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ISOLATION OF CHROMIUM RESISTANCE BACTERIA FROM LOAM SOIL SAMPLE OF RIVER GANGA

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ABSTRACT

Chromium resistance bacteria capable of reducing or transforming hexavalent chromium to trivalent chromium were isolated from soil and water. Soluble hexavalent chromium is extremely pernicious and shows mutagenic effects, due to its strong oxidizing nature. Heavy metals possess threat to the ecosystem as well as the plants animals, aquatic life and humans. At present day the bioaccumulation of heavy metals causes frequent environmental hazards. High concentration of these chromium is isolated from industrial and agricultural wastes. Chromium (VI) has physical as well as chemical contrasting characters to that of Chromium (III). The physiological reduction of Cr (VI) is highly expensive and thus the focus shifted for bioremediation of Cr (VI). Microorganisms reduces Cr (VI) to less toxic form. This reduced form Cr (III) has been reported to have dietary nutrient values. This work of review is attributed to find out how the resistance, reduction and remediation process is driven in microorganisms.

KEYWORDS: Chromium resistance bacteria, extremely pernicious and mutagenic effects, Bio–accumulation, bio–remediation, COD, BOD, FT–IR, XRD.

INTRODUCTION

Bioaccumulation of heavy metals contaminate land air and water. Industries such as rubber paints, tannery, cotton textiles are the major industries which pollute water. The major source of Chromium is tannery and leather producing industries. Chromium entering into the water bodies affect the COD as well as the BOD of water. However, to resolve the problem there are some bacteria that reduce Chromium (VI) to less harmful Chromium (III). Soil which is vigorously contaminated with Chromium (VI) reducing bacteria. These toxic metal from industrial waste water and the other human activities are directly or indirectly released into the environment. Unlike organic contaminants, these pollutants from heavy metals are not bio degradable and able to travel up the food chain via bio accumulation. Some metals like Cu, Fe, Mn, Zn are micronutrients for most of the organisms. They play a vital role in metalloenzymes. Cations which play a vital role in membrane stability and demonstrate specific role in nucleic acid structure. When the concentration of beneficial metals (Cr, Hg, Pb, Cd) in the environment are very high they become more toxic.

Figure-1: Ganga soil & Bacterium.

Distribution and Bioremediation of heavy metal chromium: $_{24}Cr^{52}$ Chromium is recalcitrant metal and is a first element in the group VI of the periodic table with electronic configuration of $[Ar]3d^5 4s^1 3d^5 4s^1$ (at ground

state). It's a hard and fragile metal with high melting point and many of its compounds are vigorously coloured and highly resistant to oxidation. Chromium is the potential soil, surface water, ground water, sediment and air contaminants, soil chromium levels are directly linked to the chromium level in the parent material.

Disease associated with Chromium: Chromium compounds initiates respiratory tract infections, and also cause pulmonary disorders. Repeated inhalation of Cr (VI) compounds increases the chance of lung, nasal as well as sinus cancer. Sometimes many people experience painless skin ulcers. It can harm immune system of human, kidney and liver damage and may cause death. Hexavalent chromium exposure in the work place can cause lung cancer in workers who breathe airborne Cr (VI).

Disease associated with other heavy metal and their sources: Huge amount of arsenic, cadmium, lead, copper, nickel originated by several industries. Arsenic evolved from phosphate and fertilizer manufacturing plant, paints and textile industries. Cadmium from electronics industries, Copper from rayon and electrical industries. Nickel from electroplating industries, iron steel industries.

In general, the harmful effects of metal ions in higher organism are due to chemical reactivity of heavy metal ions with cellular materials. There are some specific target organs which shows metal toxicities due to accumulation of heavy metals in that target organs. The effect of toxicity is also depending upon root of exposure of heavy metal.

Although we are talking about general toxicity but we have to look overpotential carcinogenicity of heavy metals. Target organs for arsenic toxicity is pulmonary nervous system, skin, it may cause respiratory cancer. Chromium is truly responsible for respiratory septum disorder, ulcer. Cadmium vigorously affects renal, skeletal, pulmonary organs.

Microbial transformation and Environmental account of heavy metal chromium: The microbial interconversion of heavy metals is of prime interest, since most of the heavy metals have entered the environment due to industrial processes. The discovery of micro–organisms that preferentially reduced hexavalent chromium has led to applications in the bio remediation of chromium contaminated ecosystem.

Environmental account:

a) Lot of Chromium eventually deposited and end up in waters and soil.
b) After that soil particles anchored with the chromium and as a result chromium can't able to move towards groundwater.
c) In water this chromium anchored on sediment under the base of water body or the basement region, these are static, immovable. Very little part of it eventually dissolves.
d) Since, the oxidation state of metal ion may determine its solubility, many scientists have been trying to use microbes that are able to oxidize or reduce heavy metal in order to remediate metal contaminated sites. Another implication of heavy metal tolerance in the environment is that it may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment. Calomiris et.al studied bacteria isolated from drinking water and observed that a high concentration of bacteria that are tolerant to metals are also antibiotic resistance.[1]

Thus, bioremediation is a noble way to remove such type of harmful contaminants of environment, because bacterial chromate reductase, a potential enzyme for bioremediation can easily transform soluble toxic form of chromium into less toxic insoluble form.

