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Bioactivity Guided Fractionation and Characterization of Waste Water Heavy Metal Degrading Bacteria From Sugar Industry Effluent

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Abstract:

Industrial waste water contains heavy metals in higher concentration and it is very harmful to living organism. So, there is a need to develop an efficient technology that can easily remove industrial waste water heavy metal elements. Mostly microbes are used heavy metals bioremediation that are present mostly in higher concentration in every industries effluent. The aim of my research work to isolate the heavy metal resistant bacteria from sugar industry waste water collected from Upper Ganges Sugar Mills Ltd., Seohara, Bijnor, U.P. in sterilized plastic bottle. Various heavy metals are detected in waste water are Cr, Zn, Cd, Mn, Pb etc. and check the minimum inhibitory concentration (MIC) of isolated bacterial strain (S1, S2, S3 and S4). Chromium concentration ranges from 0.1mg/ml to 1.6 mg/ml and MIC was determined at 1.6mg/ml concentration of chromium. The bacterial system could be the potential target for heavy metal bioremediation.

Keywords: Bioremediation, Heavy metal, Heavy metal resistant bacteria, MIC, Microorganisms.

Introduction:

Ground water is the most valuable natural resource which is a supporting system for all forms of life. Ground Water is polluted every day by various anthropogenic activities due rising human populations, urbanization and cosmetic industrialization, rapid rise in industries like Leather Industry, Pharmaceuticals industries, Sugar industries and many others that produce polluted effluents. This is the main cause of the environmental pollution [1].

Increase in industries is the main cause of environmental. With its increase, toxic heavy metals are spreading throughout the world. There are various heavy metals like Cd, Pb, Ni, and As etc. which are toxic at higher concentration. Cadmium (Cd) is a nonessential heavy metal and it is highly toxic for plants, animals, and humans. Lead (Pb) is an important pollutant existing in soil, air, water ecosystem and is cytotoxic to humans [2]. There are various methods used for Physiochemical analysis of waste water sample and various parameters like colour, pH, electrical conductivity, BOD, COD, odour, turbidity, total dissolved solids, pH and electrical conductivity have been analyzed in different experiments conducted [3].

Heavy metals are loosely defined subset of elements that shows metallic properties. Heavy metals density is greater than 5 g/cm3 and atomic number greater than 20. This includes the

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transition metals, metalloids, lanthanides and actinides. They occur naturally in the ecosystem with large variations in concentration. Biological toxicity problems and environmental pollution are the main concerns arising due to the presence of these suspended heavy metals. Many biological and industrial techniques have been developed to digest or remove these heavy metals from effluents. [4]

Few classes of heavy metals which display highly cellular toxicity but they are not involved in any cellular function. In the modern world, there are various physiochemical and bioanalytical which is used for abstraction of heavy metals from waste water. Present work engaged ininvitroisolation, specific identification & characterization of heavy metal (element) resistant bacteria. In this, there are various microbes which are used for elimination of industrial waste water heavy metal elements. Microorganisms that are present in wastewater have the capability to grow under high concentration of heavy metals and thus can be used for the abstraction of heavy metals from waste water. [5]

In various studies about removal of heavy metals by microbes, we found that metals have an association with antibiotic resistance. When stress condition arises, metals and antibiotic resistance in microbes helps to adopt easily as compared to mutations and natural selection. [6]

There are various heavy metal elements suspended in the waste water of sugar industry are Cu, Zn, Pb, As, Cr, Co, Ni and etc.The toxic eefects of heavy metals include damaging of cell membrane followed by disruption of cellular functions at the genomic level by damaging the DNA.They induces negative changes in enzyme specificity.The heavy metals also hae a potent negative effect on damaging the nervous system. Prolonged exposure of heavy metals to human system may cause severe medical complications such as neurodegenerative disorders e.g,Alzheimer'sdisease,Parkinson'sdisease,Muscular dystrophy and Multiple Sclerosis. Allergies developed due to continuous and prolonged exposure of toxic metals and their respective compounds, which may even cause cancer. [4]

Table 1: Heavy Metals and their toxic effects[8]

Heavy Metals	**Toxic Effects**
Copper	Liver cirrhosis, Wilson disease, insomnia
Nickel	Septic Dermatitis, nausea, chronic asthma, coughing, carcinogenic
Arsenic	Gastrointestinal disease, cardiovascular complications, nervous disorder, bone marrow cancer, haemolytic complications
Cadmium	Kidney complications, Carcinoma
Chromium	Headache, nausea, diarrhea, vomiting, carcinogenic
Lead	Autoimmune complications, Nausea ,Septic allergies, abdominal complications, psychological disturbances
Mercury	Body tremors, restlessness, anxiety, apnoea, mental depression,

Some heavy metal elements are highly cyto- toxic with acellular activity. [8], but there are some heavy metal elementsutilized as micronutrients are essential for sustenance of life, as they control critical physiological process of human system(Table 1). But higher dosage of

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these heavy metals is highly toxic and carcinogenic. [5].Also their elevated blood concentration may inhibit the activity of sensitive enzymes [9].

