Bioremediation of Chromium Contaminated Soil: Reduction and Optimization under Laboratory conditions

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Abstract— This study had the objective of isolation and enrich Cr(VI) potential reducing microbial strain and to determine the optimal conditions for Cr(VI) as well as to study its performance under optimal conditions. Chromium contaminated soil sample were taken from Salem Steel Plant, the physicochemical analysis of this soil showed that its pH was 9.54, the electrical conductivity was 0.36 mS, while the turbidity of this soil was found to be 3.50 NTU. Mineral salts such as Iron, Copper, Manganese, Zinc, Nickel, Cobalt, Lead, Aluminum, Vanadium, Chromium, Molybdenum, Mercury, Arsenic and Cadmium. However chromium was present at the highest amount ot 17.896 ppm. The isolation of chromium resistant bacterial strains from the soil sample collected was done followed by their characterization. Two strains Bacillus subtilis and Sphingomonas spp were found to be resistant to chromium. However Bacillus subtilis was found to be resistant at higher chromium concentration and consequently was used in this study. The determination of optimal conditions for Cr(IV) reduction by Bacillus subtilis was done using parameters such as carbon source, pH, and temperature. Results from this study showed that the reduction of Cr(VI) was more effective at pH of 7, temperature range between 30–37˚C and also when Glucose is used as carbon source. The bioremediation of soil sample by Bacillus subtilis was performed at optimal conditions. The results from this study revealed that there were a tremendous reduction of hexavalent chromium after 15 days. This confirms the ability of isolated strain for removal of Cr(VI) from soil and therefore it can be a potential candidate in insitu chromium bioremediation.

Index Terms— bioremediation, hexavalent chromium, reduction, contaminated soil

1 INTRODUCTION

THE world nowadays is facing the greatest problems in which environmental pollution. This is increasing year by year due to urbanization and industrialization, causing serious damage to the earth [18]. With industrial activities novel chemicals and heavy metals are released in the environment via effluents and these pollutant pose a threat to entire ecosystem. Among those pollutants chromium is included. Chromium was discovered later than other metals because of its relatively low concentration in the earth's crust – detected at approximately 100 ppm in chromium rich rocks. Additionally, chromium does not appear terrestrially as a native metal, but is strongly bonded to minerals in which it occurs. Naturally occurring chromium is usually present as trivalent Chromium. Hexavalent Chromium in the environment is almost totally derived from human activities (WHO, 1990).

In nature chromium occurs in more than fifty different ores. The following are some examples: Barbertonite: Mg6Cr2(CO3)(OH)16.4H2O, Brezinaite: Cr3S4; Chromite: (Mg,Fe2+)(Cr,Al,Fe3+)2O4; Chromatite: CaCrO4; Nichromite: (Ni,CoFe2+)(Cr,Fe3+,Al,)2O4. Chromium in water originates from natural sources, such as weathering of rock constituents, wet precipitation and dry fallout from the atmosphere, and run-off from the terrestrial systems. Natural sources of chromium accumulation in soil are due to the weathering of parent rock. 30-40% of chromium in atmosphere is from the natural sources [24]. Chromium is a transition metal which can exist in several chemical forms displaying oxidation numbers from 0 to VI. Only two of them, trivalent and hexavalent Cr, are, however, are the most significant to exist in environment because of their persistence and stability [27]. Chromium like other metals occur naturally in the environment in varying concentrations and are present in rocks, soil, plants, and animals [2].

