Journal of Microbial & Biochemical Technology - Open Access

www.omicsonline.org

Research Article
OPEN ACCESS Freely available online
doi:10.4172/1948-5948.1000031

JMBT/Vol.2 Issue 4

Degradation of Chromium by Using a Novel Strains of *Pseudomonas* Species

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Abstract

Microorganisms are known for their ability to metabolize a variety of chemical compound, including aliphatic and aromatic hydrocarbon, fatty acids and other environmental pollutants. These capabilities make them useful for application as bioremediation agents. The main objective of the study is to recover chromium degrading bacterial isolates from Rhizosphere soil of Amrithi Forest region, Vellore, Tamilnadu, by using enrichment method. Among the isolates recovered, two (SP2&SP8) of the isolates were selected based on their chromium degradation property. The Potential strains were identified through morphological characterization, biochemical characterization and 16S rRNA gene analysis. The sequence of SP8 and SP2 shows a sequence homology of 98.4% with *Pseudomonas putida* and 98.3% with *Pseudomonas plecoglossicida* respectively. Further these two isolates were checked for its efficiency in chromium degradation, in which SP8 show 90% of chromium degradation. Amirthi forest region was enriched with lots of novel strains in which *Pseudomonas putida* and *Pseudomonas plecoglossicida* has high potential degrading chromium. The significance of the study was to isolate the novel chromium degrading microorganisms.

Keywords: *Pseudomonas putida*; *Pseudomonas plecoglossicida*; Phylogenetic analysis; Chromium degradation

Introduction

Pseudomonas sp. is one of the most commonly encountered gram negative organisms. *Pseudomonas sp.* can be found in soil, water, plants, as well as in domestic environments such as, hot tubs, whirlpools, and contact lens solutions. *Pseudomonas* species have the ability to breakdown a variety of chemical compounds, including aliphatic and aromatic hydrocarbons, fatty acids, insecticides (Francis et al., 1976) and other environmental pollutants. These make them useful for applications as bioremediation agents. Some species suitable for this purpose such as *Pseudomonas alcaligenes,* which can degrade polycyclic aromatic hydrocarbons; *Pseudomonas mendocina*, which is able to degrade toluene; *Pseudomonas pseudoalcaligenes* is able to use cyanide as a nitrogen source; *Pseudomonas resinovorans* can degrade carbazole; and *Pseudomonas veronii* has been shown to degrade a variety of simple aromatic compounds. *Pseudomonas putida* has been shown to degrade many heavy metals like chromium, Zinc, copper etc. Chromium-resistant microorganisms, such as *B.cereus, B. subtilis, Ps.Aeruginosa, Ps.ambigua, Ps.fluorescens, Pseudomonasputida, E.coli, Achromobacter eurydice, Micrococcus roseus, Enterobacter cloacae, Desulfovibrio desulfuricans and D.vulgaris.* Among the bacterial genera with more expressed biodegradative capacity is genus Pseudomonas (Elkarmi et al., 2008).

Chromium is an essential micronutrient required for the growth of many organisms. However, at high concentration it is toxic, carcinogenic and teratogenic. Chromium has been designated as the priority pollutant by US EPA. It is released into the environment by a large number of industrial operations such as electroplating, chromate manufacturing, dyes and pigment manufacturing, wood preservation, leather tanning industry, manufacture of alloys and as corrosion inhibitor in conventional and nuclear power plants (Patterson, 1985).The effluents from these industries contain Cr (III) and Cr (VI) at concentrations ranging from tenths to hundreds of mg/l. Though chromium exists in nine valence states ranging from 2 to +6, Cr (III) and Cr (VI) are of major environmental significance because of their stability in the natural environment (Schroeder et al., 1975). The chromate anion is highly soluble and therefore can overcome the cellular permeability barrier, entering via sulphate transport pathways since it bears structural similarity with SO_4. Unless it is rapidly reduced it can oxidatively damage the DNA via the production of free radicals. It has been reported that hexavalent chromium causes lung cancer (De Flora, 2000), chromate ulcer, perforation of nasal septum and kidney damage in humans and it is also toxic to other organisms as well. It has a high tendency to bind with oxygen. Chromium in its trivalent form is an essential micronutrient for many microorganisms, relatively insoluble in water and 100 times less toxic than the hexavalent form.