Table 2: World Health Organization's Guideline Values for Heavy Metals [10]

Heavy metals	Value (mg/L)
Copper	2
Lead	0.01
Nickel	0.02
Mercury	0.001
Arsenic	0.01
Chromium	0.05
Cadmium	0.003
Manganese	0.4
Selenium	0.01
Barium	0.7

Heavy metal elements play an important role in controlling metabolic activity of many important microorganisms, as they help in various enzymatic reactions or role in regulation of biochemical processes. Those bacteria which are isolated from waste water are basically saprophytes [11].

Basically there are two types of methods which are mainly used for bioremediation of waste water effluent. (1)Biotic Method: Mainly this method was based on the heay metal accumulation in plants and microorganisms. In this method there are various bacterial species which shows the significant amount of heavy metals elimination from waste water.(2)Abiotic Method: It is based on the using the various physiochemical processes like chemical precipitation, reverse osmosis, ion exchange and heavy metalsadsorption by suitable adsorbent. The main challenge is that, the used physiochemical processes are very expensive and produce secondary products. Whereas, biological processes are cost effectives, there is secondary products generated and it is environment friendly method. Depending on the chemical properties, various heavy metals play critical role in the microbial metabolism by taking part in regulation of biomechanical processes, stabilization of cell structures or enzymatic reaction catalysis.

For the past 30 years, study on heavy metal microbial tolerance has been conducted, the last 15 years' time period has been exceptional as lot of discoveries at microbial level has been elucidated. Three useful utility of heavy metal resistance studies in the field of biotechnology has been elucidated (Table 2).Bio miming used metal resistant bacteria extraction of expensive metals (bioleaching). Metal resistant bacteria is used in bioremediation of ecosystem polluted with metal.[12] Present study is mainly focussed on assessment of chromium contamination and its microbial degradation. Chromium is an element which is denoted by Cr and its atomic number is 24.It is anodourless element with no tastelustrous metal element with high melting point. Chromium is a highly resistance to corrosion. It doesn't easily oxidized in air and is highly unstable in presence of oxygen andconverts to a thin oxide layer that is impermeable to oxygen which itself is metal protective. It is one of the most prevalent and common heavy metal pollutant which is highly toxic to the surrounding

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ecosystem including human population. Chromium is a naturally occurring element and it is found mainly in Rocky Mountains, volcanic discharge, soil system, plants, and animals and innatural gases in minimal amount. It is an essential nutrient in diet but humans need only a very small amount but in higher amount it can be toxic to humans. [7]

Table 3: General Properties of chromium [10]

Property	**Chromium (Cr)**
Elemental Group	Transition Metals (Group 6)
Element Atomic Number	24
Element Molecular Weight	51.996 $gmol^{-1}$
Element Oxidation States	+2, +3, +4, +5, +6
Element Density	7.19 gcm^{-3}
Element Melting Point	2180K
Element Boiling Point	2944K
Common Ionic forms	Cr^{2+} , Cr^{3+} , Cr^{6+}

To attain a high lustre, it can be polished and it is extremely lustrous. Chromium is a significantly active metal element and is non-reactive with water but highly reactive in presence of acids. Self-passivation is a specific property of chromium.In elemental form, it displays paramagnetic properties. Depending on the heating and cooling chromium shows different magnetic properties. Chromium compounds, like chromium dioxide has ferromagnetic property.

The concentration of chromium in air and water is not too high. Chromium concentrationin drinking water is very less but in polluted water, it's very high in dangerous Chromium-IVstate (Table 2). It is an essential human nutrientat low concentration and its deficiency can lead to heart ailments.Its elevated concentration has critical ill health effects and skin allergies. It is useful in organisms that can disrupt the carbohydrate metabolism and if the routine dose is very low then it may cause heart related problems.Chromium-IVis a very dangerous to human health and mostly who are working in the steel and textile industries. It is highly toxic to human health and it alter genetic materials that leadto cancer.

