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Isolation, identification and biochemical characterization of chromium degrading bacteria from industrial effluents and to run chromate reduction assay on the bacterial isolates along with the amplification of *chrA* and *chrB* genes.

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Abstract
The heavy metal Chromium (Cr) has been largely used in paper, pulp and textile industries worldwide. It is being used in various industrial manufacturing procedures and eventually gets released with the wastewater into the surroundings. Basically chromium exists in two stable forms. The hexavalent form of chromium is a bio-hazardous heavy metal ion. Since it is non-biodegradable in nature and persists in the environment, it holds the potential to harm the public health and put environmental protection and preservation at stack. The areas surrounding the industries which release the effluents consisting of bio-hazardous heavy metal ions like chromate have sustained several strongly resistant bacterial strains. The aim of this paper is to isolate and identify the bacterial strains from textile, paper-pulp industrial waste effluents, which degrade the toxic hexavalent chromium and further to study the biochemical properties of chromium degrading bacteria.The understanding of molecular mechanics behind chromium tolerance in microbes by applying various qualitative and quantitative analytical methods of molecular biology can help with the issues of global concern like environmental protection and bioremediation. The peculiar chromium degrading property of microbes was confirmed by this research work by growing collected bacterial samples over potassium phosphate nutrient agar and treating the pellets centrifuged from nutrient broth culture media with different concentrations of K2Cr2O7 (as chromium source) to carry out chromate reduction assay on K2Cr2O7exposure on isolated bacterial strains, the results shown the notable growth of bacteria at different concentrations proving the presence of chromium tolerance and degradation capacity within their genetic makeup.

Keywords:*Chromium toxicity, degradation of chromium, chromium degrading bacteria, "chr" gene family, chrR gene, chrA gene, chrB gene, DNA isolation and quantification, primer designing, gram staining, plasmid isolation, genomic DNA isolation, polymerase chain reaction, gel electrophoresis, Spectrophotometry, cytological function assay on chromate exposure, IMVic.*

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I. INTRODUCTION

Chromium (Cr) is the most abundant heavy metal in the lithosphere. It belongs to group VI- B transition metals of periodic table. It is also one of the most commonly used heavy metals in various types of metallurgic, tanning, leather, steel , plastic, dyeing, painting, paper and pulp treatment and manufacturing industrial processes due to its properties like anti corrosiveness, ododurlessness, high melting point, hardness etc. There are mainly two stable forms of chromium out of which one is hazardous based upon its chemical properties. The trivalent form (Cr III) of chromium occurs naturally and is essential in lipid, glucose and protein metabolism. It is found in human insulin and sugar crystals as well. On the other hand, the most stable state is the hexavalent form of chromium (Cr VI) which is mainly produced by industrial processes usually bound to oxygen as chromate (CrO42) or dichromate (Cr2O72) with a strong oxidative capacity. [1]

Toxicity: Chromium is considered to be highly toxic and carcinogenic due to its high solubility and ability to cross cell membranes to react with cellular components like proteins and genetic material of the living beings resulting in mutagenesis, genotoxicity and birth defects at developmental stages. Chromium metal can get inside the living beings through aquatic systems, groundwater absorption in plants and consumption in humans. Chromium is structurally similar to sulfate and this is the reason it gets inside the cell at the place of sulfate in cellular sulfate uptake pathway. In the environment, trivalent chromium Cr (III) is generally harmless due to its weak membrane permeability. Hexavalent chromium Cr (VI), on the other hand, is more active in

penetrating the cell membrane through passages for isoelectric and isostructural anions such as SO_4^{2-} and HPO_4^{2-} channels and these chromates are taken up through phagocytosis. [Monisha Jaishankar, Tenzin Tseten, Naresh Anbalagan, Blessy B. Mathew, and Krishnamurthy N. Beeregowda et al ., 2014] [3] It gets converted to several other harmful active intermediates as it undergoes various enzymatic and non enzymatic reductive reactions in the cell. Upon exposure to human skin it can cause serious health problems like dermatitis and ulcerations; if it gets mixed with nearby natural water body, it is a threat to aquatic life forms; it disturbs the food web and also affects the plants' growth and development. Laborers working in paper and pulp processing industries are more susceptible to severe health implications associated with chromium toxicity and in extreme cases the inhalation of chromium ions through vapors generated during various industrial processes result in bronchitis, perforation of nasal septum and increased risk of sinus and lung cancer. [2]

In areas affected and polluted by chromium contamination, there are several resistive measures adopted at molecular level by microorganisms residing and dealing with chromium toxicity. Various studies in the past have discussed and covered the isolation of several chromium tolerant or chromium degrading bacterial strains. Most of the molecular mechanisms involved in degradation of chromium include the processes like detoxification of Reductive Oxygen Species, DNA repair mechanisms, reductive conversion of (Cr VI) to (Cr III) or the extrusion of chromate ions outside of the cell. This research paper mainly focuses on identification of chromium degrading genes with the help of Polymerase Chain Reaction and DNA quantification processes along with biochemical characterization of bacterial isolates.