Chromium is one of the heavy metals whose concentration in the environment is still increasing. We do not need to be worry about global risk of chromium contamination, but for local environments it could be a serious problem [2]. Normally, Chromium is an essential micro- nutrient in the diet of animals and humans, as it is indispensable for the normal sugar, lipid and protein metabolism of mammals [17]. Its deficiency in the diet causes alteration to lipid and glucose metabolism in animals and humans. Chromium is included in the complex named glucose tolerance factor (GFC). On the other hand, no positive effects of chromium are known in plants and microorganisms [20]. However, elevated chromium concentrations are always toxic, although the toxicity level is related to the oxidation state of chromium. Cr(VI) is not only highly toxic to all forms of living organisms, mutagenic in bacteria, mutagenic and carcinogenic in humans and animals [14], but is also involved in causing birth defects and the decrease of reproductive health [12]. Cr(VI) may cause death in animals and humans, if ingested in large doses [31]. The LD50 (dose that causes the death of 50% of a defined animal population) for

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oral toxicity in rats is from 50 to 100 mg kg−1 for Cr6+ and 1900–3000 mg kg−1 for Cr(III) [8].

The respiratory tract is one of the targets for inhaled chromium following acute exposure, where Chromium (VI) may cause perforation of the nasal septum, asthma, bronchitis, pneumonitis, inflammation of the larynx and liver and increased incidence of bronchogenic carcinoma [2]. Due to the corrosive nature of some chromium (VI) compounds, dermal exposure can lead to dermal ulcers and at high doses, systemic toxicity leading to effects on the renal, haematological and cardiovascular system and death. While Skin contact of Cr(VI) compounds can induce skin allergies, dermatitis, dermal necrosis and dermal corrosion. Chromium compounds are also linked to mutagenicity where may cause chromosomal aberrations and sister chromatid exchanges in humans. The mechanism of cancer formation caused by Cr(VI) is not known for certain; however, it has been postulated that Cr(VI) binds to double stranded deoxyribonucleic acid (DNA), therefore altering gene replication, repair, and duplication[13].

The treatment of these wastes is therefore essential before discharging them to the environment. Chemical treatments in attempt to removal of Chromium IV from effluent are available but they fail to meet environmental regulations. Therefore, Bioremediation would be a better alternative to chemical treatment for this purpose as the chemical agents add to the environmental pollution [15], [11]. Based on that fact, we set the objective of this study as to isolate and enrich Cr(VI) potential reducing microbial strain and to determine the optimal conditions for Cr(VI) as well as to study its performance under optimal conditions. In attempt to contribute to the development of the bioremediation technology.

2. MATERIALS AND METHODS

2.1 Soil sample collection

Chromium contaminated soil samples were aseptically collected in screw capped sterilized bottles from industrial area(salem steel plant) district of Tamil Nadu State in India. And were transported to the ALPHA OMEGA HI-Tech bioresearch center laboratory, for further analysis. The physical and chemical properties such as, electrical conductivity, turbidity, mineral salts and pH of the soil samples obtained from this waste site were characterized and shown in Table 1.

Isolation of chromium resistant bacteria

For isolation of chromium resistant bacteria, 1.0 g soil sample was dispersed in 100 mL of the sterile distilled water. A serial dilution (Figure 4.8) was made up to 10-5 from the effluent treated soil sample. 100 µL of each dilution was spread on nutrient plates containing 10 µg potassium dichromate ($K_2Cr_2O_7$) per mL of the medium. The growth of the bacterial colonies was observed after 24 hrs of incubation at medium at 37°C. It was subcultured on nutrient agar plate containing 10 µg of potassium dichromate ($K_2Cr_2O_7$) per mL.

2.2 Identification of the isolate

The identification of the isolates was made based on both macroscopic and microscopic morphological and pigmentation characteristics of the colony. This involved culturing the isolate on nutrient agar plates for studying the appearance of colonies following which gram's staining and motility test together with biochemical tests were performed.

2.4 Screening of the potent strain

The isolated Cr resistant bacteria were further screened for maximum reducing activity. Nutrient agar medium containing different concentration range of 1µg/ml to 1000µg/ml of Cr concentration were used for screening the reduction activity. The organisms which are grown in the higher concentration were selected for further activity study.