Chromium-IV is the most dangerous state and can create health complications.Chromium suspended in water based pollutants gets absorbed on sediment and become immobile. There are no harmful effects of chromium on the bodies of fish but damages its gill. Animals have a lower ability to fight with disease. Crops have the ability to control the concentration of chromium uptake that does not cause any harm to it. High concentration of chromium in soil system leads to its acidification,which can significantly influence its uptake by crops, as soil pH is in acidic side. Plants mainly absorb only chromium (III), which is an essential kind of chromium active at low concentration and cause harmful effects when the concentration is high.

Literature Review:

Research on heavy metals like chromium, copper, zinc, lead, cadmium, nickel, iron etc. in contaminated waste water can be removed by using technical applicability of Nano filtration membrane method. They studied their properties, uses, mechanism, factors affecting the performance, advantages and limitations etc. They also observed that operating conditions like pH, dose required, initial concentration, treatment performance also play a critical role in heavy metals abstraction. There are various technologies that are used for the metal recovery

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such as reverse osmosis, ion exchange & newly developed Nano filtration. Nano-filtration method is mainly capable for the recovery of bivalent metals.It is an advanced separation method for the waste water remediation and concentration. Nano-filtration method is an inexpensive method due to its higher flow rates and low operating pressure as compared to reverse osmosis Nano filtration membrane rejecting bivalents salts but allows partial permeation of monovalent salts like sodium chloride. Heavy metals from ground water will be remove by Nano filtration method and it also lower the TDS, hardness and reduce colour of these heavy metals.[13]

Investigational studies that environmental pollution is increasing because of the extensive use of hexavalent chromium element in different industrial application. The effective process of detoxification of Cr-VI to Cr-III has increased by increasing of Cr-VI reducing microbial consortium. Pseudomonas fluorescensand Bacillus species are chromium resistant and these were isolated from the soil polluted by heavy metals were used to evaluate their tolerance and check the capability of these bacterial species to reduce Cr-VI to Cr-III.They also consider the effects of pH, time interval and initial metal concentrations on the reduction of chromium by these heavy metal resistant bacterial species. Both these bacterial isolates at 100 ppm in minimal salt broth tolerate Cr-VI. Highest accumulation rates of Bacillus species and Pseudomonas fluorescenswas 87.8 % and 93 % at 25ppm was observed. 40%, 68%, 81 %, 75 % and 60 % of Cr-VI to Cr-III was reduced by Bacillus species and 52%, 58%, 72%, 75% and 61% of Cr-VI to Cr-III by Pseudomonas fluorescensat different levels of pH mainly at 5.0, 6.0, 7.0, 8.0 and 9.0 respectively. At pH 7.0 to 8.0, there was maximal reduction of Cr-VI reduction was observed. This signifies that the detoxification of chromium polluted environment can be done by the use of microbial consortia and the mono cultures of these isolates.[14]

Zahoor et al.,(2009) aim was to check Bacillus species&Staphylococcus capitis, abilities to convert thechromium (hexavalent)to its trivalence form. The Bacillus species that can sustainCr-VI up to 4800g/ml &S. capitis up to Cr-VIto 2800 g/ml, both of these bacteria having the capability to resist Cd^{2+} at a concentration about 50 g/mL,Cu^{2+} at a concentration about 200 g/ml, Pb^{2+} concentration about 800 g/ml, Hg^{2+} concentration about 50g/ml and Ni^{2+} concentration about 4000g/ml. S. capitis have the ability to resist Zinc ions at 700 g/ml and Bacillus species having the resistance up to 50g/ml. The most optimal growth of these bacterial species was observed at pH 6 and 7 and at 37°C. After 96 hour, they observed that both these bacterial species having the capability Bacillus species JDM-(2)-(1) and (81% by S. capitis) from waste water effluent from various industries and after 144 hours the percentage is increasing i.e. 86% and 89%. Reduction of 80% and 71% concentration of 10 g/ml of Cr-VI by cell free extracts of Bacillus sp. and S. capitis was observed. To reduce hexavalent chromium (85% by induced protein) in the presence of chromium element play an important role in the chromium reduction. According to Zahoor et al., these bacterial species were having the capability to reducing the toxic form to its nontoxic form.[15]

Rajbanshi, (2008) found that pollution due to heavy metals is increasing because of industrailization in increasing. So , it's mandatory to eleminate toxic metals from waste water using heavy metal resistant microorganisms because othe methods like ion exhannge, precipitation etc. are very expensive and using heavy metals resistant microorganisms mediated bioremediation is inexpensive as compared to other costly bioanalytical methods. Tenmetal tolerant bacteriafrom wastewater effluent was isolated. They were chromium resistant Citrobacterspecies,Klebsiellaspecies,Bacillus species,cobalt resistant Methylobacteriumspecies andcopper resistant Pseudomonas species. The isolated microorganism displayed highest tolerance to toxic metals with Minimum Inhibitor