Chromate Resistance Determinants: Also known as CRDs. [Carlo Viti et al,. 2014] [35] These consist of the CHR super family of genes responsible for the transport of chromate ion. It mainly includes *chrA* gene. The Chromate Resistant Determinants are identified in *Archae, Bacteria* and *Eukarya*. [Rosa Baldiris et al., 2018] [4] ChrA gene codes for the putative chromium efflux transporter driven by membrane potential. [Pimentel et al; 2002] [5] Microbes bearing chrA gene show high variability in chromium tolerance in millimolar ranges only, from 0.35 to 200 millimoles. [Juhnke et al., 2002; Henne et al., 2009b; Monsieurs et al., 2011] [6] Microbes often show slow growth rate with the increased amount of chromium in the growth media due to the co-extrusion of sulfur during chromate efflux. The *chrA1* gene reduces accumulation of chromate. The *chrB* gene is sensitive to the presence of chromium and hence regulates its movement inside the cell. Apart from this, *chrB* gene also play positive regulatory role in *chrA* gene regulation. The Cr-metabolism rate of chrAB engineered strains was much more enhanced than those *chrA* engineered strains. [7] *chr C* gene destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems. [10] *ChrR* gene is a four electron chromium reductase which reduces (CrVI) to (CrIII). *ChrR* can also increase bacterium resistant to chromate, because it reduces chromate efficiently and no single electrons transfer is fired, so the process reduces production of ROS, and it can also increase bacterium resistant to H2O2 (Gonzalez et al., 2005)., [8] This character also protects strains against the toxicity of unavoidable ROS on its growth with chromate. (Xiangkai Li et al., 2013) Due to this particular function, the *chrR* is a dimeric flavoprotein which catalyzes the reduction of (CrVI) optimally at 70 degree Celsius. This gene along with other chromate reductases has gained importance in bioremediation of the chromium. Chromate resistance is related to the presence of chromosomal or plasmid encoded genes. [30] The most common plasmid pMOL28 of *Cuprividus metallidurans* harbors the *chrBAC* genes responsible for chromium resistance [31]. The chromate efflux system is encoded by the *chrA* gene. Thisgene is responsible for chromate resistance in *Shewanella* spp. [32]. The chrA protein belongs to theCHR super family of transporters [33]. The *chrB* gene encodes a membrane bound protein necessaryfor the regulation of chromate resistance. [34] The *ChrC* gene encodes a protein almost similar toiron-containing superoxide dismutase while the *chrE* gene encodes a gene product that is a rhodanesetype enzyme and has been detected in *Orthrobacterium tritici* 5bvI1 [34]; *chrF* most probablyencodes a repressor for chromate-dependent induction [33].

Sample Collection

To collect the sample from the desired industrial surroundings, proper sample collection steps and safety precautions given under *Environmental Protection Agency (EPA*) were followed thoroughly. [11] To locate a paper and pulp manufacturing industry Google search engine was used, the results appeared with the list of the industries with prominent paper manufacturing plants located in different industrial areas of Jaipur, Rajasthan. The hunt for the nearest textile and paper -pulp industry ended up at malviya industrial area in Jaipur, Rajasthan. *ATSDR*'s [9] safety measures and collection methods were followed. Plexiglas tubes made up of borosilicate glass were used to collect the samples from the selected locations. Those samples were stored in cleansed and sterilized plastic bottles. While collecting industrial waste water containing effluents as samples for chromium degradation analysis, proper care was taken to avoid chromium loss and sample contamination while handling, transport, transfer and storage of the sample collected. The collected samples were maintained at 4 degree Celsius while being brought back to the laboratory and then stored in refrigerator at 4 degree Celsius till further analysis. The material of the container used for the collection had been properly acid-washed before

collection and storage of sample in it. Several safety measures were taken including the avoidance of some plastic containers which can have leachable amount of chromium in them and stainless steel utensils since it can increases the level of chromium in the sample collected. Chromium may be present in soluble or insoluble forms in environmental samples and these samples may have trivalent, hexavalent or both forms based upon its oxidation state in the sample collected. Also to prevent the reduction of Cr (VI) to Cr (III) the teflon filters and alkaline solutions were used for filtration process to prevent the reduction of Cr (VI) to Cr (III) in the containers. Containers made up of polyethylene or similar material was used to prevent the adsorption of the sample in the container surface and was maintained at proper pH. A three-step sequential extraction procedure given by ASTM was followed for the analysis of hexavalent chromium in the sample collected. Deionized water was used to dissolve soluble chromium compounds such as potassium chromate, an ammonium sulfate buffer was used for sparingly soluble species, and a sodium carbonate buffer was used to dissolve insoluble chromium complexes such as lead chromate.

II. MATERIALS AND METHODS:

To execute the purpose of this research, DNA isolation (plasmid and genomic), amplification of genes of interest, DNA quantification, gram staining and biochemical tests were performed.

Growth and culture media preparation for bacteria:

Bacteria are found in large multi-species colonies in environmental samples, they live with other bacteria, protozoans, virus and fungi; therefore isolation of bacteria is the basic and crucial step to study the properties of particular bacteria. The principle of isolation lies in dilution that can be achieved either by mechanical dilution of the sample over the agar plates or diluting the inoculum in larger volume. The colony characteristics help in distinguishing between the organisms. [12] The presence of an antibiotic resistant gene on plasmids allows researchers to easily isolate bacteria containing that plasmid from bacteria that do not contain it by artificial selection (i.e. growing the bacteria in the presence of the antibiotic). [13]

Preparation of an overnight liquid bacterial culture and nutrient agar: Luria-Bertani (LB) broth is a nutrient-rich media commonly used to culture bacteria in the lab. LB agar plates are frequently used to isolate individual (clonal) colonies of bacteria carrying a specific plasmid. However, a liquid culture is capable of supporting a higher density of bacteria and is used to grow up sufficient numbers of bacteria necessary to isolate enough plasmid DNA for experimental use. The following protocol is for inoculating an overnight culture of liquid LB with bacteria as well as the Nutrient agar preparation. [14]

Calculations and compositions: (a) To make 300 ml of LB-nutrient broth (NB); weighed the following and added in 500 ml Erlenmeyer flask:

(i) Peptone = 1.25 g (5/1000 x 250 = 1.25 g)
(ii) Beef extract = 0.75 g (3/1000 x 250 = 0.75 g)
(iii) NaCl = 0.75 g (3/1000 x 250 = 0.75 g)

(Ingredients taken per half of 250 ml i.e. 125 ml)
Added 125 ml of dH20. Mixed well. Maintained it at pH= 7.