Due to leakage, poor storage and improper disposal,hexavalent chromium has become one of the most frequently detected contaminant at the waste sites.Chromium remediation is an environmental challenge. Conventionally, hexavalent chromium containing industrial effluent is treated by physico-chemical methods such as reduction, precipitation, ion exchange, reverse osmosis and electro dialysis. However, it has been observed that these processes are costly and unreliable. The maximum achievable chromate removal efficiency by conventional methods is not sufficient to attain the desired treated effluent quality standard for disposal by the industries. Recently, bioremediation is emerging as a safe and cost effective technology, an alternative to the traditional physico-chemical methods. Microorganisms can play an important role in the detoxification and removal of hexavalent chromium from the polluted sites.It has been reported that hexavalent chromium is reduced to trivalent chromium by a number of bacterial species like *Pseudomonas fluorescens LB300, Bacillus sp., Enterobacter cloacae HO1 and Enterobacter aerogenes.*

Bacteria,which can use chromium (VI) as terminal electron acceptor during oxidation of organic compounds, have attracted

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Received April 06, 2010; **Accepted** July 21, 2010; **Published** July 21, 2010

Citation: Poornima K, Karthik L, Swadhini SP, Mythili S, Sathiavelu A (2010) Degradation of Chromium by Using a Novel Strains of *Pseudomonas* Species. J Microbial Biochem Technol 2: 095-099. doi:10.4172/1948-5948.1000031

attention recently. It has been known from literature that different taxonomic group bacteria cultures can reduce chromium (VI) with Cr $(OH)_3$ forming. Also biotechnologies of galvanic wastewater treatment have been constructed and practiced.It has been shown that biotechnology process allows chromium (VI) concentration to decrease in wastewater to nominal limit 0.3 mg/l. This biological process is also more economical and ecologically effective than widely known physico-chemical treatment (Dmytrenko et al., 2007).

Chromate compounds have many industrial applications and often cause environmental pollution in marine and freshwater sediments from urban and industrial discharges. Hexavalent chromium (chromate) compounds are water soluble, toxic, and probably carcinogenic. Trivalent chromium, Cr (III), is less soluble and less toxic. Thus, reduction of Cr (VI) to Cr (III) represents a potentially useful detoxification process. Bacterial reduction of chromate has been widely reported but the enzymatic basis for chromate reduction has not been clarified. There is evidence for both aerobic and anaerobic reduction systems with different microbes. Anaerobic chromate reduction occurs with a membrane preparation. Aerobic chromate reductase activities (probably involving soluble proteins) have been found in other bacteria (Ishibashi et al., 1990).

Materials and Method

Isolation of microorganisms

The soil sample from Amrithi forest region, Vellore, Tamilnadu, was collected and 2.5g of soil sample was added to 25ml of sterilized Modified liquid Aleksandrov medium (Fang Hu et al., 2006) and it was incubated in rotary shaker for 48hrs at 200 rpm at 30°C.From the enriched sample, serial dilution was carried out and spread plated on Aleksandrov agar plates. It was then incubated at 30°C for 24hrs and the colonies were subcultured to get pure bacterial isolates.

Screening of chromium degrading microorganisms

The basal medium (g/l) without agar was used for the preparation of inoculum. The composition in (g/L) is as follows Polypeptone 5, Meat extract 5, NaCl 5, Glucose 5, Sterilized 50 ml of medium in a 250 ml conical flask was inoculated with a loop of isolated bacteria and incubated at 30°C on rotator shaker rotating at 150 rpm for 18 hours.

16s rRNA gene sequencing

Among the bacterial cultures isolated, only two strains were further subjected for 16S rRNA based on certain unique biochemical traits. Bacterial DNA from cells cultured on nutrient medium was extracted according to (Sambrook et al., 1989). The strains were amplified using the following bacteria specific universal primers. 27F (AGAGTTTGATCCTGGCTCAG), 1492R (GGTTACCTTGTTACGACTT) sequences were identified by PCR direct sequencing using the big-dye primer method (Brosius et al., 1978; Shima et al., 1994) using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan).