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Concentration (MIC) ranging from 160 µg/ml to 550µg/ml.There are six toxic metals tolerant isolates which shows multiple tolerances. All these isolates also showed antibiotic resistance (10-15%) to single antibiotic and 80-90-% were multi-antibiotic resistant.[16]

Stella *et al.,* (2012) found that environmental pollution is increasing due to the industries development.Waste water effluent from sugar mill was collected and bioremediation is done using immobilized bacterial consortium and the physico – chemical properties waste water was analysed. Beads are used for immobilization and then used for the bioremediation of waste water. Scientist inoculated the collected sugar mill effluent with immobilized beads containing bacterial consortium, and an aerator are used for aeration. After 3 and 6 months, the sample was filtered under aseptic condition after three to six month after that physico-chemical parameters are determined. The chemical properties and biological properties of the raw sugar mill effluent include elevated Biological Oxygen Demand, Chemical Oxygen Demand, TSS, TDS, Iron, Zinc, Copper, Lead and Manganese. Immobilized bacterial consortium had showed a drastic reduction in the levels of Chemical oxygen demand, TSS, TDS, heavy metals respectively.[17]

Samanta et al., (2012) aims to isolate the heavy metal tolerant and antibiotic resistantmicroorganisms from the K.M.C.'s Waste Dumping Yard, Dhapa, Kolkata. The land waterways was polluted by the effluent that was coming from various industries and by domestic waste. Based on the extent of pollutants being discharged and the land waterways. Investigational study shows that screened strain have amylase and protease activity and displays cellular growth activity in broad range of substrates and temperature ranging from 30°C-40°C and pH was 6.0-11.0. By using the 16SrDNA technique, Bacillus species was screened and has a mega plasmid andwas grown in the presence of various heavy metal elements like Nickel, Cadmium, Chromium and Cobalt. This specie of Bacillus was also resistant to a wide range of antibiotics. Such as Kanamycin (40µg/disc), Ampicillin (30µg/disc) and Methicillin (10µg/disc).Metal tolerance test showed highest tolerance of microbe towards Cadmium and lowest towards Cobalt.They observed that the bacillus species is most suitable for the bioremediation of heavy metals in the polluted environment.[18]

Silval et al., (2012) study used waste water samples from university campus,medical hospital premises and a chemistry technical school toanalyse and calculate the bacterial tolerance to metal effluent from water system. Effects of these metal effluent on pigmentation and on enzymatic activities metal tolerant bacterial strains was also observed..In their research they found that Gram positive bacteria show greater tolerance to Cr and gram negative bacteria show towards Ag and Hg. The tolerance of heavy metal was greater for Hg in the hospital discards (4.1%). Hg had the most noticeable effect on the colour of the colonies. Silver(Ag) ions tolerant Bacillus species and Hg-tolerant P. aeruginosa were not capable to produce oxidase in the presence of Ag and Hg when they consider the effect of heavy metals on respiratory enzymes. But in several bacterial strains (68% by Cr), the expression of gelatinase was inhibited. [19]

Dermentzis et al., (2011) studied that bioremediation of hexavalent chromium metal from aqueous synthetic solutions by electrocoagulation. The optimal pH was in the range between 4-8. Initial concentration of chromium (200mg/l -800mg/l) has no effect on the removing the chromium. In lesser time higher concentrations of chromium was reduced as compared to lower concentration. Their experimental results showed that the best removal of hexavalent chromium at current density 40 mA cm^{-2}. For the treatment of an electroplating wastewater sample, electrocoagulation process could be successfully applied. In 50 minutes of electro-

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processing the concentration of Cr-VI ion and COD were minimized under the permissible levels.[20]

Kumaran et al.,(2011) finds that the role of microoragnisms was very significant in adsorption of metalfrom waste water effluent. They were isolate and identify the microorganism that have the ability to absorb the metal effluent from the waste water and uses these microorganisms for the removal of heavy metals. They picked up the bacterial isolates from the estuarine waste water and they purified and identified thee bacterial isolates based on morphological and biochemical properties. They uses plate dilution method for their determination. They uses the different concentrations of Cd^{2+}, Fe^{2+}, Pd^{2+}, Ni^{2+} and Zn^{2+}(10 to 1000 g/ml). MIC to heavy metals was shown by these microorganisms.Strains of Pseudomonas sp. show the maximum resistance to these heavy metals. They used ICP-OES for the detection of heavy metals and they also analyze the pH and growth of culture after 24 hrs. This experiment showed 87.9% of Pd, 53% of Ni, 41% of Cd, 62.8% of Fe and 49.8% of Zn absorption by the Pseudomonas species and more than 55% heavy metals from estuarine water.[21]