(b) To make 250 ml of LB-nutrient agar (NA); weighed the following and added in 500 ml Erlenmeyer flask:

(i) Peptone = 1.25 g (5/1000 x 300 = 1.5 g)
(ii) Beef extract = 0.75 g (3/1000 x 300 = 0.9 g)
(iii) NaCl = 0.75 g (3/1000 x 300 = 0.9 g)
(iv) Agar = 4.5 g (15/1000 x 300 = 4.5 g) Add Agar after adding dH2O, maintaining pH=7.Boil.

(Ingredients taken per half of 300 ml i.e. 150 ml)
Added 150 ml of dH20. Mixed well. Maintained it at pH= 7.

Protocol: After measurements, closed the mouths of the flasks by covering with sterilized aluminum foil and by sealing tapes to prevent the spillage of NB and NA. Autoclaved it at 121 degree Celsius under 15 lbs of pressure for 30 minutes. Removed the flasks from the autoclave carefully wearing the heat-protective gloves. Added the appropriate antibiotics in the right concentration carefully in both the nutrient media. [14] The bacteria with antibiotic resistant genes for a particular antibiotic in its plasmid will grow specifically; hence accomplishing isolation of the desired bacteria in the nutrient media. After adding the antibiotics poured the nutrient agar on petri plates in aseptic conditions in Laminar Air Flow hood. To obtain individual colonies, inoculated the agar plates after their solidification with the sample collected by streaking method with an aseptic loop in aseptic conditions under LAF hood after cleaning the floor of the hood with 70 % ethanol. Labeled, named and dated the bottom of the petri plates. Left the growth media to incubate at 37 degree Celsius for 12-18 hours in bacteriological incubator. On the other hand, after adding antibiotic in the nutrient broth, added 50 ml of NB through a dropper to an Erlenmeyer flask under aseptic conditions under the LAF hood. Cleaned the floor of the hood with 70 % ethanol and with the help of a sterile loop, inoculated the broth simply with the sample collected

or with the appropriate bacterial sample from the incubated bacterial colonies grown over nutrient agar plates. Labeled, named and dated it. Placed the nutrient broth in the orbital shaking incubator for incubation process at 37 degree Celsius for 12-18 hours. After incubation, checked for the cloudy bacterial colonies on the nutrient agar plates and the cloudy haze in the nutrient broth; it confirmed the successful growth of the bacterial strains in the culture media with antibiotic.

Preparation of bacterial glycerol stock: Glycerol stocks are important for long-term storage of plasmids. Bacteria on an LB agar plate can be stored at 4°C for a few weeks. However, to store bacteria for a longer time, you will need to establish glycerol stocks. The addition of glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells alive. A glycerol stock of bacteria can be stored stably at -80°C for many years. [13] The bacterial glycerol stocks were formed after inoculating an overnight liquid bacterial culture.

Protocol: To make 50 ml of glycerol stock in a 50 ml Erlenmeyer flask, taken:

(i) Glycerol = 25 ml.

(ii) dH2O = 25 ml.

Mixed it well. The dilution of glycerol with dH2O gave 50 % glycerol stock with the total volume of 50 ml. Autoclaved it at 121 degree Celsius and 15 lbs for 30 minutes. Removed from the autoclave wearing heat protective gloves. Left it to cool. Stored it in 100 ml blue capped borosilicate glass bottle.

(iii) Took 500 µL of 50 % glycerol stock in an autoclaved 2 ml eppendorf tube or a cryovial and added 500 µL of nutrient broth (overnight liquid culture with bacterial growth) to it with the help of a micropipette. Added 500 µL-500 µL of bacterial glycerol stock to two cryovials each. Gently mixed. Freezed the glycerol stock tube at -80 degree Celsius. Labeled, named and dated the bacterial glycerol stock. Glycerol stocks can also be made for the purified plasmids and the same can be stores at -80 degree Celsius. It is preserved for the use in future for years and can be cultured as and when required.

Gram-staining of bacterial colonies grown over LB- nutrient agar plates: The Gram stain is fundamental to the morphological or phenotypic characterization of bacteria. The staining procedure differentiates organisms of the domain bacteria according to cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink bacteria according to cell wall structure. The performance of the gram stain on any sample requires four basic steps that include applying a primary stain (crystal violet) to a heat-fixed smear, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol, acetone, or a mixture of alcohol and acetone and lastly, counterstaining with safranin. [15] Gram-staining analysis of the bacterial colonies enables us to differentiate the bacterial strains after thorough microscopic observations.