Effect of Cr (VI) on bacterial growth

For bacterial growth curve, a volume 89ml LB broth is prepared in three Sidearm conical flasks and autoclaved. 10ml of 1000ppm Cr (VI) stock solution was added to each broth and 1ml of fresh bacterial culture of SP2 and SP8 were inoculated aseptically into each broth. The concentration of Cr (VI) in the medium was 100µg/ml. This was incubated in orbital shaker at 150rpm with 37°C and optical density was monitored on colorimeter at 600 nm for every 1hr till 12 hrs.

Minimum inhibitory concentration (MIC)

The Minimum inhibitory concentration (MIC) of Chromium was determined by the broth dilution method (Calomiris et al; 1984). Luria-Bertani broth amended with the concentrations of 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml, 600µg/ml, 700µg/ml, 800 µg/ml of Cr (VI) ($K_2Cr_2O_7$) were prepared in eight 100ml conical flask for both isolates and autoclaved. 1ml of fresh bacterial culture of SP2 and SP8 were inoculated aseptically into each broth and incubated for 24hrs at 37°C.Hexavalent Chromium in the culture supernatant was measured using S-diphenylcarbazide (DPC) method.

Effect of different pH on the chromium uptake

SP8 was used for Cr (VI) reduction study. On the basis of MIC SP8 was selected based on the cell type. 89ml of LB broth was prepared in six 100ml conical flask and pH was adjusted 5.0, 6.0, and 7.0, 8.0 and 9.0 and autoclaved.10ml of 1000ppm Cr (VI) stock solution was added to each medium and 1ml of fresh bacterial culture was inoculated into each broth. The concentration of Cr (VI) in the medium was 100µg/ml. All six flasks were incubated at 37°C on orbital shaker at 150rpm and at regular time interval after 12 and 24h the pH was analyzed.

Determination of Cr (VI) in supernatant solution

For the determination of chromium reduction, 100ml of LB broth was prepared in five 250ml conical flask and it contains chromium concentration of 200, 400, 600 and 800ppm and then it is autoclaved. 1ml of culture was added to each conical flask aseptically. It was then incubated at 37°C on orbital shaker at 150rpm for 48hrs. After incubation the cells harvested by centrifugation and the supernatant was analyzed for chromium reduction in Atomic Absorption Spectrophotometer.

Results

Prominent white color colonies were seen over the Agar plates, which were further purified and single colonies, were isolated (Table 1a). Two of the isolates were selected based on certain morphological characters and their biochemical test were performed (Table 1b).

16S rRNA gene analysis

Based on various traits of the four cultures, two strains were selected for the characterization. The 16S rRNA results suggested us that the culture SP8 and SP2 belongs to genus *Pseudomonas* of Pseudomonadaceae family having the nearest neighbor as *Pseudomonas putida* (Acc.No.1224373) with 98.4% similarity and *Pseudomonas plecoglossicida* (Acc.No.1224379) with 98.3% similarity. The phylogenetic tree (Figure 1) is done by neighbor joining tool, which shows the relation between the isolates and their respective neighbor type strains along with their respective distances.

Characteristics	SP8	SP2
Colony Morphology	Large White Colored, Water droplet, Mucoid colonies.	Round, Mucoid, Watery Colonies with a dot in the centre.
Gram Staining	Gram Negative cocco bacilli	Gram negative cocco bacilli
Capsule Staining	Non Capsulated	Capsulated
Spore Staining	Non Spore forming	Spore forming
Motility	Motile	Motile

Table 1a: Characterization of the isolates.

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Test	Sp8	Sp2
Indole	-	-
Methyl Red	-	+
VP	-	-
H2S on TSI	-	-
Acid Butt in TSI	+	+
Urea	+	+
Citrate	+	+
Gelatin	-	-
Acid production		
Sucrose	+	+
Fructose	+	+
Lactose	+	+
D-raffinose	+	+
Malate	-	-
Mannose	-	-
D-xylose	+	+
L-arabinose	+	+
Oxidase	+	-
Catalase	+	+

Table 1b: Biochemical Tests for sp8 and sp2.