Literature Summary: In biodegradation of Chromium soils contain organic and other reducing agents which help to reduce Cr^{6+} to Cr^{3+}.[2– 3] As we know Cr^{6+} is more toxic than Cr^{3+} by 100 times, however it is proven that water and soil which is contaminated with Cr shows a diverse range of Cr resistant bacteria promoted by natural selection. Heavy metals which contain industrial effluents pose threat to human life, animal life and also to the natural fauna (Robin et al 2012). These effluents have a wide impact on the biological oxygen demand and also the chemical oxygen demand. The wastes generated from the tannery industries contain heavy metals like Manganese, Chromium, Cadmium. Chrome powder and chrome liquor is used in the tanning industries that cause water pollution, and as it enters the cell membranes of the living organisms it causes hazards to the living organisms.[4] Bioreduction procedure of Cr can occur aerobically or anaerobically. This procedure of bioreduction of chromate involves microbial metabolism or through bacterial metabolism such as H_2S.[5]

However chemically it is proven Cr^{6+} is a strong oxidizing agent and can also be reduced by electron donors. Iron can be used as an electron donor in this biochemical reduction. Low oxygen concentration is also responsible for the reduction of Cr^{6+} to Cr^{3+} by H_2S released, diffuses out in the media and reduces Cr^{6+} to Cr^{3+}. Trivalent Cr formed reacts with hydroxide and forms $Cr(OH)_3$. This is an *in– situ* method done for contaminate soil.

In removing Cr from ground water– Chromite ore processing involves Cr^{6+} reducing microbes and the use of organically rich acidic manures produced layer. This layer is placed just below the rich Cr layer and the leaching of Cr^{6+} is reduced. Thus, ground water is protected this way.

Engineered strains have been developed by cloning two bacterial encoding the genes (Class 1 and Class 2). These classes of genes Cr^{6+} to Cr^{3+}. This was done by Gonzale. Pseudomonas strains have been reported to have been able to reduce Chromate dichromate. It is also shown scientifically that pure culture of bacteria which were previously not exposed to Cr^{6+} was capable of reducing it. This might have taken place because these bacteria may have degraded similar compound in the environment and hence possess the enzymes for degradation.

Natural attenuation is a process in which Cr^{6+} is reduced to Cr^{3+} majorly in soil and water without any human intervention. Chromium resistant bacteria helps in this process. Cr can be reduced through direct or indirect method.

In the direct method Cr resistant bacteria take up the waste containing Cr and produce enzymes within themselves. These enzymes help in degrading the microbes. In the indirect method oxidation or reduction of microbes is carried on which is mediated by H_2S.

As we know Cr^{6+} is a strong oxidizing agent and can be reduced by electron donors. In anaerobic environment Cr^{6+} depended on dihydrogen (H_2) for growth and chromate reduction. When Cr^{6+} is not present dihydrogen accumulated. When appropriate electron donors are added along with H_2, it speeds up the process of the reduction of Cr. Reduction of Cr^{6+} also depends on the pH. Nonmetabolising bacteria reduces Cr^{6+} in nutrient deprived environment–which is important in biogeochemical distribution of Cr. A biological way to reduce Cr (VI) to Cr(III) is by making a more reducing or low pH environment. Cr(VI) has an ability to switch on p53 by reactive oxygen species (ROS) conciliate free radical reactions that happen during oxidative reduction of hexavalent chromium within the cell. Oxidative damage is considered to be an principal mechanism in the genotoxicity of Cr(VI). Thus, the requirement emerge to remediate chromium before being discharged.[6]

In marine environments sulphate reducing bacteria produce sulphide which too helps in the reduction of Cr^{6+}. Cr^{6+} is a mutagen and carcinogen of all living organisms. Another interesting fact is Cr^{6+} can be dissolved by sweat produced on the surface of the skin. Such people are sensitized to allergic dermatitis.

Cr^{6+} present in soil causes reduction in the growth of the plants due to damage in the roots. It also delays sprouting. Under Aerobic conditions Cr^{6+} reduction is associate with soluble proteins that use Nicotinamide Adenine Dinucleotide (NAD) as an electron donor or as a important component for growth (Ishibashi et. At 1990) or for intensify activity (Horitsu et.al 1987). as shown in the following reaction:

$2\ CrO_4^{2-} + 13\ H^+ + 3\ NADH \rightarrow 2\ Cr^{3+} + 3\ NAD^+ + 8\ H_2O$.[7]

The United States environmental protection agency (US EPA) has identified $CrVI^+$ one of the 17 chemicals initiates a greatest threat to human. An enormous variety of bacteria have been found that can remediate chromium like other heavy metals under aerobic and anaerobic condition e.g. *Intrasporangium* sp., *Bacillus* sp., *Escherichia coli, Bacillus* sp., *Enterobacter cloacae* etc.[8]

In both aerobic and anaerobic condition *Enterobacter cloacae* has the capability to grow in chromium rich environment.[9]

Where normal bacteria cannot grow in presence of 10mM potassium chromate concentration, *Enterobacter cloacae* successfully grown in a nutrient broth in presence of 10 mM potassium chromate concentration. This chromate resistance capacity may or may not be plasmid determined.[10]

Existing methods of waste treatment to reduce chromium count: There are several techniques of removal of chromium. By using low– cost adsorbents such as cornelian cherry, apricot stone and almond shell batch removal of chromium form aqueous solution takes place under various experimental condition was carried out by Damirdas et.al in the research on kinetics of adsorption for the removal of chromium.[11]

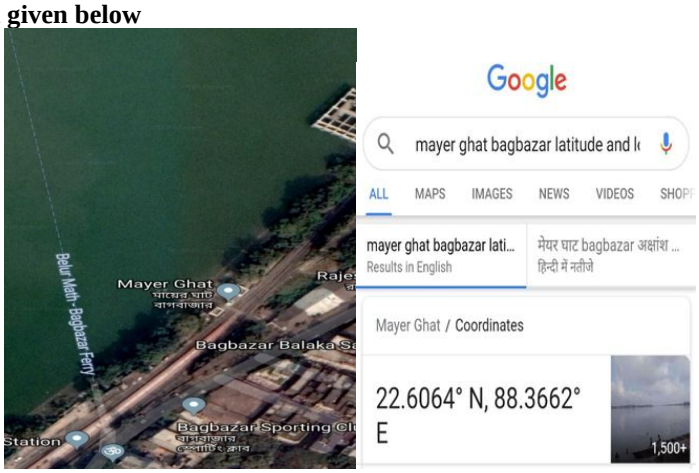
Khudbudin Mulani et al. 2013 studied that aromatic compound present in coffee beans in very large amount are phenolics, presumably obtained from chlorogenic acid and melanoidins. Coffee melanoidins are derived on roasting coffee beans and they have the potentiality to chelate metal ions. In the present study, adsorbent resins were prepared from condensation of coffee polyphenol and formaldehyde for Cr(VI) removal. Application of vesicular basalt volcanic rock for adsorption of chromium from aqueous solution was scrutinized. Different physical properties of powdered rock were explored using Fourier transform infrared spectroscopy (FT– IR), powered X– RAY diffraction (XRD) and by using SEM. A series of batch experiments were performed for analysis of various experimental parameters (pH, Ionic strength, & contact time) on chromium adsorption.[12]