2.5 Cr(VI) reduction assay

LB broth with 1000µg/ml of chromium concentration was prepared and 50ml of broth is taken into 100ml of Erlenmeyer flasks. Isolates those are capable of grown in 1000µg/ml were selected and loopful of inoculum was inoculated in to Erlenmeyer flasks containing 50 of LB broth and kept for incubation. After incubation cells were collected after centrifugation at 10,000rpm for 10 minutes. Then supernatant was analyzed for residual chromium by 1, 5- Diphenyl Carbazide method by measuring absorbance at 540 nm using a spectrophotometer. Similarly in order to observe chromium reduction, sediment of bacterial cells were washed twice with Phosphate Buffer Solution (PBS) and resuspended in water and presence of Chromium was observed Diphenyl Carbazide DPC method.

2.6 Diphenyl carbazide assay

Reagents required

1N HCL: mix 86 ml of concentrated HCL in 914 ml of distilled water. Diphenyl carbizide solution : 24 ml 0f 85% H_3PO_4 to 56 ml distilled H_20, this solution was mixed with 0.076g of DPC reagent previously dissolved in 20 ml of 95% ethanol.

Procedure

To 1ml of Cr solution, 1ml of 1N HCl solution was added and mix the solution and then 1ml of Diphenyl Carbazide solution was added. The content was mixed well, wait for 5min to develop purple color. The optical density of the obtained color was measured at 540nm against reagent blank. The procedure was repeated for different aliquots of standard Cr solution and calibration curve was constructed using concentration against absorbance forum. The calibration curve was used for estimation of Cr in samples.

2.7 Optimization of parameters for chromium bioreduction

Optimization based on carbon source

Glucose, starch and cellulose were used as carbon sources, was taken and added in nutrient medium prepared in a conical flask of 50 ml at difference ratio of concentration, the amount of $K_2Cr_2O_7$ were sterilized separately and added to the media. The incubation period was 5 days at 37℃. Each day the culture was assayed for residual chromium by DPC method.

Optimization based on pH

Different pH value such as 3.0, 5.0, 7.0, 9.0, and 11.0 were selected and adjusted in the nutrient medium prepared and ster-

ilized pH was adjusted using NaOH and HCL All the media contained 50 ppm chromium in the form of K2Cr2O7 which was separately sterilized and added to prevent its reduction by media components. The incubation temperature was maintained at 37℃ for 5 days chromium estimation by DPC method was performed each day to estimate the residual chromium.

Optimization based on temperature

Nutrient medium in conical flask of 50 ml was prepared and sterilized, 50 mg/l of chromium concentration was added into a conical flask, and *bacillus sp* as inoculum was added and incubated at different temperature i.e. 30℃, 37℃ & 40℃ Incubation period was 5 days. OD values of Chromium content was taken each day by DPC method.

Bioremediation of chromium from the soil sample

Bioremediation was carried out in a period of 15 days. Non polluted soil samples was taken from the surface (5-15 cm) and soil was kept at 10-15 ℃ in the dark until use. 100 gm Soil sample was taken into 2 clean glass conical flask of 250 ml (control and sample) and kept 20% humidity with $K_2Cr_2O_7$ solution to obtain a final concentration of 50mg/kg dry weight of soil. The above soil sample was steam sterilized (3 successive sterilization at 24 hrs intervals at 100℃ for 1 hr each) *Bacillus* sp. was grown in nutrient broth, the culture was incubated for 3 days at 30℃ . The soil samples was inoculated with the precultured *Bacillus* sp. At a concentration of 0.5mg/kg dry weight of soil glass conical flask was incubated at 37℃ for 2 weeks and the OD values for Cr(IV) was recorded each 2 days till completion of incubation period 1g of soil in each conical flask was taken to 9ml of distilled water and make a serial dilution and centrifuged at 3000rpm for 5 mins and collect the supernatant which was measured by DPC method for the chromium removal.

3. RESULTS AND DISCUSSION

3.1 Characteristics of collected soil sample

The collected soil sample were subjected to physicochemical characterization. Those Physicochemical parameters of contaminated soil estimated are shown in Table 1 and Table 2.

