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Bioremediation of chromium by unique chromium reductase activity in *Proteus* sp. isolated from waste water

Puja Jhunjunwala, Rini Roy, Santanu Roy and Aditi Nag Chaudhuri

Abstract

This is the first time where *Proteus* sp. has been isolated from waste water which has higher potential for bioremediation. Chromium (Cr) compounds are used in dyes and paints and in the tanning of leather. So they are found in soil and ground water at abundant industrial sites, now needing environmental clean-up and remediation. More toxic Cr(VI) is reduced by the chromium reducing bacteria to Cr(III) which is less toxic. Isolation of chromium reducing bacteria from water samples from East Kolkata Wetland leather complex was done to observe the effect of Chromium on them, to study their growth curve characteristics and for remediation assay of heavy metal contaminated industrial wastes by evaluating their Cr(VI) reducing ability through chromate reductase activity. Potassium dichromate ($K_2Cr_2O_7$) and potassium chromate (K_2CrO_4) were employed for the growth of these bacteria. Attempts were made to isolate the genomic DNA of the organism and to amplify its 16S rRNA gene for identification of the organism using Bioinformatics tools. The organism identified was *Proteus* sp. Fourier transform infrared (FT-IR) spectroscopic study was performed to obtain information of the possible cell-metal ion interaction. This study has application in terms of bioremediation of metal contaminated industrial waste water with the bacteria isolated from waste water. This process is environment friendly and cost effective. Moreover the chromate reductase enzyme from isolated bacteria can be purified and immobilized which can be further used to detoxify of Cr from tannery wastes.

Keywords: Chromium reducing bacteria, chromate reductase, bioinformatics, FTIR

1. Introduction

Wetlands are considered the most biologically diverse of all ecosystems, serving as home to a wide range of plant and animal life. East Kolkata Wetlands ($22^{\circ}27' N 88^{\circ}27' E$), are a complex of natural and human-made wetlands lying adjacent to Eastern part of Kolkata and borders on the Salt Lake Township on one hand and the new township of the Rajarhat on the other. The multifunctional wetland ecosystem consists of an area of 12,500 hectares, in the districts of 24 Parganas North & South, West Bengal in India. It includes salt marshes and salt meadows, as well as sewage farms and settling ponds. The wetlands are used to treat Kolkata's sewage, and the nutrients contained in the waste water sustain fish farms and agriculture. It comprises a large number of water bodies (Kundu *et al.*, 2008) [8].

Chromium belongs to the 6th group and 4th period of the block d series in the periodic table. Chromium occurs in oxidation states Cr(0) to Cr(VI), but only Cr(III) and Cr(VI) are biologically significant. Trivalent chromium, Cr(III) is less soluble than the hexavalent chromium and is hundred times less toxic, less soluble and less mobile, mostly found as oxides, hydroxides or sulphates, generally bound to organic matter in soils and is an essential micronutrient for humans (Rahman and Rahman, 2005).

Chromium is an essential trace mineral (~200 mg/day) required by humans for health. It is involved in the metabolism of carbohydrates, fats, and proteins whereas the high valence hexavalent chromium Cr(VI), usually found as oxyanions (Mistry *et al.*, 2010) [11] is toxic, mutagenic, carcinogenic (respiratory tract cancer, listed as class A human carcinogens by the US-EPA), teratogenic and corrosive (causes chronic ulceration and perforation of nasal septum).

Hexavalent chromates are strong oxidizing agents. Chromate oxyanions, analogous in structures with sulfate and phosphate ions, can readily permeate through biological

membranes and their intracellular reduction results in the dire consequences of the chromate induced toxicity. The intracellular reduction of Cr(VI) generates Cr(V), Cr(III) valence states and reactive oxygen species (ROS), the molecular mechanisms of mutagenesis involve the formation of ternary adducts of intracellular Cr(III) with DNA, proteins and oxidative damage of DNA by Cr(V) and ROS (Desai *et al.*, 2008) [1].

Thus, metal also brings about serious environmental pollution, threatening human health and ecosystem. Sources of chromium pollution (Desai *et al.*, 2008; Edward Raja *et al.*, 2009) [1,2] include metallurgy (finishing of metals), dyes and pigment, petroleum refinery, inorganic chemical production, pulp producing industry, chromium metal production and leather tannery. Untreated effluents from these industries have an adverse impact on the environment (Desai *et al.*, 2008) [1].