A process namely, IERECHROM (Ion Exchange REcoveryCHROMium) has been established for removal of Cr (III) ionic complex from wastewater.[13]

MATERIALS AND METHODS

Collection on soil sample from riverbed of ganga: soil sample collected from bagbazarghat located at north Kolkata.

Place of sample collection given below



Figure–2: Location of soil samples.

Preparation of liquid culture: After collection of soil sample, 1 gram of sample was taken and added the sample into 20 ml liquid media of Luria broth [REF–M575– 500G]. Where 20.0 grams Luria broth dissolved in 1000ml distilled water. Then heated could be given if necessary to dissolve the medium completely. Then sterilized by autoclaving at 15 lbs. Then incubated the conical overnight with shaking, and growth observed. Then again inoculated this culture into fresh Luria broth with 15 mM potassium chromate. It is a nutritionally rich medium primarily used for the growth of bacteria. Its creator, Giuseppe Bertani, intended LB to stand for lysogeny broth, but LB has also come to colloquially mean Luria broth, Lennox broth, life broth or Luria–Bertani medium.

Isolation of single colony by spread plate method: Now the preparation of single colony in an agar plate. Agar is made up of Luria broth and 2.5% bacteriological agar in 4 different conical, where 10–, 15–, 20– and 25–mM potassium chromate was added respectively and after pouring agar into autoclaved glass plate, 20 µl of culture is added into it, then spread the plate with glass spreader. The whole procedure should be within LAF [laminar air flow, with HEPA (high efficiency particulate air) filter]. After incubation in the bacteriological incubator, we observe eleven different isolated single colony from the plate where 15 mM potassium chromate was given.

Biochemical analysis of samples

Voges Proskauer test

Materials required:

a) tryptone – 0.35%
b) peptone – 0.35%
c) Dipotassium hydrogen phosphate – 0.5%
d) Dextrose – 0.5%
e) Baritt reagent A (alpha naphthol 5 gm, & absolute ethanol 95 ml)
f) Baritt reagent B (KOH=40 gm, creatine=0.3 gm, $D.H_2O$=1000ml)
* pH=6.9

Media should be autoclaved properly and make 11 test tubes for inoculation of respective samples, and one test tube for control (standard)

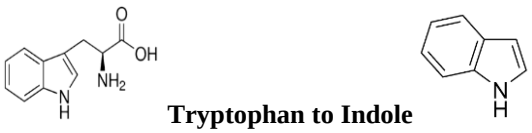
Aim and principle: This test depends on the digestion of glucose to acetylmethylcarbinol. If glucose is being broken down it is reacting with alpha naphthol (VP reagent 1) and potassium hydroxide (VP reagent 2) to form a red colour. alpha–naphthol and KOH are chemicals that detect action. This test is used to differentiate enteric bacteria based on their property of fermentation when glucose is supplied to the media.

Indole test

Materials required:

a) Tryptone – 1%
b) NaCl – 0.5%
* pH = 7.5

Aim and principle: The indole test is a biochemical test performed for bacterial species to determine the ability of the organism to convert tryptophan into the indole. This decision is performed by a chain of a number of different intracellular enzyme, a system generally referred to as tryptophanase.



Tryptophan to Indole

Stuart's test (Urea broth test)

Media composition:

a) Yeast extract – 0.01%
b) Potassium phosphate monobasic – 0.9%
c) Potassium phosphate dibasic anhydrous – 0.95%
d) Urea – 2%

e) Phenol red – 0.001%
f) Agar – 1.5%
* pH= 6.8

Aim and principle: Urease broth is a differentiated medium that test ability of an organism to produce and exoenzyme called urease that hydrolyses urea to ammonia and carbon dioxide. If the urea in the broth is degraded and ammonia is produced and alkaline environment is created and the media turns pink.

Citrate Agar test
Media composition:
a) Magnesium Sulphate – 0.2 gm
b) Ammonium phosphate monobasic – 1 gm
c) Dipotassium phosphate – 1gm
d) Sodium citrate – 2gm
e) Sodium chloride – 5gm
f) Bromothymol blue – 0.08 gm
g) Agar – 15 gm
* pH=6.8
(Media composition in 1000 ml D.H_2O)

Aim and principle: Simmons citrate agar is a defined enrichment that tests for an organisms ability to use citrate a sole carbon source and ammonium ions as sole nitrogen source. Use of citrate result in creation of the carbonates and bicarbonates.

Bi – products thus increasing pH of the medium. Increasing pH then causes colour change. Bromothymol blue in the indicator.

Motility test
Media components:
a) Tryptone – 1%
b) NaCl – 0.5%
c) Agar – 0.5%
d) Yeast extract – 1%
pH=7

Aim & principle: Motility test medium is use to test the motility of organism. Although there is function test medium, motility test is multi-interest media used in the different Enterobacteriaceae.