Congeevaram et al., (2007) studied the specific role of microorgnisms for it's bioremediation potential to abstract heavy metal polluted soil and wastewater system. They isolated the heavy metals resistant fungi and bacteria from electroplating industry waste water these isolated microorganisms to check their ability to bio remediate waste water heavy metal pollutant. The optimal range ofpH for fungus was (5 -5.2) and for bacteria was (7). Optimal temperature should be provided for growth and for removal of heavy metals. They observed the effects of pH on cellular physiology because all the experimental results of cell growth was associated with removal of heavy metals. For solid retention time (SRT) design, batch and tolerance experiment provide the information and lethal tolerance limit for isolated microorganisms. As per experimental data that expanded SRTs (stationary phase) can be recommended but they use the fungal and bacterial Cr-resistant isolates for removing chromium from waste water effluent. As per tolerance data high range of heavy metal concentration exposed the Chromium-resistant isolates particularly for fungal and could tolerate chromium toxicity up to 11,000 mg/l chromium. TheMicrococcus and Aspergillus species having the capability to bio remediate chromium and nickel from wastewater effluent.[22]

Materials & Methods:

MaterialsRequired:Nutrient Agar (28 gm in 1.0 L), Pseudomonas Isolation Agar,EMB Agar, Mannitol Salt Agar,And Violet Red Bile Agar.

Sample Collection: The effluent sample was collected in dry sterilized plastic bottle from discharge area of Upper Ganges Sugar Mills Ltd., Seohara, Bijnor, U.P.The waste water sample was collected in dry sterilized polyethylene bottles (500-1000ml) from effluent discharge area of sugar industry wastewater.

Research Methodology:

Today industrialization increasing day by day and causing various environmental issues and modern society has been concerned about environmental issues caused by effluent produced by these industries. So, industries follow more and more strict rule and regulation about environmental issues caused by waste water and other polluting waste produced by industries

Sugar industry produces waste water that contains toxic metals which are cytotoxic for the living organism and causing serious diseases and disorders. Therefore, to remove the toxic

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metal pollutants from the waste water, various technologies are used for removing heavy metals like chemical precipitation, ion exchange, adsorption, electro dialysis but most of these techniques are ineffective and expensive. Recovery and removal of metal pollutant from the waste water effluent using microorganism is a cost effectives, sustainable, environment friendly method. So, most research has been running using various microorganisms for removing toxic heavy metals from industries waste water.

For the detection of heavy metals in the sugar industry waste water effluent sample, sample was sent to Punjab Agricultural University, Ludhiana. The various metals and metal effluent in the waste water sample of sugar industry were detected by using technique Inductively Coupled Plasma- Mass Spectrometry(ICP-AES) are mainly Zinc (Zn), Sodium (Na), Manganese (Mn), Cadmium (Cd), Lead (Pb), Iron (Fe), Chromium (Cr), Potassium (K), Copper (Cu), Nickel (Ni), Arsenic (As), Boron (Bo) and phosphorus (P). These metal effluents detected are measured in g/l (gram per liter).

For the isolation of bacterial species from waste water, I have used nutrient agar medium (2.8 gm in 100 ml each) were prepared in a conical flasks. The pH for the medium was adjusted accordingly and then media and all the glass wares such as petriplates, tip of micropipette and test tubes were sterilized at 15 lb/in² pressure and 121°C for 30 minutes. Prepared nutrient agar was autoclaved and allowed to cool for few minutes. Then media is now poured into the petriplates and allowed to solidify in the laminar air flow chamber. Then I prepared five dilution of waste water sample. After that the diluted waste water sample was taken in different test tubes, then with the help of micropipette, take 0.1ml diluted sample one by one and put it on the petriplates and then with the help of spreader, spread it on the whole petriplates containing nutrient agar. Then these different petriplates were kept maintained at 37°C for 48-72 hrs for the isolation of bacterial species.