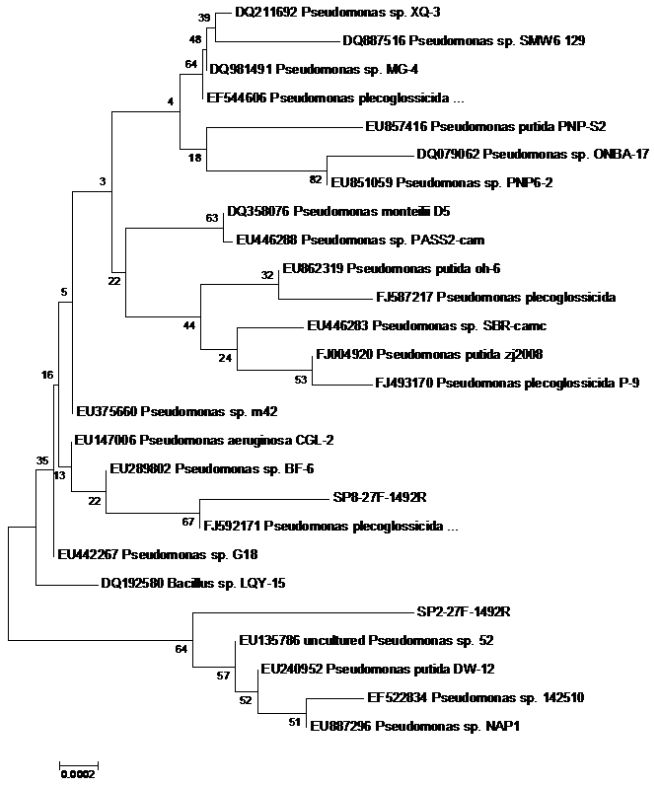

Figure1: Distance tree from Blast search SP 8 and SP 2.

Incubation (hrs)	ODat 600nm[without Cr(VI)] SP2	ODat 600nm [with Cr(VI)] SP2
1	0.06	0.03
2	0.13	0.05
3	0.19	0.08
4	0.30	0.13
5	0.38	0.19
6	0.45	0.24
7	0.49	0.29
8	0.56	0.35
9	0.62	0.40
10	0.67	0.44
11	0.69	0.49
12	0.71	0.56

Table 2: Growth of SP2 without Cr (VI) and With Cr (VI).

Effect of Cr (VI) on bacterial growth

The growths of the isolates were studied in the presence and the absence of Cr (VI). The optical density of SP2 and SP8 were determined at 600nm are listed in the Table 2 and Table 3 The growth of both isolates is much better in Cr (VI) free medium which is clearly shown in the Figure 2 and Figure 3.

Minimum inhibitory concentration (MIC) of chromate

After incubation cell density of the broth cultures was monitored at 600nnm. The growth responses of the strains in potassium dichromate were found to be varying in concentration. At high concentration of potassium dichromate, growth of both the isolates declined, but growth responses of both the strains at lower

Incubation(hrs)	OD at 600nm[without Cr(VI)] SP8	OD at 600nm[with Cr(VI)] SP8
1	0.21	0.04
2	0.27	0.09
3	0.32	0.17
4	0.46	0.31
5	0.53	0.42
6	0.59	0.51
7	0.67	0.57
8	0.72	0.62
9	0.76	0.66
10	0.81	0.69
11	0.86	0.72
12	0.92	0.74

Table 3: Growth of SP8 without Cr (VI) and With Cr (VI).

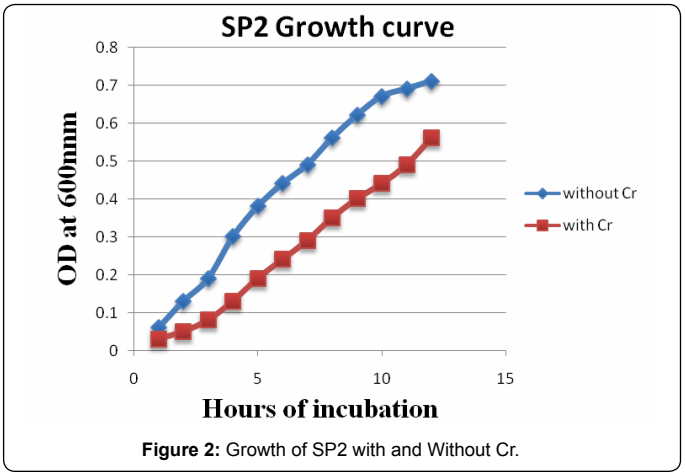

Figure 2: Growth of SP2 with and Without Cr.