TABLE 1
PHYSICAL PROPERTIES OF SOIL SAMPLE

Sl.No.	Physical properties	Values
1	pH	9.54
2	Electrical conductivity	0.36 Ms
3	Turbidity	200 NTU=0.35 20 NTU=3.50
4	Temperature	32.1°C

3.2 Isolation and characterisation of chromium resistant bacterial

3.3 Strains and Cr(VI) tolerance study

Soils contain a very large number of micro-organisms which can include a number of Cr(VI) utilizing bacteria. Two chromium resistant bacterial strains were isolated from the soil sample by serial dilution technique. The isolates are tested for their chromate tolerance at different concentrations (25- 250µl/ml) in solid agar medium. The microbial strain showed the resistance against chromium were found to be *B. subtilis* and *Sphingomonas* sp.

Furthermore these two strain were sub cultured on medium with high concentration of chromium. Bacillus subtilis strain showed the ability to grow in high concentration of chromium. Therefore it was subcultured on NA agar slants and stored for further studies. The figure 1 shows screening of isolated bacteria on chromium resistance and table 3 depicts the results of biochemical characterization of the isolated bacteria.

TABLE 2
CHEMICAL PROPERTIES OF SOIL SAMPLE

S. No	Element analysed	Amount(ppm)
1	Iron (Fe)	12.93
2	Copper (Cu)	0.5796
3	Manganese (Mn)	0.478
4	Zinc (Zn)	0.523
5	Nickel (Ni)	0.0969
6	Cobalt (Co)	0.049
7	Lead (Pb)	0.248
8	Aluminum (Al)	7.1647
9	Vanadium (V)	0.3571
10	Chromium (Cr)	17.896
11	Molybdenum (Mo)	0.8613
12	Mercury (Hg)	0.0039
13	Arsenic (As)	0.0043
14	Cadmium (Cd)	0.0771

TABLE 3
BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED BACTERIA

Biochemical test	*B. subtilis*	*Sphingomonas* sp
Gram staining	+	-
Methyl Red	+	+
Oxidase	-	-
Catalase	+	+
Indole	-	-
Urease	-	+
Nitrate reduction	-	-
Voges- Proskauer	+	+
Glucose fermentation	-	-
Motility	+	+

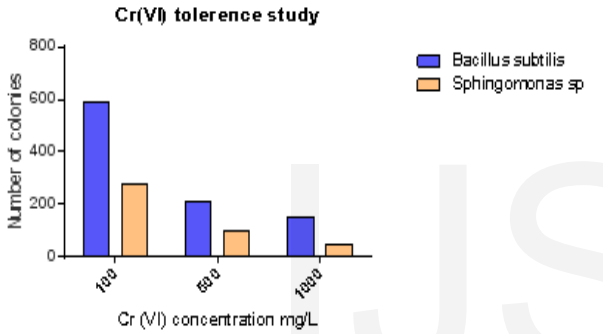

Fig. 1. Cr (VI) Tolerence Study.

The isolated bacterial strain Bacillus subtilis and Sphingo-monas spp showed capability to grow under different concen-trations of Chromium(VI), however Bacillus subtilis showed the highest re-sistance in higher concentration of Chromium(VI).

3.4 Optimization of Cr(VI) reduction based on carbon source

The figure 2. Shows the results of optimization study of biologi-cal based reduction of (Cr VI) by *Bacillus subtilis* under laborato-ry conditions.

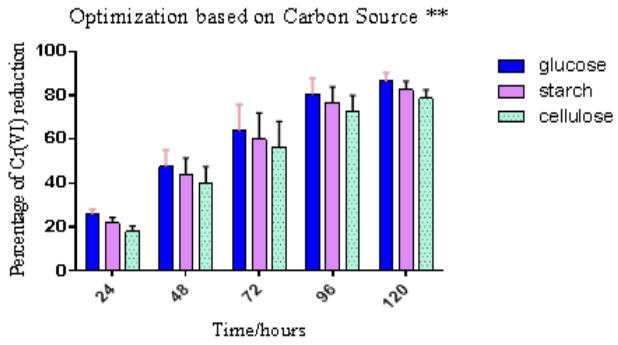

Fig. 2. Optimization based on Carbon source.