Isolation of toxic metals tolerant bacterial species using several selective media was performed.Four different selective media were used for the isolation of different bacterial species. Pseudomonas isolation agar, Eosin methylene blue (EMB), Mannitol Salt Agar and Violet Red Bile Agar (VRBL) were used and was prepared in four batches and the pH for the medium was normalized accordingly and then the media and all the glasswares like petriplates and test tubes were sterilized at 15 lb/in² pressure and 121°C for 30 minutes. Prepared media was autoclaved and allowed to cool for few minutes.Single colony from the petriplates containing nutrient agar was isolated with the help of inoculation loop, streak on the plates one by one containing different media and then these petriplates were kept at 37°C for 48-72 hrs for the isolation of bacterial species.Analyze the isolated bacterial colonies on the petriplates after 48-72 hours of incubation at 37°C. For the preparation of pure culture of different bacteria, I have used nutrient broth in four conical flasks. Loop full of isolated single bacterial colony from different selective media containing colonies was taken and put in the nutrient broth in the four different conical flasks one by one. Then these different flasks were incubated in a rotary shaker for 2-3 days at 37°C for the proper growth of bacteria in four different conical flasks. And I have check OD of cultures for 7 days to check the growth of the microorganisms.
Gram's Staining for the morphological identification of bacteria.Catalase test was performed. Microbial colony from the different plates containing selective media were loaded on slide in laminar flow chamber. Then one drop of hydrogen peroxide put on slide and oxygen gas will evolved due to this evolution of oxygen gas causes the formation of bubbles and is indicative of a positive test. When there is no formation of bubbles then it indicates the negative test.Oxidase test has been performed using filter paper. In this test, four small piece of filter paper were taken and then the microbial colony was picked up from different petriplates

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containing different selective media and smear it on filter papers one by one in all four.One drop of *tetra methyl* p-*phenylenediaminehydrochloride* 1 %(1gm in 100ml) was poured on filter papers and observed after some time.If the filter paper containing bacterial colony turnS blue then it indicates a positive test. If no colour change then it indicates a negative test.**Citrate utilization test-**This test is basically used to see that if microorganism can use the citrate as it its single source of carbon and energy for the growth of the microorganism. If a microbe can grow on Simmons citrate agar then the pH of medium will increase and pH indicator change the colour. There are some microbes that use citrate as it its single source of carbon and energy. So, to do this test, I prepared Simmons citrate agar in eight slants tubes. In these eight test tubes four were used as test and four are used as control. MR-VP broth also used in this test. And this broth was transferred into the slant tubes in laminar air flow and streaked with four different microbes on the surface of four different slants tubes(Test) and remaining four are kept as control. Then put all these slants tubes were in incubator at 37 ^{0} C for 24 - 48 hours. If the colour on the surface of medium was blue then it will give positive test but if there is no growth or no blue colour then it will indicates negative test.**Indole Test-** There are not so many bacteria that can produce indole from amino acid tryptophan by using typtophanase enzyme. This test is performed to determine that if bacteria can breakdown the amino acid tryptophan into indole.Peptone broth is needed to perform this test. I will take eight test tubes and 5ml broth was transferred to each test tubes and four test tubes (Test) are inoculated with four different culture inside the laminar air flow and remaining four (Control) are kept as control. And then these eight test tube were transferred to incubator at 37 ^{0} C for 24 hours. After incubation 0.2 ml of Kovac's reagent was added to test tubes. Indole reacts with the aldehyde in the Kovac's reagent to give red colour and a red colour ring will form at the top. It indicates the positive result if not then negative result will appear**.Methyl Red (MR) Test**-This test is mainly used to determine the ability of microorganisms to produce and maintain stable at high concentration of acid from the glucose fermentation. In this test, methyl red is used as pH indicator that remains red in colour at pH 4.4 or less than 4. To do this test, MR-VP broth was needed and I was prepared broth and transferred into eight test tubes inside the laminar air flow. Four out of eight test tubes were inoculated with the four different test cultures and remaining four were kept as control. After that put all these test tubes in incubator at 37 ^{0} C for 48 hrs. After 48 hrs. Add 5 drops of methyl red solution then red colour indicates the positive test and no change in colour indicates a negative test.

VOGES-PROSKAUER (VP) Test -In this test also MR-VP broth was needed. So first of all I prepared MR-VP broth and eight sterilized test tubes. Then broth is transferred to these test tubes. After that four test tube are inoculated with four different test cultures and remaining four are kept as control. Put all of these test tube in incubator 37^0 C for 48 hours. Post 48 hrs of incubation, add 1ml of 40% of potassium hydroxide and 5% solution of alpha naphthol in ethanol. Then tubes are allow to stand for 15 minute. If red colour will appear than it will indicates positive result. And if after 30- 40 minute, there is no change in colourwhich indicates the negative result.

