus pumilus* and *Bacillus thuringiensis* from the broth medium having 100 µg/ml chromium in the form of Cr^{+6}

withstand 0.04 mg/L [46]. Both the chromium resistant strains isolated belong to genus Bacillus as several strains were isolated earlier [47]. The highest chromium resistant strain isolated from the effluents of leather processing unit was over 2500 mg/L [48]. Minimum inhibitory concentration for chromium of both the strains, *Bacillus thuringiensis* and *Bacillus pumilus* isolated is 500 µg/ml and 400 µg/ml respectively showing similar results in another study [49,3].

The genomic DNA was isolated of both the chromium resistant strains, *Bacillus thuringiensis* (Cr-S1) and *Bacillus pumilus* (Cr-S2) and partially amplified. This amplified 16S rRNA gene by PCR having size of about 0.5 kb. The DNA band was removed from gel and after gene clean sent to CEMB for sequencing. After the partial sequence of 16S rRNA gene of Cr-S1 strain showed 97% homology with *Bacillus thuringiensis* and the partial sequence of 16S rDNA sequence of Cr-S2 showed 99% homology with *Bacillus pumilus*.

In the recent study, *in vitro* chromium reduction from the broth media, contain 100 µg/ml chromium concentration, was observed 87.04% and 90.1% by *Bacillus thuringiensis* and *Bacillus* *pumilus* respectively after 24 hours. Similar results have also been reported chromium reduction by bacillus sp. [50,7,21].

The *Bacillus thuringiensis* and *Bacillus pumilus* are extremely tolerate against poisonous heavy metals and survive in the presence of high concentration of chromium. These two chromium resistant bacterial strains isolated from the tannery waste effluent may be used for bioremediation of industrial waste containing several types of heavy metals especially chromium.

5. CONCLUSION

From this research work, it can be concluded that the bacterial strains of *B. thuringiensis* and *B. pumilus* can be used in bioremediation. These can be useful in removal of different heavy metals including chromium, thus decreasing level of these metals in water being polluted with industrial wastes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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