Starch hydrolysis test
Media composition:
a) Nutrient agar/LB agar
b) Starch – 0.3%

Aim and principle: Starch agar is a differential medium that test for the ability of an organism to produce the extra– cellular enzymes alpha amylase and oligo– 1,6–glucosidase that are secreted out of the bacteria differentiated into starch by breaking glucosidic linkages between glucose subunit and allow the products of starch hydrolysis to enter the cell. This test is used for differentiating different species like *Streptococcus, Clostridium, Corynebacterium, Fusobacterium, Enterococcus pseudomonas and bacillus.*

Triple Sugar Iron test
Media composition:
a) Triple sugar iron agar
Aim and principle: TSI is a microbiological test roughly used to test micro–organisms immunity to ferment sugars and produce hydrogen sulphide. This test is used to differentiated enteric bacteria like *Salmonella* and *Shigella*.

Carbohydrate test
a) Tryptone – 1%
b) Carbohydrate – 0.05%
c) NaCl – 0.05%
d) Phenol red – 0.001%
pH=6.8

Six different carbohydrate is used–
1. L – arabinose
2. D – arabinose
3. Lactose
4. D – mannitol
5. Sucrose
6. Glucose

Genomic DNA isolation
Introduction: DNA was isolated for the first time more than 100 years ago and today it is isolated as a regular process.

Distinctive challenges can still arise, but depending upon nature or type of organism and tissue challenges are different.

We have isolate DNA in two different procedures
1st method:
1. Preparation of liquid broth and inoculate it with culture obtained by streak plate.
2. Then placed conical in bacteriological incubator. Growth observed after 24 hrs incubation.
3. Taking 1 ml of liquid culture in 11 different Eppendorf and centrifuge with 10000 rpm for 7 min.
4. Then add 500 µl CTAB buffer preheated at 60^0C.
5. Then incubate the Eppendorf at 60^0C for 20 mins.
6. After that add 1 ml PCI [phenol:chloroform:isoamylalcohol=25:24:1] and

invert the Eppendorf gently, incubated at RT (37^0C) for 10 min.
7. Then centrifuge for 10 min at 4^0C.
8. Now we have to collect the supernatant (aqueous phase) without disturbing the middle layer (interphase) and then add equal volume of chilled absolute ethanol.
9. Then incubate it for overnight.
10. After incubation, centrifuge for 8 min at 10000 rpm at 4^0C. then remove supernatant and add 70% ethanol.
11. Again, centrifuge for 8 min at 10000 rpm at 4 ^{0}C.
12. Remove the supernatant and complete evaporation of ethanol should takes place and then add 1X TE buffer.

2nd method:
1. Take liquid culture in Eppendorf and placed it in water bath for 20 min with 80^0C temperature,
2. Then centrifuge the sample with 10000 rpm for 10 min.
3. Now collect the supernatant and add proteinase k (2– 3 µl) and incubate for 5 min.
4. Then add phenol chloroform mixture in a ratio 25:24, with 1 ml in each Eppendorf and incubate for 15 min.
5. Now centrifuge Eppendorf with 10000 rpm at 4^0C for 10 min.
6. Then collect aqueous phase without disturbing interphase.
7. After that equal volume of absolute alcohol chilled (–20^0C)

Agarose gel electrophoresis: It is a method of gel electrophoresis used in biochemistry, biotechnology, molecular biology, medical chemistry to distinguish several macromolecules such as DNA, proteins in agarose matrix. Agarose is a polysaccharide, evolved from certain seaweed, it has repeating unit of agarobiose. It is not only for size separation of macromolecules, fractionization of nucleic acid is also happened for future analysis and it is also used to separate protein and nucleic acid on basis of their shape. Agarose gel electrophoresis is a technique where circular DNA with different supercoiling pattern could be reconcile.

To prepare agarose gel, we have to prepare 50X TAE buffer and then make 500ml 1X TAE by adding 10 ml 50X TAE and 490 ml distilled water.

Prepare 1 % agarose gel (adding 1gram agarose in 100 ml distilled water and microwave three times for 30 sec until the agarose is totally dissolved add EtBr (ethidium bromide) into the gel, then wait for solidification in proper container. In order to visualize DNA, EtBr is added with the 1X TAE buffer. It binds with DNA and irradiate in presence of UV light.

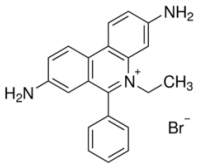


Figure-3: Chemical formula of ethidium bromide.

After the preparation of gel, comb is removed, it is placed into gel electrophoresis chamber and load DNA sample in pre–cast wells in the gel with tracking dye bromophenol blue and xylene cyanol. 1X TAE used as a buffer, and current applied. Due presence of phosphate backbone, DNA is negatively charged, it moves towards positively charged anode with placed into electric field. Bromophenol blue also has some negative charge thus moves towards positive terminal with DNA, it is mainly used to identify the movement of DNA or position of DNA inside the gel. It is also a pH indicator.

If a DNA strand contain approximately 300 base pair, it moves at same rate with bromophenol blue, but xylene cyanol which migrates at about at an equal rate as a DNA strand which has approximately 3500 base pairs.

Smaller fragments move faster inside the gel, when electrical field removed, DNA including tracking dyes stop.

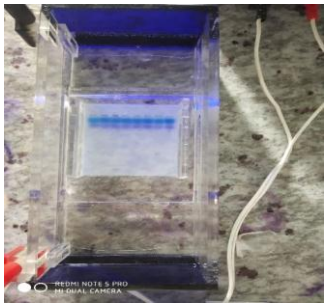


Figure-4: Agarose gel electrophoresis.

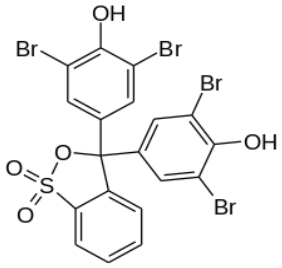


Figure-5: Bromophenol blue.