Cr(VI) (chromate) is a widespread environmental contaminant. Bacterial chromate reductases can convert soluble and toxic chromate to the insoluble and less toxic Cr(III). At the sites of East Kolkata Wetland it is the most common heavy metal contaminant, ranging in concentration between 3.200-0.520 mM. Several bacteria possess chromate reductase activity that can convert chromate to Cr(III), which is much less toxic and less soluble, and thus reduction by these enzymes affords a means of chromate bioremediation and is effective in removing chromate from the environment (Park *et al.*, 2000) [14].

Environmental clean-up strategies for Cr(VI) removal involve physicochemical and biological detoxification. Major limitations of the first procedure are the high energy inputs, different chemical treatments and generation of unnecessary sludge, reactive chemical species as secondary wastes whereas the second means is more ecofriendly and an economically feasible technology. Bioreduction and biosorption of Cr(VI) using bacterial, fungal, yeast or plant biomass are amongst the most lucrative strategies currently employed for removal of chromium by biological means (Johncy *et al.*, 2010) [6].

Bioreduction of Cr(VI) has been demonstrated in several bacterial species including *Pseudomonas* sp., *Escherichia coli*, *Bacillus* sp., *Desulfovibrio* sp., *Microbacterium* sp., *Shewanella* sp., *Achromobacter* sp. and *Arthrobacter* sp. Possible application of a locally isolated environmental isolate, *Acinetobacter haemolyticus* to remediate Cr(VI) contamination in heavy metal contaminated water system was demonstrated appearing to favour the lower concentrations (10–30 mg/L) (Zakariaa *et al.* 2007) [17]. Hexavalent chromate reductase activity was localized and characterized *in vitro* in cytosolic fraction of a newly isolated *Pseudomonas* sp. G1DM21 from Cr(VI) contaminated industrial landfill. The suspended culture of the bacterium reduced 99.7% of 500 μ M Cr(VI) and 93.06% of 1000 μ M Cr(VI) in 48 h. The suspended culture repeatedly reduced 100 μ M Cr(VI) within 6 h up to four consecutive inputs (Desai *et al.*, 2008) [1]. Direct bacterial reduction of Cr(VI) to Cr(III) is the most promising practice with proved expediency in bioremediation. *Lysinibacillus fusiformis* ZC1 isolated from Cr contaminated wastewater of a metal electroplating factory displayed high chromate [Cr(VI)] resistance. It almost completely reduced 1 mM K_2CrO_4 in 12 h. The large numbers of NADH-dependent chromate reductase genes may be responsible for the rapid

chromate reduction in order to detoxify Cr(VI) and survive in the harsh wastewater environment (Hea *et al.*, 2011).

The objective of this particular experiment is to isolate chromium reducing bacteria from the waste water samples from East Kolkata Wetlands (22°27' N, 88°27' E) and this experiment explores the Cr reducing bacteria having higher bioremediation capacity. These Cr(VI) resistant bacteria can be used for environmental clean-up and bioremediation of heavy metal contaminated industrial wastes. These type of bacteria can reduce toxic Cr(VI) to less toxic Cr(III). This may improve the local ecology and minimize the toxic effects of Cr(VI). Purified chromate reductase if properly immobilized can be used repeatedly for bioremediation of Cr(VI) from leather industry waste.

This experiment deals with the study of the growth pattern of the isolated bacteria in the presence as well as in the absence of K_2CrO_4 (1 g/L) and its metal remediation assay by measuring the chromium reductase activity. For instance growth curve was done to see whether the growth of the microbe is slowed down by the effect of such substances. Such knowledge can only be obtained if we study the growth of the microbe from time of inoculation till it reaches the stationary phase. Such a comparative study will throw light upon very important aspects regarding the antimicrobial activity or how the presence of the interacting substances affects the growth of the microbe. The study also aims to observe the effect of chromium compounds on bacteria isolated. FTIR study was performed to obtain information of the possible cell-metal ion interaction. Lastly the genomic DNA of the organism was isolated and its 16S rRNA gene was amplified for identification of the organism using Bioinformatics tools.

2. Material and Methods

2.1 Sample collection

Water samples were collected from different sites of East Kolkata Wetland in a sterile stopper bottle in summer. They were then brought to laboratory for further analysis. They were labeled as leather complex inside (LCI), leather complex outside (LCO) and log gate (LG) at Kultighat.