Protocol for the gram-staining analysis of the bacterial colonies: Took 5-6 autoclaved and sterilized microscopic slides to heat fix the bacterial inoculum upon it. Marked the center of the slides with a wax pencil. Entitled the slides as *K1*, *K2*, *K3*, *G2* and *G3*. To this marked space, added the drops of dH2O on each slide. Aseptically transferred the well grown bacterial inoculum from the LB-nutrient agar plates with the help of an aseptic loop under the LAF hood. Air dried and heat fixed the bacterial inoculum over the slide with the help of a Bunsen burner keeping the face of the slide with bacterial inoculum up. Followed the general gram staining protocol given by American Society of Microbiology, US. [15]

Air dried the floor, heat-fixed smear of cells for 1 minute with crystal violet staining reagent. Washed slide in a gentle and indirect stream of tap water for 2 seconds. Flood slide with the mordant: Gram's iodine. Waited for 1 minute. Washed the slide in a gentle and indirect stream of tap water for 2 seconds. Flood slide with decolorizing agent. Waited for 15 seconds or added drop by drop to slide until decolorizing agent running from the slide runs clear. Flood slide with counter stain, safranin. Waited for 30 seconds to 1 minute. Washed the slide in a gentile and indirect stream of tap water until no color appeared in the effluent and then blot dried with absorbent paper. Observed the results of the staining procedure under oil immersion using a Bright field microscope. At the completion of the Gram Stain, Gram-negative bacteria stained pink/red and gram-positive bacteria stained blue/purple. The observations were recorded in an observation table in results. [15]

Plasmid isolation: Principle:Plasmids are circular double stranded bacterial DNA. Plasmids specify traits that allow the host to persist in environments that would otherwise be either lethal or restrictive for growth. For example antibiotic resistance and protein expression. Antibiotic resistance genes are often encoded by the plasmid, which allows the bacteria to persist in an antibiotic containing environment, thereby providing the bacterium with a competitive advantage over antibiotic-sensitive species. [16] The plasmid often has the gene of expression which adds to its peculiarity by developing its persistence in the environment. The expression of "chr" genes for chromium degradation aids in the bacterium's tolerance mechanism to survive in toxic conditions. The isolation of plasmid DNA from bacteria is a crucial technique in molecular biology and it is an essential step in many procedures such as cloning, DNA sequencing, transfection, and gene therapy. These

manipulations require the isolation of high purity plasmid DNA. The purified plasmid DNA can be used for immediate use in all molecular biology procedures such as digestion with restriction enzymes, cloning, PCR, transfection, in vitro translation, blotting and sequencing. [16]

General compositions for laboratory stock solutions for plasmid isolation: [17]

(i) **50% Glucose (200 ml):** To be dissolved in 100 g of glucose (dextrose) in 150 ml of distilled water. To be made upto 200 ml in volume and autoclaved.

(ii) **1M Tris-HCl pH 8.0 (500 ml):** To dissolve 60.55 g of Tris in 400 ml of distilled water. pH should be adjusted to 7.5 with concentrated HCl. To be made up to 500 ml as total volume.

(iii) **0.5 M EDTA pH 8.0 (100 ml):** To dissolve 16.8 g of anhydrous disodium salt of EDTA in 70 ml of distilled water. To adjust the pH to 8.0 using NaOH pellets. More EDTA will dissolve as the pH approaches 8.0. To Make up the volume to 100 ml and autoclaved.

(iv) **10% SDS (100 ml) (w/v):** To dissolve 10 g in 100 ml distilled water. To heat gently to get SDS into solution.

(v) **1 N Sodium Hydroxide (100 ml):** To dissolve 4 g in 100 ml distilled water.

(vi) **5M Potassium Acetate (500 ml):** To dissolve 245.5 g in 400 ml distilled water. pH to be adjusted to 5.5 with glacial acetic acid. To make up the volume to 500 ml.

Composition for Resuspension solution (P1):

(i) 1M glucose- 50 ml.

(ii) 1 ml TE buffer- 20 ml. (1 ml Tris HCl + 0.5 M EDTA) (pH=3)

(iii) dH2O- 25 ml.

Composition for Lysis buffer (P2):

(i) 1 N NaOH – 20 ml.

(ii) SDS (1%) – 20 ml.

(iii) dH2O- 25 ml.

Composition for Neutralization solution (P3):

(i) Glacial Acetic Acid- 10 ml.

(ii) 5 M potassium Acetate- 50 ml.

(iii) dH2O- 25 ml.

Calculations and formulae for calculating compositions of chemical compounds for plasmid isolation:

For Resuspension solution (P1):

(i) 1M glucose in total volume of 50 ml.

∵ Molarity = weight x 1000/molecular weight x volume

∴ 1M of glucose = weight x 1000/180 x 50 = 9 g of glucose.

To make total of 50 ml volume; added 41 ml of dH2O to 9 g of glucose.

(ii) 1M Tris HCl in total volume of 20 ml.

∵ Molarity = weight x 1000/molecular weight x volume

∴ 1M of Tris = weight x 1000/121 x 20 = 2.42 g.

To make total volume of 20 ml; added 17.58 ml of dH2O to 2.42 g of Tris HCl.

(iii) 0.5M EDTA in total volume of 20 ml.

∵ Molarity = weight x 1000/molecular weight x volume

∴ 0.5M EDTA = weight x 100/292 x 20 = 2.92 g of EDTA.

To make total volume of 20 ml; added 17.8 ml of dH2o in 2.92 g of EDTA.

For Lysis buffer (P2):

(i) 1N NaOH in total volume of 20 ml.

∵ Molarity = weight x 1000/molecular weight x volume

∴ 1N of NaOH= weight x 1000/40 x 20 = 0.8 g of NaOH.