** Error bars indicate 6.02% deviation from mean

From the figure 5.10, at each time interval the highest Cr (VI) reduc-tion is observed where glucose was used as carbon source, starch is higher than cellulose.

3.5 Optimization based on temperature

The Cr(VI) reduction capability by bacterial strain is greatly influenced by incubation Temperature. The figure 5.12 shows the results of the study of optimization based on the tempera-ture of biological based reduction of Cr(VI) by *Bacillus subtilis*

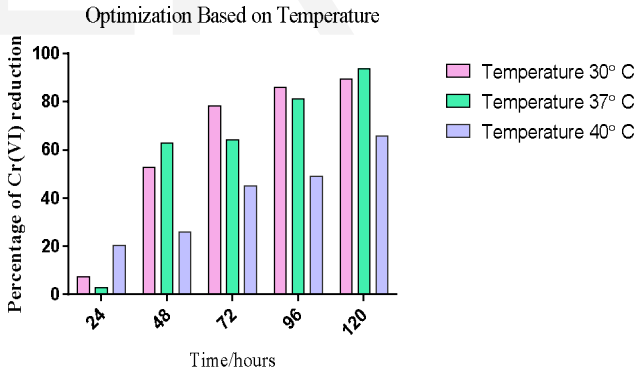

Fig. 3. Optimization based on temperature.

The highest chromium reduction was recorded between temperature of 30 and 37˚C. at temperature of 40˚C the amount of chromium recorded was slightly lower than those recorded at 30 and 37˚C.

3.6 Optimization of Cr(VI) reduction based on pH

All biochemical reactions are enzyme catalyzed which are pH sensitive. Therefore the optimization of biological Cr(VI) re-duction by the isolated *Bacillus subtilis* were carried out to find out the optimal pH of this strain. The figure 5.11 shows the

effect of pH on the reduction of hexavalent chromium by bacteria strain *Bacillus subtilis.*

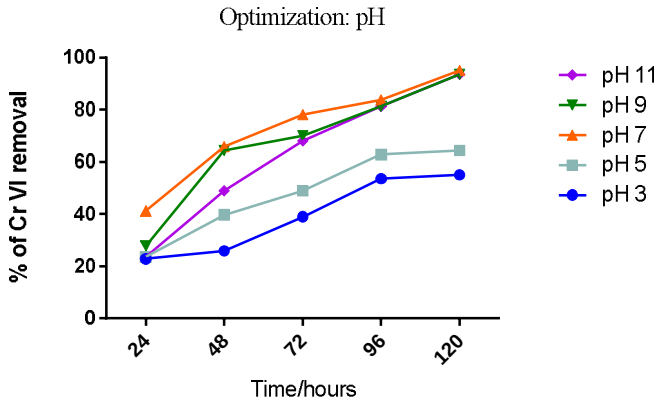

Fig. 4: Optimization of hexavalent chromium reduction based on pH

Chromium reduction is being observed at each pH value set. However at pH of 7 the significant amount of Cr(VI) reduction was recorded though alkaline pH values also the amount of chromium reduced was higher than acidic pH values.