2.2 Materials required

Different media like Luria Bertani (L.B) Broth and agar, Nutrient Broth (N.B) of analytical grade were used for the growth of the isolated microorganisms. Potassium Chromate (K_2CrO_4) and Potassium Dichromate ($K_2Cr_2O_7$) were used as Cr(VI) salts for observing the effect of chromium on the microorganisms. Concentrations of both the salts were taken from 1 g/l to 10 g/l. The intermediate concentrations are 1.5 g/l, 1.75 g/l, 2 g/l, 2.5 g/l and 5g/l.

2.3 Screening and isolation of microbes in the collected samples

While culturing the three samples were screened separately. 1 ml each of the samples (LCI, LCO and LG) was suspended in three flasks containing 50 ml autoclaved L.B media, respectively. They were then incubated at 37 °C for 48 hours. Loopful of inoculums from the above the three culture flasks were streaked on prepared autoclaved L.B agar plate. The plates were then incubated for growth of organism at 37 °C for 24 hours. 0.1 ml of inoculums from each of the culture flasks was suspended in three flasks containing 50 ml autoclaved L.B media, respectively. They were then incubated at 37 °C for 48 hours. Loopful of

inoculums from the above the three sub-culture flasks were streaked on prepared autoclaved L.B agar plate. The plates were then incubated for growth of organism at 37 °C for 24 hours. Further work was done with the three selected colonies.

LCO (leather complex outside) streaked plate showing marked isolated colonies and were marked as area -1, 2, 3 and used for replica-plating on the L.B agar plate containing $K_2Cr_2O_7$ (10 g/l). The plates were then incubated at 37 °C for 24 hours. Three L.B agar plate containing $K_2Cr_2O_7$ (10 g/l) was streaked from the subculture broth. The plates were then incubated at 37 °C for 24 hours. Marked isolated colonies were grown on different concentrations of $K_2Cr_2O_7$ (Desai *et al.*, 2008; Shakoori *et al.*, 1999) [1, 16] such as 1g/l, 2.5g/l, 5g/l on L.B agar plate and incubated as above. Above steps were performed for all the 3 colonies thus the experiment was performed in the sets of three.

To determine the growth limiting concentration of $K_2Cr_2O_7$, L.B agar plates containing different concentration of $K_2Cr_2O_7$ were prepared (1.5g/l, 1.75g/l, and 2g/l) and streaked with the inoculums from the 3 plates of 1g/l of each colony respectively. The plates were then incubated at 37 °C for 24 hours. Isolation of the selected bacterial culture was done from L.B agar plate of 1.75g/l $K_2Cr_2O_7$ of colony-3 & was used to inoculate L.B Broth -100 ml for further experiments. Inoculums from above broth were streaked on L.B agar plates and pure isolated colonies obtained and preserved.

2.4 Effect of potassium chromate on isolated culture

Two L.B broths were prepared which served as the control-one without K_2CrO_4 and the other with 1 g/l K_2CrO_4 . Two more L.B broths were made where selected inoculum was added to respective flasks - one without K_2CrO_4 and the other with 1 g/l K_2CrO_4 and then incubated at 37 °C for 24 hours.

To see the effect of K_2CrO_4 (1 g/l) on different media the above set up was repeated with four Nutrient broths. The pH of the media was checked.

2.5 Gram character and morphology

Characteristics study of colonies like morphology, Gram character and shape was recorded using Gram staining method under light microscope. Phase contrast microscopy was performed with the isolated colonies.

2.6 Determination of minimum inhibitory concentration (MIC) and growth curve

Media were prepared in duplicate for different concentrations of K_2CrO_4 . One set kept as control of different concentrations to compare the results with and the other set to which 0.1 ml of inoculum was added and incubated at 37 °C for 24 hours following the method of Edward Raja *et al.* (2009) [2]. Readings were taken at 600 nm after certain interval of time. A graph was plotted (O.D. v/s Time).

To compare the growth curve (Zolgharmein *et al.*, 2010) of the organism grown in presence and absence of K_2CrO_4 (1 g/l), two sets were prepared-one containing plain L.B broth and the other containing L.B Broth + K_2CrO_4 (1g/l). The tubes were then maintained at 37 °C for the respective time interval. Readings were taken after each ½ hour but due to time limit only 5 hours could be maintained and O.D. at 600 nm was recorded.