To make total volume of 20 ml; added 9.2 ml of dH2O to 0.8 g of NaOH.

(ii) 1 % SDS in total volume of 20 ml. (1/100 x 20= 0.2 g)

To make total volume of 20 ml; added 19.8 ml of dH2O in 0.2 g of SDS.

For Neutralizing buffer (P3):

(i) 5M Potassium Acetate in total volume of 50 ml in 10 ml of Glacial Acetic Acid.

(ii) ∵ Molarity = weight x 1000/molecular weight x volume

∴ 5M Potassium Acetate= weight x 1000/ 98.15 x 50 = 24.53 g of Potassium Acetate.

To make total volume of 50 ml; added 25 ml dH2O in 24.53 g of Potassium Acetate.

Principle method for plasmid isolation: Alkaline lysis is a method used in molecular biology, to isolate plasmid DNA or other cell components such as proteins by breaking the cells open. Bacteria containing the

plasmid of interest is first grown, and then allowed to lyse with an alkaline lysis buffer consisting of a detergent sodium dodecyl sulfate (SDS) and a strong base sodium hydroxide. The detergent cleaves the phospholipid bilayer of membrane and the alkali denatures the proteins which are involved in maintaining the structure of the cell membrane. Through a series of steps involving agitation, precipitation, centrifugation, and the removal of supernatant, cellular debris is removed and the plasmid is isolated and purified. [16] Purification of plasmid DNA from bacterial DNA using is based on the differential denaturation of chromosomal and plasmid DNA using alkaline lysis in order to separate the two.

Procedure: The basic steps of plasmid isolation are disruption of the cellular structure to create a lysate, separation of the plasmid from the chromosomal DNA, cell debris and other insoluble material. Bacteria are lysed with a lysis buffer solution containing sodium dodecyl sulfate (SDS) and sodium hydroxide. During this step disruption of most cells is done, chromosomal as well as plasmid DNA are denatured and the resulting lysate is cleared by centrifugation, filtration or magnetic clearing. Subsequent neutralization with potassium acetate allows only the covalently closed plasmid DNA to reanneal and to stay solubilized. Most of the chromosomal DNA and proteins precipitate in a complex formed with potassium and SDS, which is removed by centrifugation. The bacteria is resuspended in a resuspension buffer (50mM Tris-Cl, 10 mM EDTA, 100 µg/ ml RNase A, pH 8.0) and then treated by 1% SDS (w/v) / alkaline lysis buffer (200mM NaOH) to liberate the plasmid DNA from the E. coli host cells. Neutralization buffer (3.0 M potassium acetate, pH 5.0) neutralizes the resulting lysate and creates appropriate conditions for binding of plasmid DNA to the silica membrane column. Precipitated protein, genomic DNA, and cell debris are then pelleted by a centrifugation step and the supernatant is loaded onto a column. Contamination like salts, metabolites, and soluble macromolecular cellular components are removed by simple washing with ethanolic wash buffer (1.0 M NaCl, 50mM MOPS, pH 7.0, isopropanol (v/v) 15 %). Pure plasmid DNA is finally eluted under low ionic strength conditions with slightly alkaline buffer (5 mM Tris / HCl, pH 8.5). [17]

Protocol: Took autoclaved eppendorf tubes and added 4.5 ml of the culture broth from the LB- nutrient broth inoculated with the sample and transfer these to the eppendorf/centrifuge tubes. Marked the tubes as K1 (1.5 ml NB), K2 (1.5 ml NB) and K3 (1.5 ml NB) respectively. Centrifuged these tubes at 10,000 rpm for one minute. Discarded the supernatant and drained the tubes properly on the paper towel. Filled the tubes with more bacterial culture and repeated the previous step. Added the 200 µL of ice-cold resuspension solution (P1) to the tubes as well as 50 µL of lyzozyme. Incubated. Re-suspended repeatedly with the help of a micropipette. Added 400 µL of lysis buffer (P2) to the tubes closed the cap of the tubes and mixed by inversion. Kept at room temperature for five minutes. Added 300 µL of ice cold neutralization buffer (P3) to the tubes and mixed by inversion; incubated the tubes on ice for 10 minutes. Centrifuged the tubes at 10,000 rpm for 5 minutes and transferred the supernatant from centrifuge tubes to the fresh tubes. Discarded the tubes which had pellet/precipitate. Took 600 µL of supernatant and added phenol: chloroform in 1:1. Took supernatant and transferred to another vial. Added phenol: chloroform isoamyl alcohol (PCI) in 25: 24:1. Centrifuged at 3000 rpm for 2 minutes. Took 600 µL of supernatant and mixed with 600 µL of isopropanol in new tubes and maintained at room temperature for 20 minutes. Centrifuged at 13,000 rpm for 15 minutes and discarded the supernatant. Allowed the pellet in the tubes to air dry at room temperature. Added dH2O.Added 50 µL of TE and dissolved the plasmid.