RESULTS

Isolated single colony by spread plate method

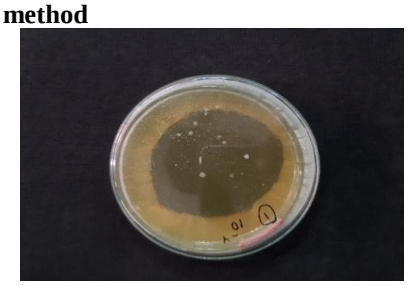


Figure-6: 11 different single colony observed.

Isolated single colony by streak plate method

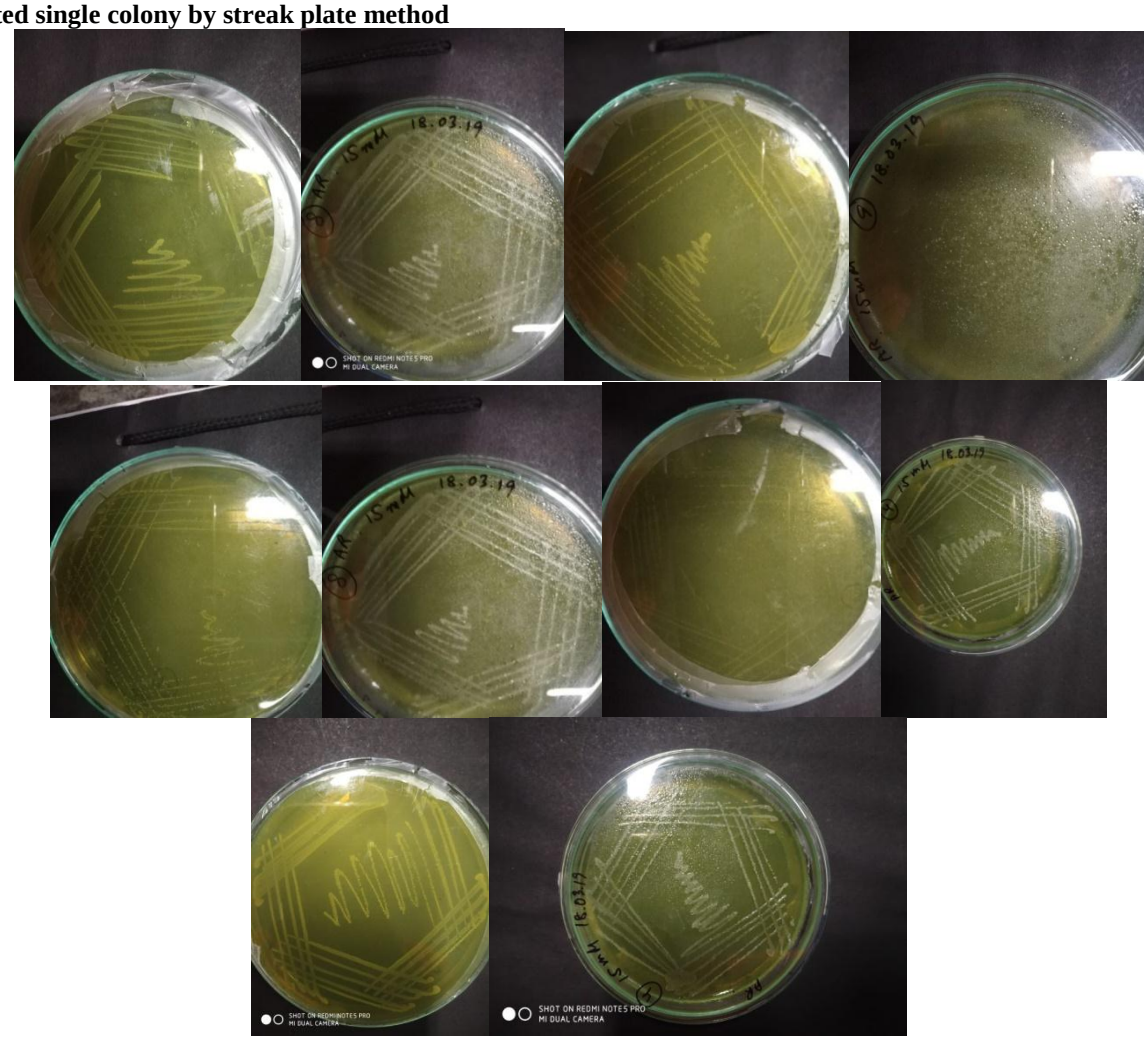


Figure-7: 11 single colony from spread plate transformed into new agar plate containing 15 mM potassium chromate medium, inoculation by streak plate method.