2.7 Chromate reductase assay

Bacterial chromate reductase can convert soluble and toxic chromate to the insoluble and less toxic Cr(III). NADH acts as coenzyme for enzyme activity (Rahman and Rahman, 2005). For the enzyme assay, stock solutions of the following chemicals were prepared- 0.5 mM K_2CrO_4 , 1 M H_2SO_4 , 1% Diphenylcarbazide (DPC dissolved in acetone and distilled water, 1:1 [v/v]), 1 mM NADH and 0.1 M Tris-HCl buffer (pH 8). The standard curve was prepared by varying the concentrations of K_2CrO_4 from 0 to 25 μ M along with 0.1 M H_2SO_4 (0.5 ml), 0.01% DPC (0.5 ml) and volume was made up to 5 ml with distilled water. The resulting pink colour formed by reaction of Cr(VI) with DPC was observed at 540 nm in spectrophotometer. Resting cell extract and spheroplast suspension was prepared using the method described in Desai *et al.* (2008) [1]. NADH reductase assay was performed with both the suspensions following the method of Ishibashi *et al.* (1990) [5]. The Chromate reductase assay involves (Ishibashi *et al.*, 1990) [5] incubation of cell suspensions with 100 mM Tris-HCl buffer, 0.1 mM NADH and 25 μ M K_2CrO_4 for 5 hours and the reaction was then stopped with 0.1 M H_2SO_4 . Then 0.01% DPC was added for colour development along with addition of distilled water and the absorbance was taken at 540 nm. The control was prepared without Cr(VI) and the experimental tube contained Cr(VI).

2.8 Genomic DNA isolation and PCR and Bioinformatics

Isolation of genomic DNA was performed with MB505: HiPur A™ bacterial and yeast genomic DNA Miniprep Purification Spin Kit. The isolated genomic DNA was then stored at -20 °C. PCR amplification of this DNA was performed with common universal primers like 16S-27F and 16S-1492R at 2 mM $MgCl_2$ concentration and 44 °C annealing temperature.

DNA purification was done with Hi PurA™ Agarose Gel DNA Purification Spin Kit. The purified DNA can be used for further downstream applications. To do the sequencing, the entire PCR product was subjected to agarose gel electrophoresis. After the completion of electrophoresis, gel extraction was performed. The DNA bands were excised from the ethidium bromide stained gel with a clean razor blade, using UV light and then placed in a 2.0 ml collection tube. The gel slice was then weighed and accordingly 3 volumes of Gel Bind Buffer (HG) (DS0023) (yellow color signifies the pH to be ≤ 7.5) was added per slice volume. It was then incubated at 50 °C for 5-10 minutes in a water bath. The content is mixed every 2-3 minutes so that the agarose is completely dissolved (100 mg- 300 μ l which means 100 mg of gel slice is dissolved with 3 volumes i.e. 300 μ l of gel bind buffer). 1 gel volume of isopropanol was added to the sample and mixed. The sample was loaded onto the Hi Elute Miniprep Spin Column (DBCA02) and centrifuged at 10,000 rpm for 1 minute. The column was then placed in a new collection tube. 750 μ l of Gel Wash Buffer was added and centrifuged for 1 minute at 10,000 rpm. The flow through was discarded and the column was placed in the same collection tube and spin for an additional 1 minute at 10,000 rpm to remove excess of ethanol. 50 μ l of Elution Buffer (Bangalore Genei) was added directly onto the center of the Hi Elute Miniprep Spin Column (Borosil). It was then incubated at room temperature (15 °C – 25 °C) for 1 minute. It was then centrifuged at 10,000 rpm for 1

minute to elute DNA. The eluted DNA was then sent for sequencing to the company XCELRIS LIMITED. By the use of the universal primers 1.5 kb sequence of amplified 16S rRNA gene fragment was determined. The purified PCR product was sent for sequencing by automated DNA Analyzer. BLASTn program at NCBI server was used to identify and download the nearest neighbor sequences from the NCBI database. All the sequences were aligned using ClustalW2 program at <http://www.ebi.ac.uk/Tools/msa/clustalw2/> (Larkin *et al.*, 2007; Nicholas *et al.*, 1997)^[9, 13]. The phylogenetic tree was constructed using aligned (Goujon *et al.*, 2010)^[3] sequences by the neighbor joining algorithm. Biochemical characteristics of the bacterial strain were studied.