Bacterial genomic DNA isolation: Principle and procedure: The lysis of the bacteria is initiated by re-suspending the bacterial pellet in a buffer containing lyzozyme and EDTA. The EDTA disrupts the outer membrane of the gram-negative envelope by removing the Mg2+ from the lipopolysaccharide layer and additionally inhibits DNases. This allows the lyzozyme access to the peptidoglycan. After partial disruption of the peptidoglycan by the enzyme, a detergent such as SDS is added to lyse the cells. Most gram-negative cells are lysed after this treatment and many can even be lysed without lyzozyme. Once the cells are lysed, the solution should be treated gently to prevent breakage of the DNA strands. Subsequent steps involve the separation of the DNA from other macromolecules in the lysate. Both phenol (that has been equilibrated with Tris buffer) and chloroform (with isoamyl alcohol as a de-foaming agent) are commonly used to dissociate protein from nucleic acids. These reagents also remove lipids and some polysaccharides. Proteolytic enzymes such as pronase or proteinase K are often added to further remove protein. Proteinase K is a particularly useful enzyme; it is not denatured by SDS and in fact works more effectively in the presence of SDS. The nucleic acids (including RNA) may then be precipitated in ice cold ethanol if the ionic strength of the solution is high. This is followed by RNase treatment to degrade the RNA. The solution may then be re-precipitated with ethanol. In this precipitation, the ribonucleotides from RNase treatment will remain in solution leaving purified DNA in the pellet. The pellet can then be dissolved in an appropriate buffer. Alcohol precipitations of DNA and RNA are widely used in molecular biology and are valuable because they allow the nucleic acids to be concentrated by removing them from solution as an insoluble pellet. If concentrations of DNA are relatively high (> 1 µg/ml) DNA can be effectively precipitated in 10-15 minutes by shielding the negative charge with monovalent cations

(0.3 M sodium or 2.5 M ammonium ions are commonly used) followed by the addition of 2 volumes of 95% ethanol. A major consideration in any DNA isolation procedure is the inhibition or inactivation of DNases which can hydrolyze DNA. The buffer in which the cells are suspended should have a high pH (8.0 or greater) which is above the optimum of most DNases. EDTA is also included in the re-suspension buffer to chelate divalent cations (such as $Mg2+$) which are required by DNases. The SDS also reduces DNase activity by denaturing these enzymes. DNase activity is further controlled by keeping cells and reagents cold, using proteolytic enzymes such as pronase or proteinase K, and a heating step that will thermally denature DNase (but not hot enough to denature the DNA). The procedure used here is useful for isolating DNA from a large variety of gram negative bacteria. It yields partially purified DNA of sufficient quality for most techniques, such as restriction digestion, ligation and cloning. Further purification by additional solvent extraction may be required for experiments needing purer DNA (example – physical and chemical studies, such as melting curves). [17]
General compositions of stock solutions for bacterial genomic DNA isolation: [17]

(i) **10% SDS (100 ml):** To dissolve 10 g SDS in 100 ml of distilled water. To heat gently to get SDS into solution.
(ii) **Proteinase K (20 mg/ml):** To dissolve 20 mg proteinase K in 1 ml of distilled water.
(iii) **Phenol-Chloroform (1:1):** To mix 50 ml of buffered phenol with 50 ml of chloroform.
(iv) **3 M Sodium Acetate pH 5.2 (500 ml):** To dissolve 123 g in 450 ml of distilled water. To adjust the pH to 5.2 with glacial acetic acid. To make the volume up to 500 ml.

Calculations for genomic DNA isolation:

(i) 1M Tris base-EDTA buffer in total volume of 25 ml.
∵ Molarity = weight x 1000/molecular weight x volume
∴ 1M of Tris-HCl = weight x 1000/121 x 25 = 3.025 g.
To make total volume of 25 ml; added 21.975 ml of dH_2O to 3.025 g of Tris HCl. Taken 400 µL of TE buffer for DNA isolation.
(ii) 0.5M EDTA in total volume of 20 ml.
∵ Molarity = weight x 1000/molecular weight x volume
∴ 0.5M EDTA = weight x 100/292 x 25 = 3.65 g of EDTA.
To make total volume of 20 ml; added 21.35 ml of dH_2o in 3.65 g of EDTA.
(iii) Phenol:Chloroform:Isoamyl alcohol (25: 24: 1)
∵Formula= (Ratio to be taken/Total x Volume in ml)
∴Phenol = 25/50 x 4 = 2 ml
∴Chloroform = 24/50 x 4 = 1.92 ml
∴Isoamyl alcohol = 1/50 x 4 = 0.08 ml
Taken 4 ml of the total PCI composition made.
AND
Stepwise formulation: (a) Phenol: Chloroform (1:1)
∵Formula= (Ratio to be taken/Total x Volume in ml)
∴Phenol= ½ x 2 = 1 ml; Chloroform= ½ x 2 = 1 ml. Mix both to get total volume of 2 ml P: C.
(b) Chloroform: Isoamyl alcohol (1:1)
∵Formula= (Ratio to be taken/Total x Volume in ml)
∴Chloroform: ½ x 2 = 1 ml; Isoamyl alcohol = ½ x 2 = 1 ml. Mixed both to get total volume of 2 ml C: IA.
(c) Phenol: CIA (24:1)
∵Formula= (Ratio to be taken/Total x Volume in ml)
∴Phenol= 24/25 x 4= 3.84 ml; CIA= 1/25 x 4= 0.16 ml. Mixed both to get total volume of 4 ml P: CIA.
(iv) 10% SDS in 2 ml of dH_2O. Taken 250 µL of it for DNA isolation.
10% SDS = 10/100 x 2= 0.2 g
Added 1.8 ml of dH_2O to 0.2 g of SDS.
(v) 500 µL of 70% ethanol has to be taken.
(vi) Took 10 mg in 1 ml dH_2O. Added 50 µL of it for cell lysis.