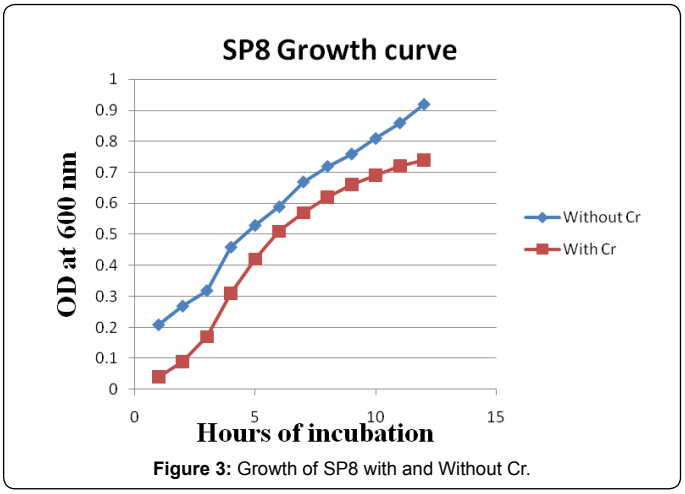

Figure 3: Growth of SP8 with and Without Cr.

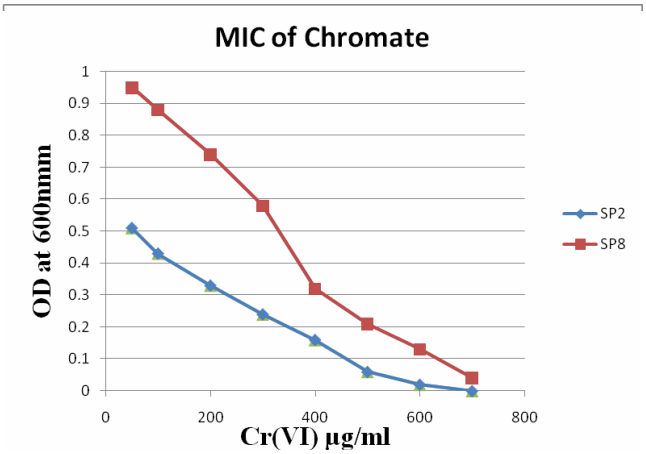

Figure4: Minimum inhibitory concentrations (MIC) of Cr (VI).

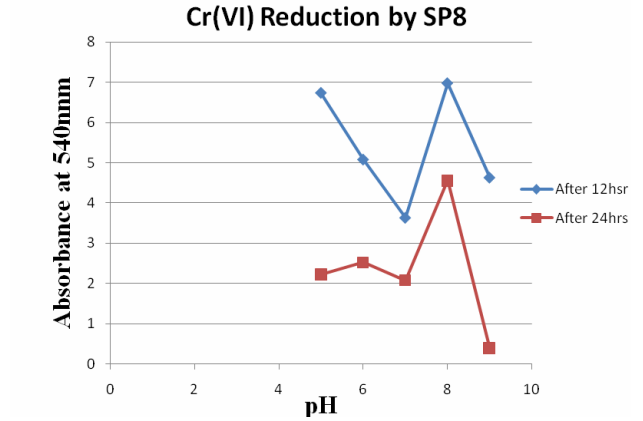

Figure 5: Reduction of Cr (VI) at different pH by SP.

Conc. Of Cr(VI) µg/ml	OD Value at 600nnm	
	SP2	SP8
50	0.51	0.95
100	0.43	0.88
200	0.33	0.74
300	0.24	0.58
400	0.16	0.32
500	0.06	0.21
600	0.02	0.13
700	0	0.04

Table 4: Minimum inhibitory concentrations (MIC) of Chromium (VI).

ISOLATE	pH	INITIAL Cr(VI) CONC. µg/ml	AFTER 12 hrs Cr (VI) µg/ml	%of REMOVAL	AFTER 24hrs Cr(VI) µg/ml	%of REMOVAL
SP8	5	100	6.74	93.26	2.22	97.78
	6	100	5.08	94.92	2.52	97.48
	7	100	3.63	96.37	2.08	97.92
	8	100	6.98	93.02	4.56	95.44
	9	100	4.36	95.37	0.38	99.62

Table 5a: Reduction of Cr (VI) at different pH by SP8.

concentration of chromium salts varied. When compared to both the strains SP8 showed relatively better growth in high salt concentration, at 50µg/ml, Growth of both the strains was much better but chromium concentration increased, the growth decreased. The concentration at which no growth was inhibited was taken as MIC of isolates. The minimum inhibitory concentration of Cr (VI) by broth dilution method are represented in Table 4 and Figure 4.