2.9 Fourier transform infrared (FT-IR) spectroscopy

Three L.B agar plates prepared - one without salt and the other two with 1.5g/l K₂CrO₄ and 1.75g/l K₂CrO₄ respectively. The plates were streaked with the culture organism and incubated at 37 °C for 24 hours. The bacterial growth culture obtained was suspended in 30 µL double distilled water and was used for FTIR (Kamnev, 2008; Mangaiyarkarasi *et al.*, 2011; Naumann, 2008)^[7, 10, 12].

3. Results and Discussion

3.1 Screening and isolation of microbes in the collected samples

Heavy growth was observed in the culture flasks as the sample was heavily contaminated. Due to heavy growth on all plates no proper isolated colonies observed. Growth and turbidity observed in all three flasks due to subculture. The streaked plates from the sub-culture excluded LCI and LG due to lawn type of heavy growth. Three isolated colonies were marked from LCO as colony 1, 2 & 3 which were used for further experiments.

The nature of growth of the marked isolated colonies on different concentrations of K₂Cr₂O₇ is given in Table 1. From the table it is evident that the growth range of the isolated organism lies between 1-2.5 g/l. Isolation of the selected bacterial culture was done from L.B agar plate of 1.75g/l K₂Cr₂O₇ of colony-3 (Fig. 1) since colony-1 and 2 showed limited growths.



Fig 1: Isolation of the selected bacterial culture from L.B agar plate of 1.75 g/l K₂Cr₂O₇ of colony-3

Table 1: Growth of the marked isolated colonies on 1 g/l, 1.5 g/l, 1.75 g/l, 2 g/l, 2.5 g/l, 5 g/l K₂Cr₂O₇

Concentration/ Colony	1g/l K ₂ Cr 2O ₇	1.5g/l K ₂ Cr 2O ₇	1.75g/l K ₂ Cr ₂ O ₇	2g/l K ₂ Cr 2O ₇	2.5g/l K ₂ Cr ₂ O ₇	5 g/l K ₂ C r ₂ O ₇
Colony 1	+++	-	-	-	+	-
Colony 2	+++	+	-	-	+	-
Colony 3	+++	++	+++	-	++	-

Legend: +++indicates heavy growth, ++ indicates medium growth, + indicates low growth

3.2 Effect of potassium chromate on isolated culture

The organism was able to grow well in K₂CrO₄ medium. The organism grew well even in different media of Nutrient Broth. As the pH of the media rises, thus the organism makes the medium alkaline (pH 9.0 in case of L.B and pH 8.0 in case of N.B without K₂CrO₄) compared to control (pH 7.5 in L.B and pH 7.0 in N.B without K₂CrO₄). This effect was combated in the presence of potassium chromate due to the presence of chromic acid. Thus the pH of the L.B medium changed to 7.5 from pH 8.0 (control) in presence of 1g/l K₂CrO₄ and that of N.B changed to pH 7.0 from pH 7.5 (control) in presence of 1g/l K₂CrO₄.

3.3 Gram character and morphology

The morphology of the isolated colony 1 was white, flat, rough and non-glossy whereas colony 2 and 3 were off-white, raised, glossy and smooth. The gram staining is shown in Fig. 2A and 2B. The organism obtained is gram-negative in character and rod shaped. Presence of potassium chromate affects the cell division property of the cell thus resulting in the formation of ribbon like structures. Under phase contrast microscope, the organism was found to be motile, as they were living cells.

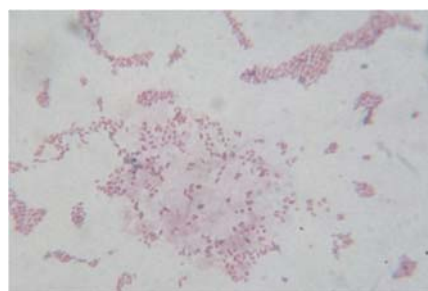


Fig 2A: Gram staining of the organism grown in plain L.B under 400X magnification



Fig 2B: Gram staining of the organism grown in L.B with K₂Cr₂O₇ showing ribbon like structure due to the effect of chromium metal ions under 400X magnification

3.4 Determination of minimum inhibitory concentration (MIC) and growth curve

From the graph in Fig. 3 it was noted that the microbe remained unaffected till 1.5 g/l concentration of potassium chromate after which the microbe was affected by higher concentrations. The growth of the microbe was slowed down and the slope was of gentle type as is evident in Fig. 4. The isolated microbe can tolerate up to 10 g/l Cr(VI) but we have taken the concentration of Cr(VI) in the range between 1 g/l to 2.5 g/l in subsequent experiment because growth curve was not affected in this range.