Protocol: Grown cells overnight in nutrient rich broth (LB broth). Transferred 4.5 ml of culture to a micro-centrifuge tube and centrifuged at 10,000 rpm for 2 minutes. Collected the pellet and repeated with another 1.5 ml of culture containing cells. Drained the tubes on a paper towel briefly. Re-suspended the pellet in 450 µL of TE buffer. Added 45 µL of 10% SDS and 5 µL of 20 mg/ml proteinase K mixed and incubated for 1 hour at 37°C. Added 500 µL phenol-chloroform and mixed well by inverting the tubes until the phases are completely

mixed. Centrifuged the mixture at 10,000 rpm in a microfuge for 2 minutes. Transferred the upper aqueous phase to a new tube and re-extracted by adding an equal volume (about 500 µL) of phenol-chloroform. Again centrifuged the mixture at 10,000 rpm in a microfuge for 5 minutes. Transferred the upper aqueous phase to a new tube. Added 50 µL of sodium acetate and mix. Added 300 µL of isopropanol and mix gently to precipitate the DNA. DNA can then be isolated by spooling out or centrifuge. [17]

Polymerase Chain Reaction (PCR) of the isolated bacterial DNA

General method: PCR is carried out in a test tube with the DNA template, primers specific for the region that is desired, DNA polymerase, and reagents that stabilize the reaction. Once the reaction is put together, it will go into a thermocycler (PCR machine) that will create the conditions for DNA replication to occur. Each round of PCR requires three steps, denaturation, annealing, and elongation, each of which doubles the amount of DNA template present in the reaction. By repeating this process multiple times, usually 30, this will amplify the DNA exponentially. [18] The choice of the target DNA is, of course, dictated by the specific experiment. However, one thing is common to all substrate DNAs and that is they must be as clean as possible and uncontaminated with other DNAs. Naturally, if the source material is an environmental sample such as water or soil, then the researcher must rely upon the specificity of the PCR primers to avoid amplification of the wrong sequence.

Specificity in the choice of PCR primers can be an issue in any PCR amplification. The on-line IDT SciTools software OligoAnalyzer 3.0 and PrimerQuest are invaluable aids both in primer design and validation. PrimerQuest will assist in primer design and will permit the researcher to directly assess primer specificity via a direct BLAST search of the candidate sequences. Taking candidate primer sequences into OligoAnalyzer will allow for each primer sequence to be assessed for the presence of secondary structures whether these are hairpins or homo- and hetero-dimers. [19] [20] [21] [22]

Protocol: PCR kits are available for amplification of isolated DNA.

A general reaction setup and compositions of PCR are added below for laboratory composition.

Component	**25 µL reaction**	**50 µL reaction**	**Final Concentration**
10X Standard *Taq* Reaction Buffer	2.5 µL	5 µL	1X
10 mM dNTPs	0.5 µl	1 µL	200 µM
10 µM Forward Primer	0.5 µl	1 µL	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	1 µL	0.2 µM (0.05–1 µM)
Template DNA	variable	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl	

Table 1.1.Protocol for Polymerase Chain Reaction taken from BioLabs Inc, New England international website. [24]

General compositions:

Template:

DNA(template)	**Amount**
Genomic	1 ng–1 µg
plasmid or viral	1 pg–1 ng

Table 1.2.Protocol for Polymerase Chain Reaction taken from BioLabs Inc, New England international website. [24]

Guidelines to be followed for PCR:

Primers: Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer program BLAST was used to design and analyze primers. The final concentration of each primer in a reaction may be 0.05–1 µM, typically 0.1–0.5 µM. [24]

Mg^{++} and additives: Mg^{++} concentration of 1.5–2.0 mM is optimal for most PCR products generated with *Taq* DNA polymerase. The final Mg^{++} concentration in 1X Standard *Taq* Reaction Buffer is 1.5 mM. This supports satisfactory amplification of most amplicons. However, Mg^{++} was further optimized in 0.5 or 1.0 mM increments using $MgCl_2$. Amplification of some difficult targets, like GC-rich sequences, was improved with additives, such as DMSO or form amide. [24]

Deoxynucleotide: The final concentration of dNTPs is typically 200 µM of each deoxynucleotide.

Taq DNA polymerase Concentration: Used *Taq* DNA polymerase at a concentration of 25 units/ml (1.25 units/50 µL reaction). However, the optimal concentration of *Taq* DNA polymerase may range from 5–50 units/ml (0.25–2.5 units/50 µL reaction) in specialized applications. [24]

Thermo cycling conditions for PCR:

STEP	**TEMP**	**TIME**
Initial Denaturation	95°C	30 seconds
30 Cycles	95°C 45-68°C 68°C	15-30 seconds 15-60 seconds 1 minute/kb
Final Extension	68°C	5 minutes
Hold	4-10°C	

Table 1.3.Protocol for Polymerase Chain Reaction taken from BioLabs Inc, New England international website. [24]

Designing of primers: (i)Nucleotide sequence for *chr A* gene in *Bacillus sp. SJI:*

5'ATGAATACATGGCTTGTTCTTGTTATCAGCTTAGCTTACCCACTGAAAATGC……
……..
AGTGCATTACCTCGATGTCGGATGTCGGCGGCGGTGTACAACCGCCTGAATCGGTCGGCATTGAAAGCG TA 3'
Forward primer: *5' ATGAATACATGGCTTGTTC 3'*
Reverse primer: *5' TACGCTTTCAATGCCGACCG 3'*