Biochemical tests: Voges Proskauer test

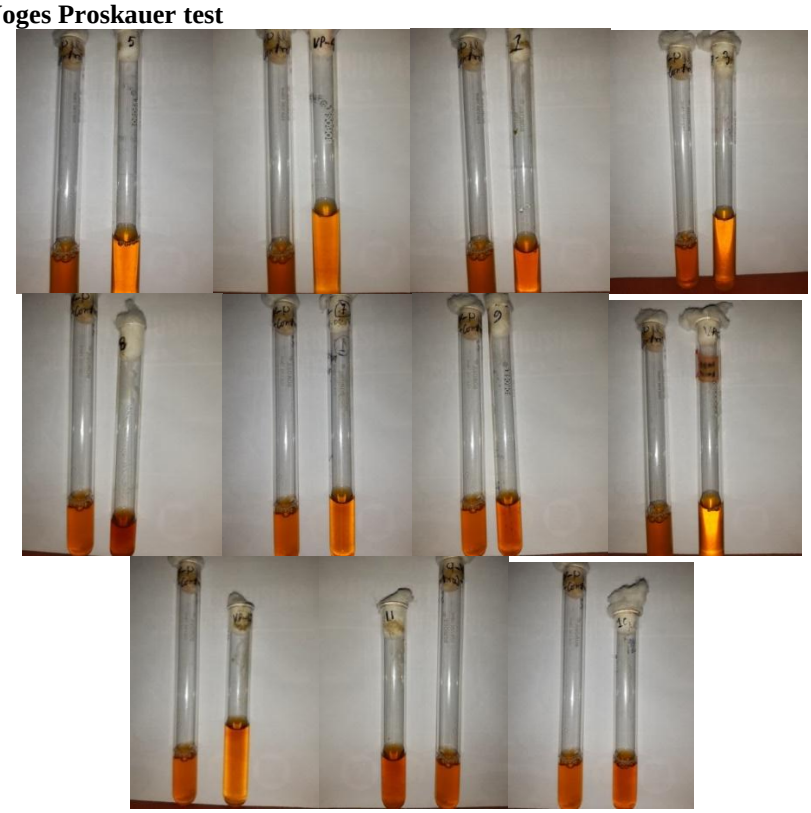
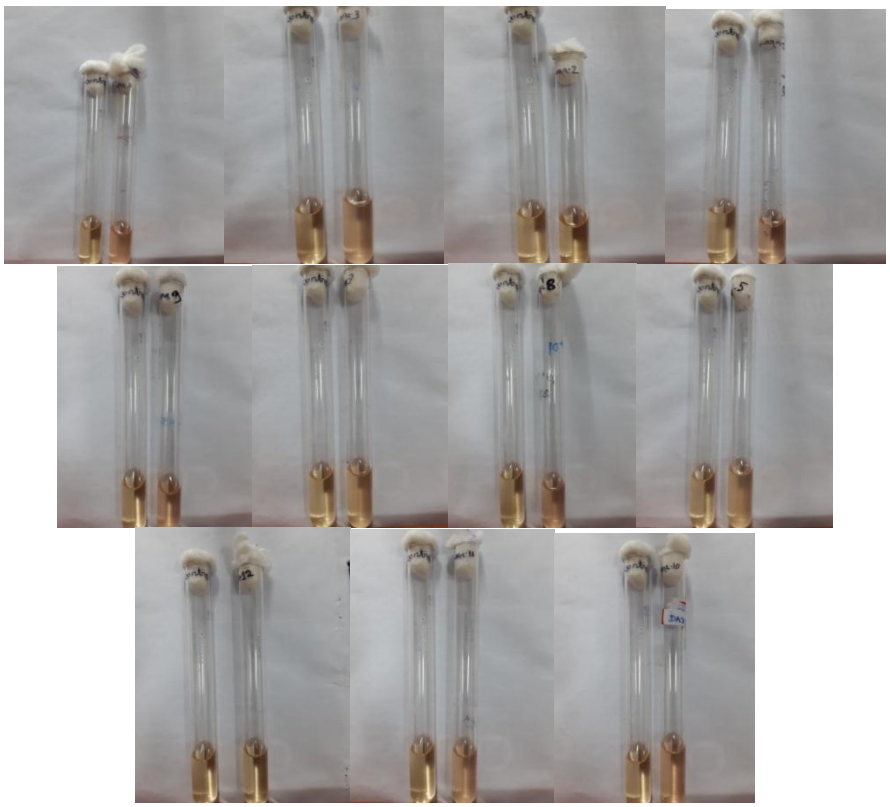
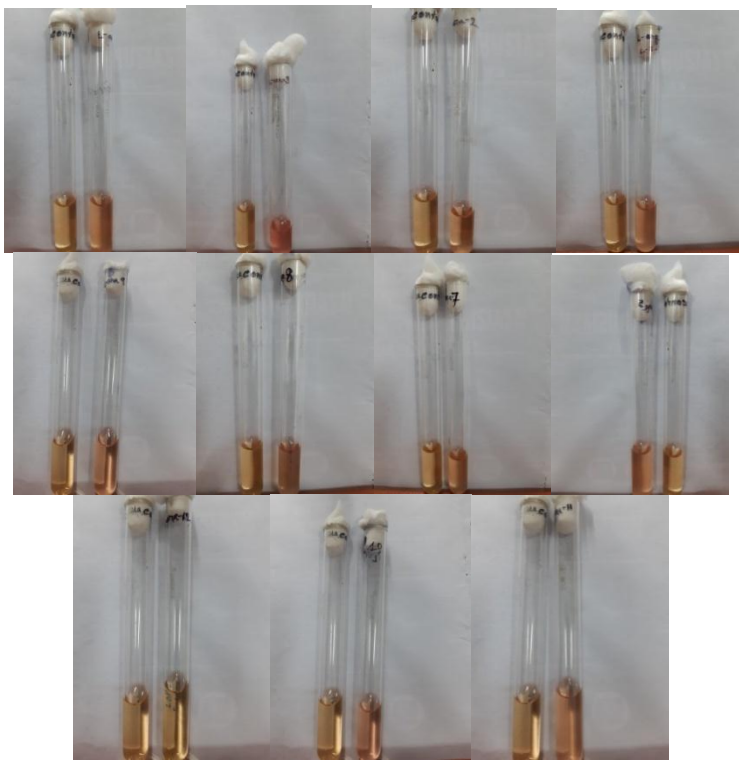


Figure-8: All negative test results found and micro-organism failed to break down the glucose.