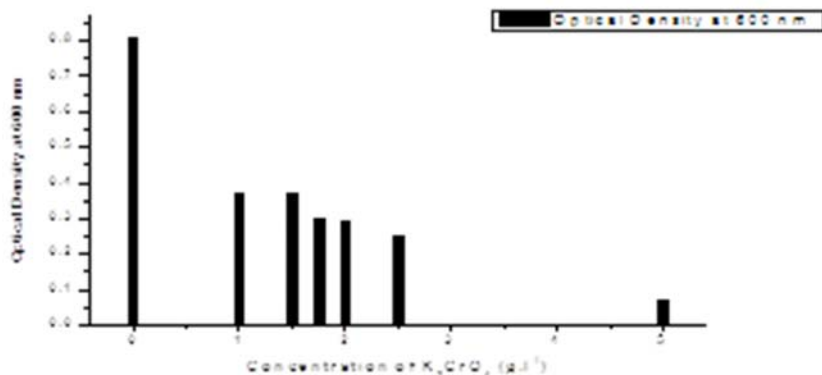


Fig 3: Determination of minimum inhibitory concentration

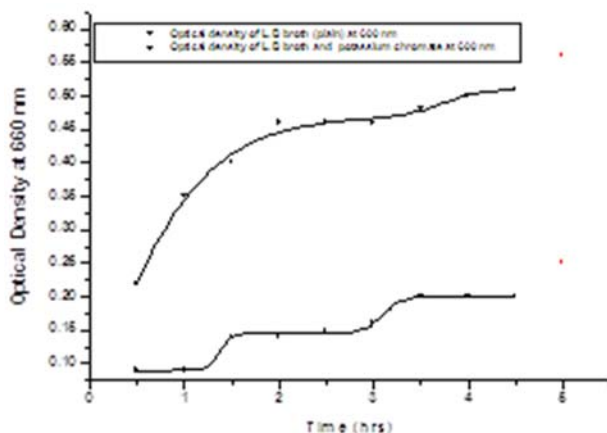


Fig 4: Growth curve of the isolated microorganism

3.5 Chromate reductase assay

The resting cell extract and spheroplast suspension showed the tenfold reduction in chromate reductase assay and the activity of the enzyme was found to be 0.075 $\mu M/min$.

NADH reductase assay revealed that the chromate reductase enzyme requires NADH as coenzyme. The result of the chromate reductase assay is given in Table 2.

Table 2: Chromate reductase assay with resting cell and spheroplast suspension extract

Cell suspension		Amount of Chromium, μM	%	Fold reduction
1. Resting cell extract	Initial	25	100	0
	After experiment	2.5	10	10
2. Spheroplast suspension extract	Initial	25	100	0
	After experiment	2.5	10	10

Legend: Control: without Cr (VI), Experiment: with Cr(VI)

3.6 Genomic DNA isolation and PCR and Bioinformatics

The genomic DNA was successfully isolated from the bacterial sample and was visualized on agarose gel as shown in Fig. 5. PCR amplified DNA was visualized in 0.8% agarose gel as shown in Fig. 6.

The sequence of the 16S rRNA gene of the isolated organism was obtained with forward and reverse primers. The nucleotide sequence of 1000 bp DNA was submitted to GenBank (accession number KC480056).

The BLAST results and the phylogenetic tree are given Table 3a and 3b and Fig. 7A and 7B. Thus the organism isolated was found to be *Proteus* sp. After the knowing that the given organism was *Proteus* sp., its properties were compared to the one isolated in the experiment. Following were the points found to be similar: Both are gram-negative, facultative anaerobic, rod shaped bacterium. Swarming motility is observed in both. *Proteus* sp. inhabits the

intestinal tracts of humans and animals. It can be found in soil, water and fecal matter. Due to its ability to make the growing media alkaline, it is used as identification tool in many biochemical reactions. *Proteus* sp. has bioremediation potential.