Table 4: Biochemical characteristics of isolated bacteria:

S.No.	Biochemical Test	S1	S2	S3	S4
1	Citrate Utilization Test	(+)	(-)	(-)	(+)
2	Indole Test	(-)	(+)	(-)	(-)
3	Catalase Test	(-)	(+)	(-)	(+)

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4	Oxidase Test	(+)	(+)	(-)	(-)
5	Methyl Red Test	(+)	(-)	(-)	(+)
6	Voges-Proskauer Test	(-)	(-)	(-)	(-)

Determination of resistance level of isolated bacterial species against different concentration of heavy metal was performed using **Minimum Inhibitory Concentration test (Table 4) .**Nutrient Agar medium(2.8 gm in 100 ml distilled water each) was prepared for different concentrations of heavy metal, (Cr).$K_2Cr_2O_7$ as a source of heavy metal, chromium at different concentrations of 100ug/ml, 200ug/ml, 400ug/ml, 800ug/ml and 1600ug/ml in a distilled water were poured in a prepared nutrient agar medium. The medium containing different concentrations of heavy metals were sterilized in an autoclave at 15 lb/in² pressure and 121°C for 30 minutes. The autoclave sterilized medium were poured on the petriplates in a laminar air flow chamber and allowed to solidify for 10 -15 minute. After that medium get solidified, the pure culture was taken from the incubator.O.1ml of culture was taken by micropippete from inoculum flask and put on the nutrient agar plates and then spread it with the help of spreader whole the plates one by one to obtain colonies at different concentrations of heavy metals. Plates were kept in the incubator at 37°C for 48-72 hrs to determine the resistance level of isolated bacterial species at different concentrations of heavy metal.

Result & Discussion:

Table 5: Physiochemical analysis of sample collected:

PARAMETERS	**OBSERVATION**
Appearance	LIGHT BROWN
Odour	SWEET ODOUR
pH	5.17
Nickel	0.0180 mg per litre
Cadmium	0.003 mg per litre
Chromium	0.154 mg per litre
Copper	1.879 mg per litre
Iron	4.922 mg per litre
Manganese	0.702 mg per litre
Lead	0.180 mg per litre
Zinc	1.493 mg per litre
Sodium	9.533 mg per litre
Potassium	57.43 mg per litre
Boron	0.230 mg per litre
Arsenic	0.001 mg per litre
Phosphorous	5.976 mg per litre

Table 6: Detection of Heavy metals in the waste water sample

The sugar industry waste water sample was sent to Punjab Agricultural University, Ludhiana for the detection of heavy metals by ICAP-AES. The heavy metals such as Lead (Pb), Cadmium (Cd), Chromium (Cr), Zinc (Zn) detected in the waste water sample are shown in the report shown below:

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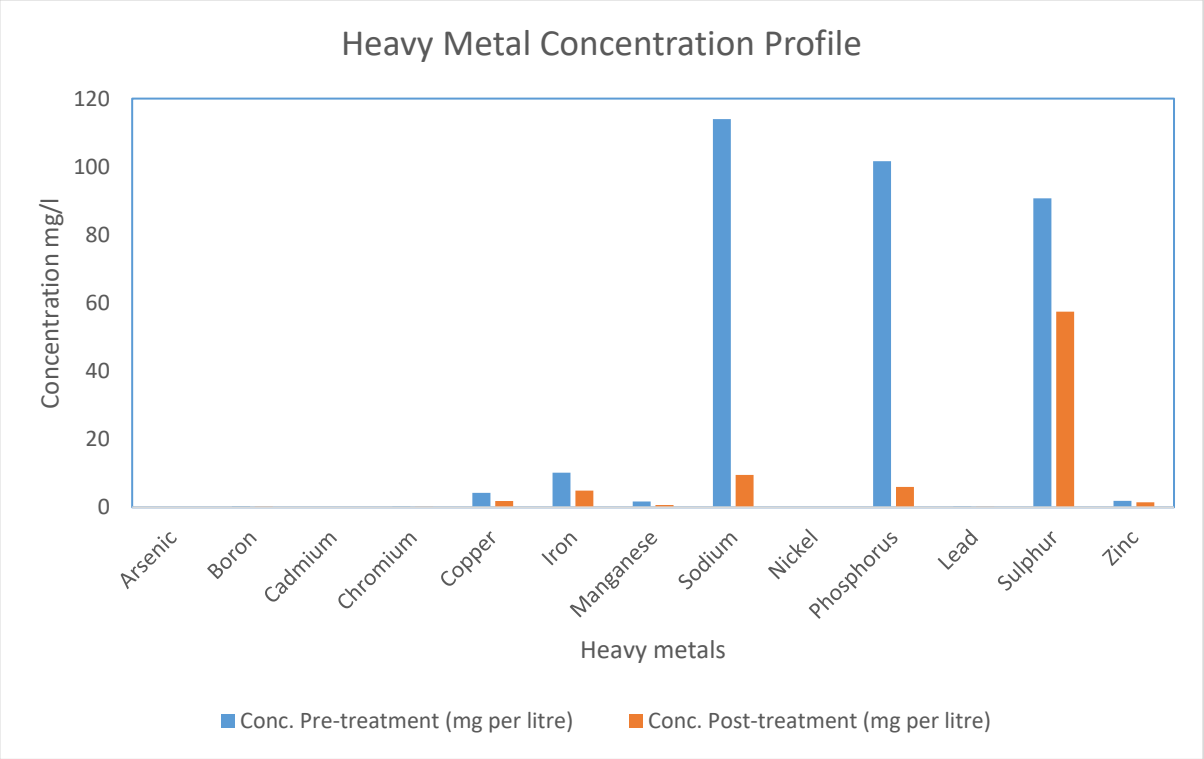
Waste water Elements	Conc. Pre-treatment (mg per litre)	Conc. Post-treatment (mg per litre)
Arsenic	0.012	0.001
Boron	0.370	0.230
Cadmium	0.004	0.003
Chromium	0.257	0.154
Copper	4.239	1.879
Iron	10.18	4.922
Manganese	1.716	0.702
Sodium	114.0	9.533
Nickel	0.037	0.018
Phosphorus	101.6	5.976
Lead	0.293	0.180
Sulphur	90.73	57.43
Zinc	1.885	1.493