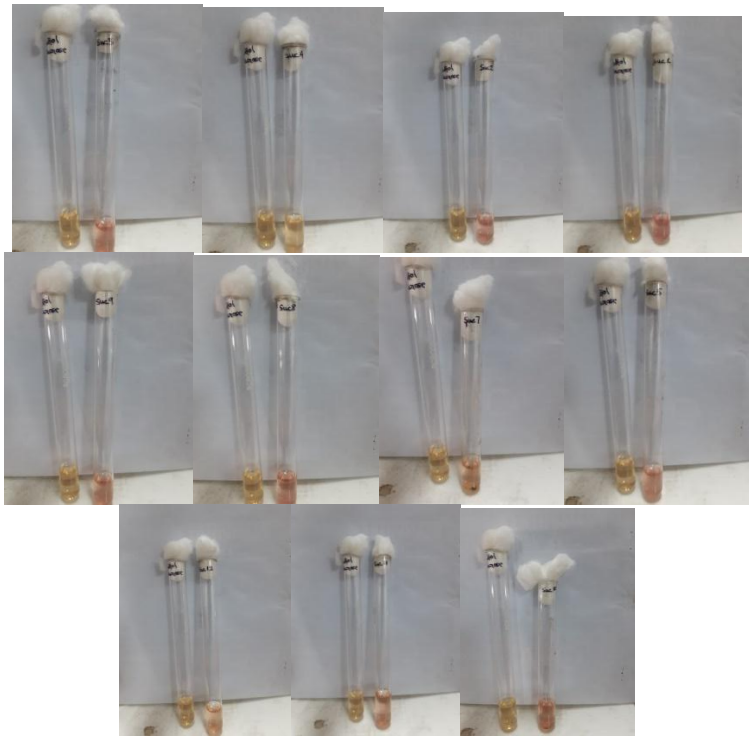
Carbohydrate test
D–arabinose



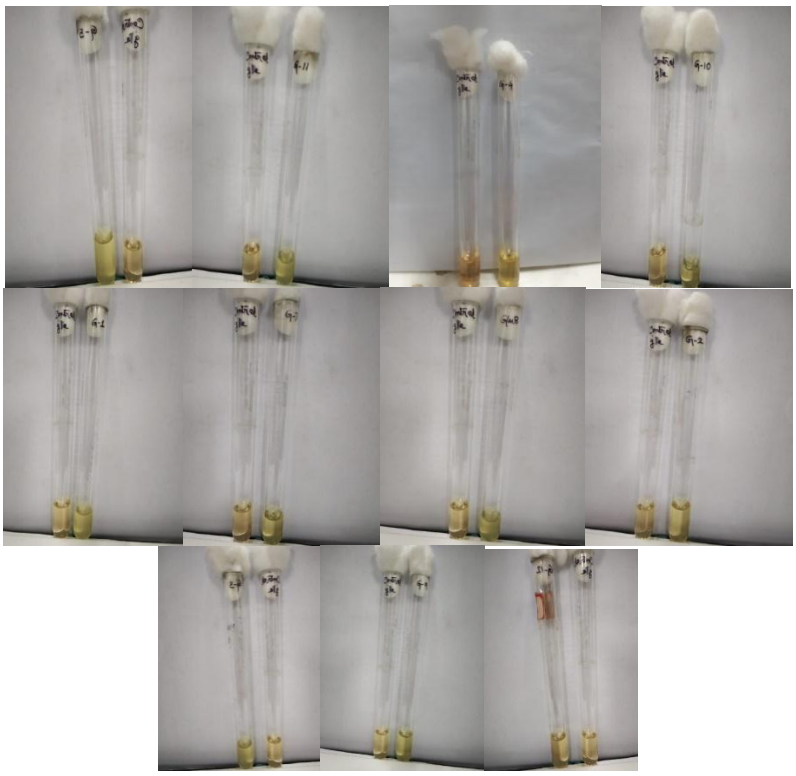
L–arabinose



Sucrose



Glucose



Lactose

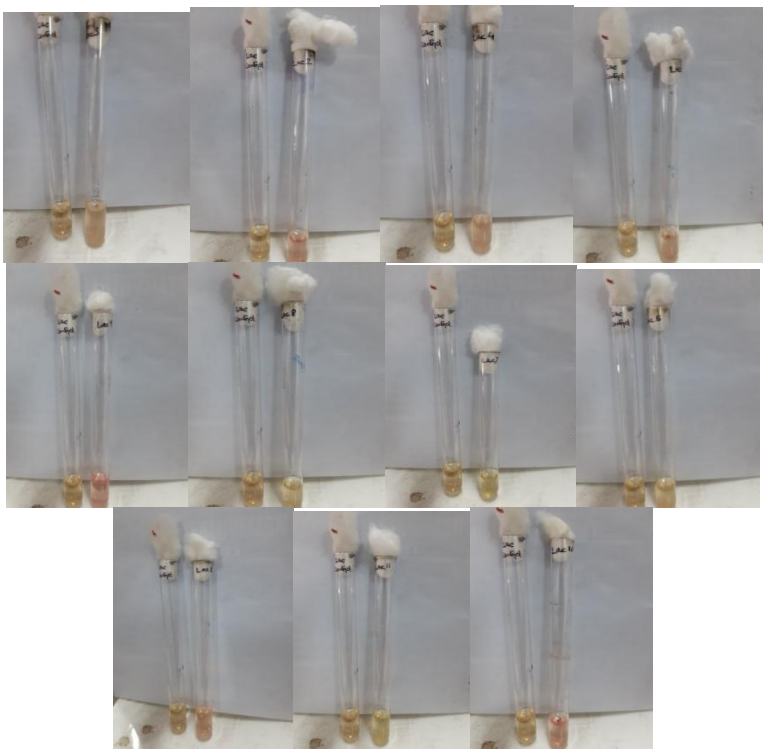


Figure-9: Test results on various carbohydrates.

Carbohydrate Test Results

(+) = means micro– organisms growth takes place.