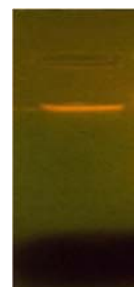


Fig 5: Isolated genomic DNA band in lane 1 of 0.5% agarose gel.

1 2 3 4

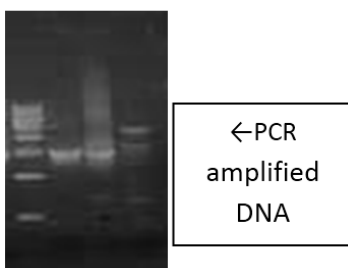


Fig 6: PCR amplified DNA shown in lane 4 compared with DNA ladder in lane 1 in 0.8% agarose gel. Lane 1→DNA ladder (From top in kilobases – 2.5, 2.0, 1.5, 1.0, 0.5, 0.25), Lane 4→ PCR amplified DNA

Table 3: BLAST results with forward primer (FP) and reverse primer (RP) sequences

a) Blast result with FP sequence:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF946783.1	<i>Proteus</i> sp. P242 16S ribosomal RNA gene, partial sequence	1500	1500	99%	0.0	95%
JN222368.1	<i>Proteus</i> sp. pro1 16S ribosomal RNA gene, partial sequence	1498	1498	99%	0.0	95%
JN092590.1	<i>Proteus mirabilis</i> strain FFL2 16S ribosomal RNA gene, partial sequence	1498	1498	99%	0.0	95%
JF946807.1	<i>Proteus</i> sp. A729 16S ribosomal RNA gene, partial sequence	1498	1498	99%	0.0	95%
JF947362.1	<i>Proteus mirabilis</i> strain 2115 16S ribosomal RNA gene, partial sequence	1498	1498	99%	0.0	95%

b) Blast result with RP sequence:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB626123.1	<i>Proteus mirabilis</i> gene for 16S rRNA, partial sequence, strain: JCM 1669	1565	1565	98%	0.0	97%
JN092590.1	<i>Proteus mirabilis</i> strain FFL2 16S ribosomal RNA gene, partial sequence	1565	1565	98%	0.0	97%
HQ407312.1	<i>Proteus mirabilis</i> strain HH137 16S ribosomal RNA gene, partial sequence	1565	1565	98%	0.0	97%
HQ169118.1	<i>Proteus mirabilis</i> strain FUA1240 16S ribosomal RNA gene, partial sequence	1565	1565	98%	0.0	97%
AY820623.1	<i>Proteus mirabilis</i> 16S ribosomal RNA gene, partial sequence	1565	1565	98%	0.0	97%

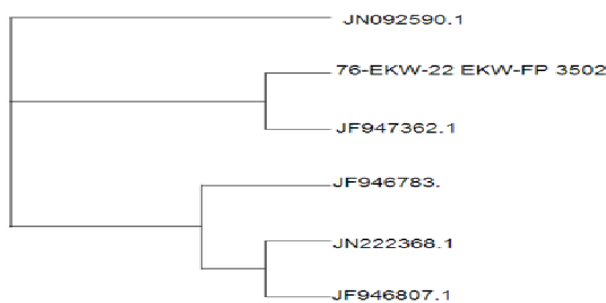


Fig 7A: Forward Primer

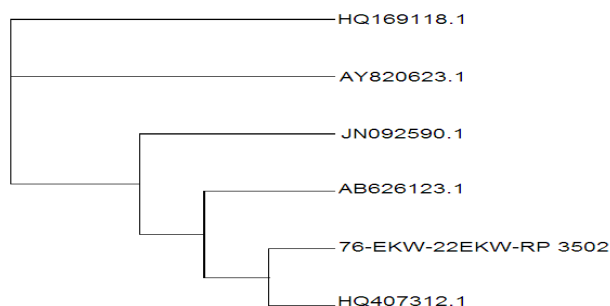


Fig 7B: Reverse primer

Fig 7: Phylogenetic tree with forward primer (A) and reverse primer (B) sequences