Isolate	Initial pH	Change of pH after 12hrs	Change of pH after 24hrs
SP8	5.0	6.38	7.02
	6.0	6.57	7.52
	7.0	7.29	7.63
	8.0	7.55	7.91
	9.0	8.81	8.26

Table 5b: pH changes during Cr (VI) reduction by SP8.

Initial concentration (ppm)	Final concentration (ppm)	Reduction (ppm)
200	32.49	165.51
400	62.54	377.46
600	187.8	422.2
800	234.4	565.5

Table 6: Determination of Cr (VI) in supernatant solution.

Chromium (VI) reduction study at different pH

Table 5a shows the result of Cr (VI) reduction experiment using the bacterial isolates in the medium at initial chromium concentration of 100µg/ml potassium dichromate and different pH from 5 to 9, after incubation of 12 and 24 hours cultures were harvested and Cr (VI) reduction was studied by calorimetrically (540nm) with S-diphenylcarbazide method. The purpose of this experiment was to establish an optimum pH for maximum chromium reduction values by the isolate. From the two isolates, one isolate (SP8) was used for Cr (VI) reduction experiment based on their MIC. In Sp8, chromium reduction occurred maximum at pH 7 in 12hrs culture but in 24hrs culture reduction high at pH 9. Cr (VI) reduction at different pH is represented in the Figure 5 and Table 5a.The changes in pH during Cr (VI) reduction by SP8 is listed in Table 5b.

Determination of chromium (VI) in supernatant solution

The supernatant obtained from the centrifuged broth is determined for chromium concentration using Atomic Absorption spectrophotometer. The amount of chromium reduction by the culture is listed in Table 6.

Discussion

Isolation of *pseudomonas* bacteria and their characterization is the foremost step in this track. Rhizosphere soils from Amrithi forest plated on the Aleksandrov medium. Based on the Biological characterization, 2 strains were selected. These strains were further subjected for the phylogenetic analysis by 16S rRNA gene analysis. Thus based on the 16S r RNA analysis the two strains were identified as follows: SP8- *Pseudomonas putida* and SP2- *Pseudomonas plecoglossicida* However, when the sequences were subjected to BLAST search, this SP8 and SP2 shows a sequence homology of 98.4% with *Pseudomonas putida* and 98.3% with *Pseudomonas plecoglossicida.*

The present study focus on the ability on microbes isolated from the rhizosphere soil of Amrithi forest capable of reducing the Cr (VI), batch culture experiments were carried out to study the reduction of Cr (VI) with LB medium. The one potential chromium (VI) resistant isolates namely SP8 were capable to reduce Cr (VI) which may be done by various mechanisms such as adsorption, uptake, methylation, oxidation and reduction and this process affected by various factors such as pH and temperature. The optimum pH for Cr (VI) reduction of the isolates was 7-8 and the pH of the medium was changed during Cr (VI) reduction. The optimum temperature was 30-37°C. One chromium resistant bacteria it could tolerate up to 1000µg/ml of $K_2Cr_2O_7$ on LB broth. The strains grew well both on solid as well as in liquid medium in the presence of Chromate.

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doi:10.4172/1948-5948.1000031
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The degradation of Chromium was estimated by the presence of the SP8 and SP2 in the media. It was observed that Chromium volumetric degradation rates decreased when the microorganism grew in media with two or three carbon components. The decrease was more pronounced in the presence of SP8 than in the presence of SP2. The isolate SP8 could reduce 90% of Cr (VI) in 12hrs. The monoculture of the isolate can be useful for Cr (VI) detoxification in chromium contaminated environments.

The study demonstrated that rhizosphere soil of Amirthi forest region was enriched with lots of novel strains in which *Pseudomonas putida* and *Pseudomonas plecoglossicida* has high potential degrading chromium. The diversity of microorganisms in Amirthi forest was less studied. This is the first report on *Pseudomonas plecoglossicida* which enables chromium degradation. Further research in above organisms is going on.

Acknowledgement

The authors thank the VIT management for providing necessary facilities to complete this work.

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