Carbohydrate	1	2	3	4	5	7	8	9	10	11	12
Lactose	+	+	+	+	–	–	–	+	+	–	+
D – Mannitol	–	–	–	–	–	+	–	–	–	–	–
Sucrose	+	+	+	–	+	+	+	+	+	+	+
Glucose	–	–	–	–	–	–	–	–	–	–	–
D – Arabinose	+	–	+	+	+	+	+	+	+	+	–
L – Arabinose	+	+	+	+	+	+	+	+	+	+	–

Starch hydrolysis

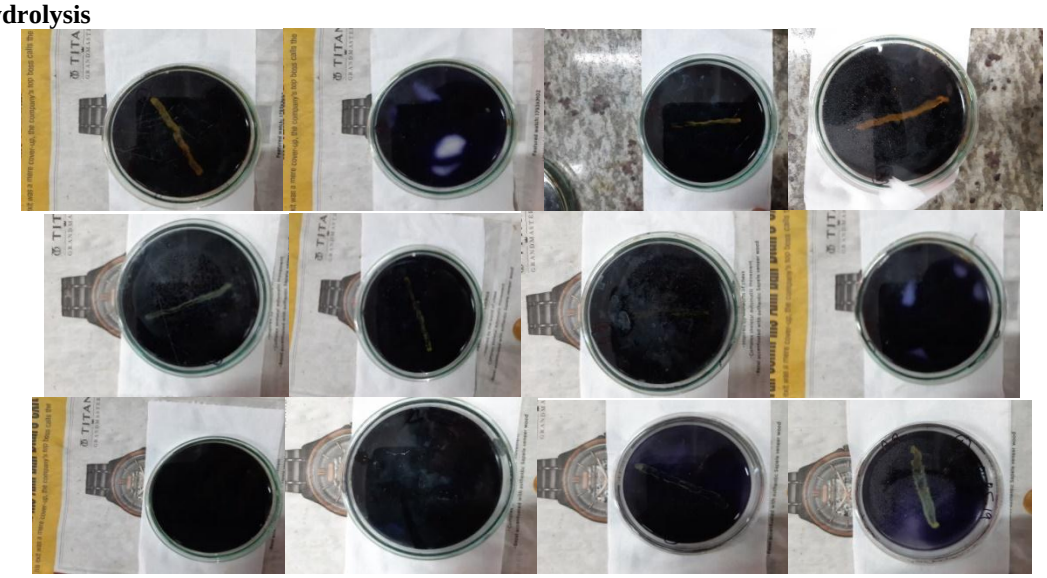


Figure-10: All negative test results found, the organism is not able to produce the extra–cellular enzymes alpha amylase and oligo – 1,6– glucosidase.

Indole test

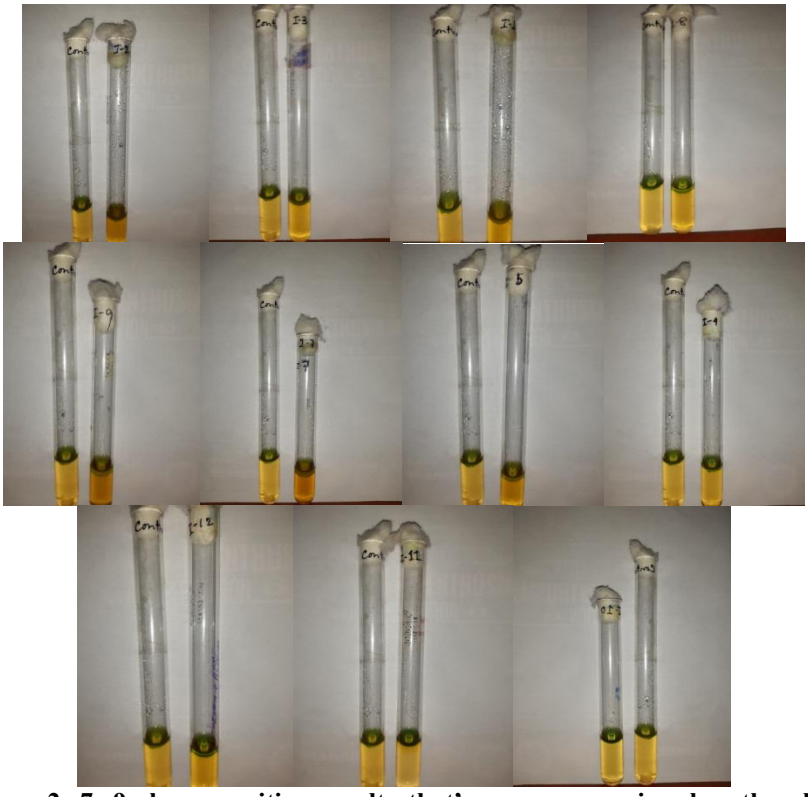
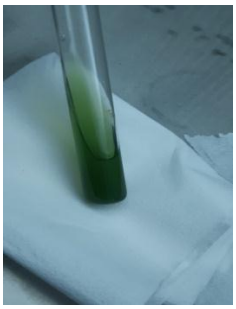


Figure-11: Sample no– 2, 7, 9 shows positive results that's means organism has the ability to convert the tryptophan into the indole.

Citrate Agar Test: All negative test results found; no colour change appeared. All samples look like control after 24 hours incubation. Following is the image of control.



Motility Test: All positive results observed, only control and one sample displaying below.

Figure-A: control.

Figure-B: sample.

CONCLUSION

Chromium has been designated as a priority pollutant due to its carcinogenicity in animals and mutagenicity in a number of bacterial species.[13] Chromium is discharged into the environment mainly due to industrial effluents from metal finishing industry, petroleum refinery, leather industries[14] and many more that causes a health hazard threat to humans as well as animals (Beszedits, 1988). River Ganga harbours several such industries in and around its banks that discharges waste into the river directly. This resulted in development of resistance to chromium in microorganisms for their proper survival.

The untreated sewage water dumping and effluents from the industries in the water has been the major reason behind the findings of high concentration resistant microorganisms from the soil sample taken from the banks of the River Ganges. Further studies may be required to come to the conclusion of how these organisms isolated can be used for bioremediation of chromium contaminated areas and how these will affect the environment and the surrounding habitat.

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