3.7 Fourier transform infrared (FT-IR) spectroscopy

FTIR result is given in Fig. 8A and Fig. 8B. Fig. 8B displayed a broad stretching peak around 3400.66 cm⁻¹ characteristic of N-H and O-H stretching from polysaccharides and proteins compared to control in Fig. 8A. Cell wall is the probable site for the direct interaction of OH and Cr. This shows involvement of hydroxyl groups in Cr reduction. Significant shift in frequency from 1643.55 cm⁻¹ to 1650.88 cm⁻¹ and 1644.54 cm⁻¹ in Fig.8B indicate

protein C=O stretching in the Cr binding by the species. The peak at 1077 cm⁻¹ frequency is prominent and significant on exposure to Cr (VI) thus suggesting the involvement of either phosphate moiety or the C=O group in the interaction with Cr. The phosphate linkage appeared with 1.5 g/l of Cr (VI) in Fig. 8B. This is the evidence of the sufficient participation of phosphate groups in the chromium reduction. Stretching of the peak at 1650.88 cm⁻¹ from the control in Fig. 8A on exposure to 1.5 g/l Cr(VI) implied the

involvement of carboxylate groups in chromium adsorption on the biomass. FTIR in presence of 1.75 g/l K_2CrO_4 was similar to that of control in Fig. 8A.

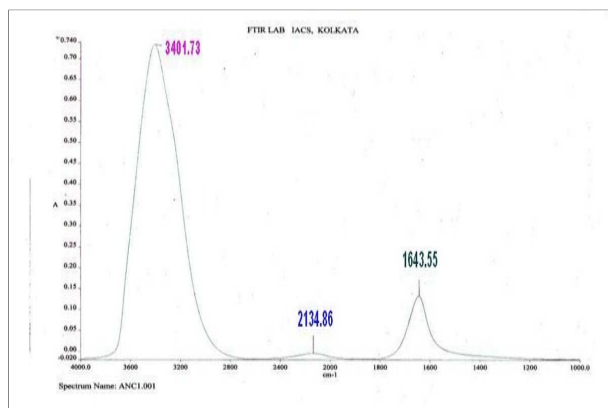


Fig 8A: FTIR of bacterial culture suspension in distilled water- Control without metal ion

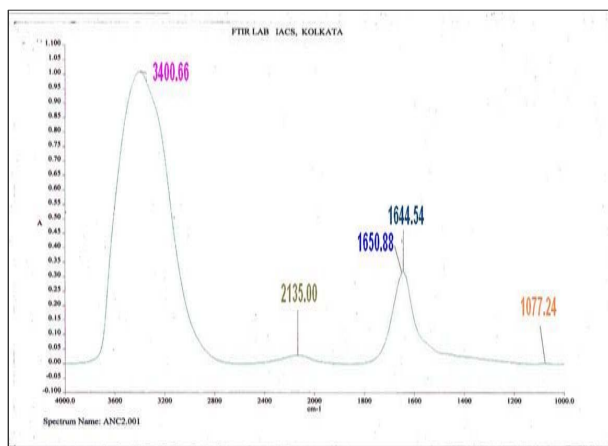


Fig 8B: FTIR of bacterial culture suspension in presence of 1.5 g/l K_2CrO_4

4 Conclusion

The significance of the study is that the isolated organism was able to survive well up to 1.75 g/l concentration of potassium dichromate and 1.5 g/l concentration of potassium chromate. Due to presence of chromate, the cell division property of the cell was affected and thus ribbon like structure observed in the cell. Thus, the growth of the microbe is slowed down in the presence of the metal ions as the microbe tries to cope with the adverse effect of the metal ions on it, but at higher concentrations the cells are not allowed to fight back and thus the growth is inhibited as seen in MIC experiment. Chromium reductase assay showed that the organism is responsible for 10 fold reduction of chromium present. Not much change in NADH reductase activity in cell free extract shows the obvious relation of NADH reductase activity with chromate reductase activity in the organism. FTIR study showed phosphate group and carboxylate group to be interactive with the metal ion. The organism was identified to be *Proteus* sp. from bioinformatics study. The proposed process is environment friendly and cost effective. The chromium reducing bacteria has been successfully isolated from waste water. Leather factory is situated beside the waste water system. The finally released chromium in the waste from the factory is

detoxified by microorganisms by their chromate reductase activity. If the chromate reductase enzyme is purified and immobilized then the immobilized enzyme can be used to purify the waste water.

5. References

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