3.7 bioremediation of chrmomium contamited soils

The study of removal of Chromium from soil sample were carried out using the isolated bacterial species *Bacillus subtilis* the figure 5.13 depicts the results obtained from this study

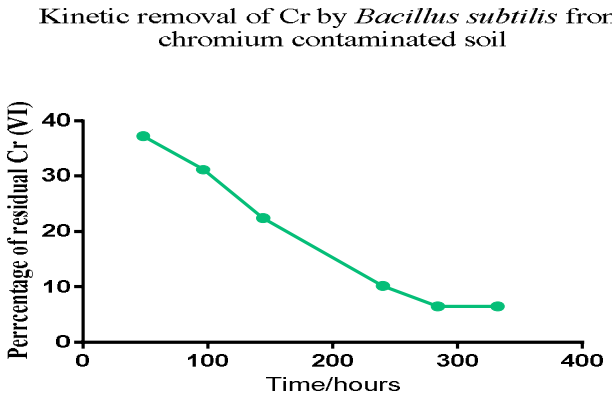

Fig. 5: Optimization of hexavalent chromium reduction based on pH

After inoculation of the strain in chromium contaminated soil under optimal conditions a significant reduction of chromium was observed each day. After 15 days of incubation the amount of chromium was significantly removed from soil used in this study.

4. DISCUSSION

The objective of this study was to isolate and enrich Cr(VI) potential reducing microbial strain and to determine the optimal conditions for Cr(VI) as well as to study its performance under optimal conditions. Therefore there were a need of organism, which is able to resist to chromium concentration. To fulfill this, chromium polluted soil samples were collected form industrial area (salem steel plant) district of Tamil Nadu State in India, hoping that the possibility of getting Cr(VI) reducing bacterial strain will be more.

The physical characterization results table 5.4 shows the pH value of 9.54 which indicates the alkalinity of soil from which the sample was taken. High alkaline pH levels are associated with slowed organic matter mineralization or poor microbial activity. Additionally, some crops are unable to grow in higher alkaline soils [24]. The electrical conductivity is the measure of salinity. Oftentimes, it is defined as the ability of material to transmit an electrical currents. The results reported in table 5.4 showed that the electrical conductivity of soil sample collected is 0.36 mS. Soils with minerals salts is said to be able to conduct electricity, in addition to this the low electrical conductivity of soil is observed in sandy soil [33]. The turbidity depict the cloudiness of a fluid, this is caused by usually invisible total suspended solids. The turbidity of soil sample collected was reported to be 3.50 NTU. According to WHO, turbidity should be ideally below to 1 NTU. High turbidity is associated with reducing of dissolved oxygen which is a threat to aquatic life. The suspended solids responsible for turbidity help the attachment of heavy metals and so increasing heavy metals toxicity.

Chemical characterization of the sample soil was done by estimating the amount of mineral salts such as: Iron (Fe),Copper (Cu),Manganese (Mn), Zinc (Zn), Nickel (Ni), Cobalt (Co), Lead (Pb), Aluminum (Al), Vanadium (V), Chromium (Cr), Molybdenum (Mo), Mercury (Hg), Arsenic (As), And Cadmium (Cd). From the results table 2, the analysed chemicals were presents in adequate amount. However, chromium showed the highest amount of 17.896 ppm(17896 ppb) which exceeds the standard amount of 100 ppb according to US EPA standards. Once this reaches water will make it unfit for drinking and other applications [32].

For isolation of the microorganisms serial dilution and pour plate method are best suited, because by using these techniques we can get minimum number of microorganisms population after each dilution. Microorganisms isolated were characterized based on biochemical tests (table 3), found to be *Bacillus subtilis* and *Sphingomonas* spp. Furthermore these two strain were sub cultured on medium with high concentration of chromium. *Bacillus subtilis* strain showed the ability to grow in high concentration of chromium figure 1. The isolated bacteria isolated was used in optimization of Cr reduction and soil Cr(VI) bioremediations.

The results of this study shows that there is a significant

Cr(VI) reduction by microbial strain used when glucose is used as carbon source. In the study Detoxification of hexavalent chromium by an indigenous facultative anaerobic Bacillus cereus strain isolated from tannery effluent, Neha Singh *et al*. (2012), it was reported that a 78% of chromium reduction was observed at 0.5% glucose. Compared to other carbon source employed, glucose was the most effective carbon source while lactose was the least carbon source. Also Pal *et al*. (2005) demonstrated that glucose act as electron donor and hence it significantly increase the reduction of Chromium by *Bacillus sp.* The same results was observed on *Ochrobacterium Sp. CSCr-3*[10] and *Streptomyces griseus* [23].