(*Test Report: Elemental composition by ICAP-AES [Central Testing Laboratory, Department of Soil Science, Punjab Agricultural University, Ludhiana*)

Graph 1:



Minimum Inhibitory Concentration test

Minimum Inhibitory Concentration test was done to determine the resistance level of isolated bacterial species at different concentration of heavy metals. The isolated bacterial species showed a great degree of variations at different concentrations of chromium. Different petriplates were prepared contains different concentrations of chromium 100ug/ml, 200ug/ml,

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400ug/ml, 800ug/ml and 1600ug/ml in distilled water incorporated into nutrient Agar medium by pour plating method. These plates were incubated at 28°C for 72 hours.

Bacterial growth was observes at Chromium concentration of 1600ug/ml. There are four bacterial strains (S1, S2, S3 and S4) and in all petriplates one was control and other was experimental. There were not a single colony of S1, S3 and S4 were observed. But there are few colonies white colour colonies of S2 were observed. It means that S2 bacterial strain was resistant to chromium concentration at a 1600ug/ml in nutrient agar medium but all other bacterial strains (S1, S3 and S4) were not resistant to chromium at a concentration of 1600ug/ml in nutrient agar medium.

Hence it was observed that bacterial growth was decreasing when increasing the concentration of chromium concentration. The growth of bacteria was observed at 100ug/ml, 200ug/ml, 400ug/ml, and 800ug/ml. Thus, we examine that growth of bacteria was inhibited at 1600ug/ml of chromium concentration. So, Minimum inhibitory concentration of Chromium resistant bacterial species S1, S3 and S4 was determined between 800ug/ml - 1600ug/ml in nutrient agar media. But for S2 bacterial species was determined at 1600-3200ug/ml.

Conclusion:

Removal of heavy metals from waste water using various microorganism is an environment friendly method and is highly efficient than several conventional methods like precipitation, ion exchange etc. Because it is a low cost and very effective technique to reduce the toxicity caused by heavy metal to the environment.

Mostly bacterial species have the ability to remove heavy metals from waste water by bio sorption or by bioaccumulation or by both mechanisms. In my research work, waste water sample was collected from sugar industry and in that waste water several heavy metals were detected by technique called ICP-AES.

Four heavy metals resistant bacterial strain were isolated (S1, S2, S3 and S4) by pour plating method using various selective media. Then inoculums were prepared from the isolated bacterial species. Morphological and various biochemical tests were conducted. Then, took chromium (Heavy metal) to check the minimum inhibitory concentration of these isolated bacterial species. At 1.6mg/ml concentration of chromium, the growth of four out of three bacterial species (S1, S3 and S4) was inhibited and other specie (S1) growth was not inhibited at this concentration. So, it concludes that the bacterial species are very efficient in removing of heavy metals from industrial waste water. And it is a cost effective and eco-friendly method of removal/recovery of heavy metals. After recovery, these heavy metals are used in various industries for several applications due to their useful properties.

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