From the figure 3, even though at all pH values set the bacteria showed ability to reduce Chromium, significant amount of Cr(VI) reduction was observed at pH 7. Optimal pH for growth of Cr(VI)-resistant bacteria was evidenced at 7.0 to 7.8 [14], but Cr(VI) forms are soluble over a wide pH range and generally mobile in soil–water systems). It was suggested that Cr(VI) reduction by *Enterobacter cloacae* occurred at pH 6.5 to 8.5 and was strongly inhibited at pH 5.0 and 9.0 [30]. It can be therefore inferred that this bacteria grew well in neutral to alkaline pH and shown noteworthy Cr(VI) reduction efficiencies in those conditions. Other studies also showed the same results [4]. Since the pH of the sample soil tested falls in neutral range, the Cr(VI) reducing potent bacteria will not confront any problem in reducing Cr(VI) and if Cr(III) is formed after bio-reduction, there bioavailability will be reduced by forming hydroxide. Cr(VI) is highly soluble in water and forms divalent oxyanions [chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$)], whereas Cr(III) readily forms less soluble hydroxides at neutral pH [1]. While in acidic pH, bacterial growth and Cr(VI) reduction were inconsequential. Insignificant Cr(VI) reduction in acidic pH may be accounted to negligible bacterial growth.

Effect of temperature on chromium reduction was studied at pH 7 and 50 mg/l Cr(VI) concentration. From the figure 4, it is demonstrated that maximum Cr(VI) degradation occurred at temperature range of 30˚ and 37˚C, with the later showing the highest percentage of Cr(VI) removal. The most possible reason is the effect of temperature on the activity of chromium reductase. Similar results were also reported in other studies [4] and [30] respectively. The optimal temperature of 30–37˚C reducing Cr(VI) reduction was observed in[14]. It was reported that optimum Cr(VI) reduction was observed at 30˚C and Cr(VI) reduction was severely affected by temperatures above 30˚C [9]. It can be concluded that the optimal temperatures for Chromium reduction by this strain fall in the range between 30-37˚C higher temperatures may reduce the Cr(VI) reduction.

Results from figure 5 shows that there were a significant removal of Cr(VI) form soil sample inoculated with Bacillus subtilis after 15 days. Previous studies determined that greatest removal efficiency was observed in soil supplemented with an organic substrate [28] and [29]. It was demonstrated that indigenous microbes in a shaker flask supplemented with yeast extract aerobically reduced 100 mg l^{-1} of Cr(VI) to Cr(III) after 15 days at room temperature [7]. It was reported from the study on Bioremediation of chromium (VI) contaminated soil by *Streptomyces sp. MC1*, that there were a reduction of the Cr(VI) concentration in soil sample inoculated *with Streptomyces sp. MC1* after 3 weeks of incubation additionally, the authors' analyses revealed that Cr was not present in its hexavalent form, indicating that it was metabolically reduced by *Streptomyces sp. MC1*[16]. Therefore it can be inferred that the reduction of Cr(IV) in our soil sample is due to metabolic conversion into Cr(III) and hence the strain isolated Bacillus subtilis shows the capability of removing Cr(VI) from soil.

5. CONCLUSION

The bioremediation of soil sample by Bacillus subtilis was performed at optimal conditions. The results from this study revealed that there were a tremendous reduction of hexavalent chromium after 15 days. This confirms the ability of isolated strain for removal of Cr(VI) from soil and therefore it can be a potential candidate in insitu chromium bioremediation.

Although this research showed positive results about Cr(VI) bioremediation based on biological reuction under laboratory conditions. Challenges still remain in the form of elevated concentration of Cr(VI) in ground water and in deeper soil profile which needs further research in the field of bioremediation.

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