(ii)Nucleotide sequence for *chr B* gene in *Alishwanella sp. WH-16-1:*

5'AATGGATGGATGAAAAAAAGTTATGCGGATTTAGTTGCGTTATGTGAGTTTTTACCTGGCCCTGCTAGTA GTCA……GCGGATAACGATGAAGCGAAACTAAGTTTCCCAATCTCCAGAAA GTTAGGAGTAATTTGTTTATCACTATTTTTTGGATACTTATTTCCCTTCCAATTCTAAGAGAGGTTACATCGT TAAATTGGATTGCAATGTTNGATAGT 3'
Forward primer: *5' TTAGTTGCGTTATGTGAG 3'*
Reverse primer: *5' GATGTAACCTCTCTTAG 3'*

(iii)Universal primers nucleotide sequence for 16s rRNA:
Forward primer: *5' AGAGTTTGATCCTGGCTCAG 3'*
Reverse primer*: 5' CCGAATTCGTCGACAAC 3'*

Protocol:
For known strain: PCR for *B.cereus, E.coli K12, B. subtilis* and genomic DNA (unknown strain) in 20 µl PCR vials with 1-10 µl of buffer, 7.5 µl of dNTPs, 5 µl of primer, 3 µl DNA ladder and 2.5 µl of *Taq* DNA polymerase.
For the unknown isolated DNA samples (K1, K2, K3, G1 and G2): To run the PCR, five PCR vials of 20 µl capacity each were taken. 14 µl of each DNA sample as template DNA was added. To this 2 µl of 10 x Taq buffer, 1 µl-1 µl of each primer (forward – backward) of each DNA sample was added along with 1 µl of dNTPs and 0.5 µl of Taq polymerase. For nucleotide sequence of gene of interest; Computer based BLAST application was used to generate nucleotide sequence for *chrA* and *chrB* genes along with pubMed application and to design forward and reverse primers against desired nucleotide sequence in DNA template, In-silico PCR was used to check the feasibility of the designed primer against the DNA strand through the long sequence study of the particular strain. Points considered while designing a primer against the gene of interest: Primer with length of 18-24 bases, 40-60% G/C content which started and ended with 1-2 G/C pairs, melting temperature (Tm) of 50-60°C, primer pairs had a Tm within 5°C of each other and primer pairs had complementary regions.

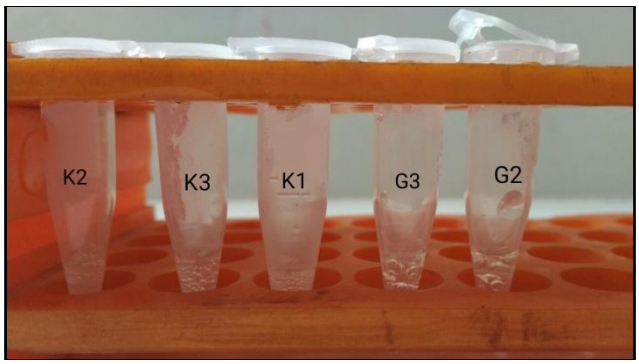

Fig1.1.The vials contain the amplified PCR products.

After getting amplified DNA sample for the gene of interest in the PCR vials, the results of PCR were subjected to undergo Gel Electrophoresis for DNA quantification and separation.

Gel Electrophoresis: Because nucleic acids are negatively charged ions at neutral or alkaline pH in an aqueous environment, they can be moved by an electric field. Itis a technique used to separate charged molecules on the basis of size and charge. The nucleic acids can be separated as whole chromosomes or as fragments. The nucleic acids are loaded into a slot at one end of a gel matrix, an electric current is applied, and negatively charged molecules are pulled toward the opposite end of the gel (the end with the positive electrode). Smaller molecules move through the pores in the gel faster than larger molecules; this difference in the rate of migration separates the fragments on the basis of size. The nucleic acids in a gel matrix are invisible until they are stained with a compound that allows them to be seen, such as a dye. Distinct fragments of nucleic acids appear as bands at specific distances from the top of the gel (the negative electrode end) that are based on their size. A mixture of many fragments of varying sizes appear as a long smear, whereas uncut genomic DNA is usually too large to run through the gel and forms a single large band at the top of the gel. [25]
Protocol: A gel electrophoresis kit has been used to run the genomic DNA sample along with other known and unknown bacterial strains. To make 1 % of agarose gel, 1 g of agarose was weighed and this agarose powder was added in 100 ml 1x TAE buffer. Microwaved this for 2-3 minutes. Left the agarose gel to cool to 40-50 degree Celsius. Added ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µL of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light. [26] Poured the agarose in the gel tray with well comb in place. Placed newly poured gel at 4 °C for 10-15 mins until it was completely solidified. Added loading buffer to each of the DNA samples. On solidification, placed the agarose gel into the gel box. Filled the gel box with 1xTAE until the gel was covered. Carefully loaded DNA samples into the additional wells of the gel. In first well, DNA ladder was added, in second well DNA sample of *Bacillus cereus* was added, in third well, DNA sample of *E.coli K12* was added, in fourth well genomic DNA was added and in fifth well DNA sample of *Bacillus subtilis* was added. Made to run the gel at 80-150 V until the dye line was approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage. Turned OFF the power, disconnected the electrodes from the power source, and then carefully removed the gel from the gel box. Using any device that has UV light, visualized DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel. To analyze gel, used the DNA ladder in the first lane to infer the size of the DNA in sample lanes. To conduct certain procedures, such as molecular cloning, DNA is required to be purified away from the agarose gel through DNA extraction kit. [26]

Fig.1.2. Gel Electrophoresis set up to quantify amplified DNA isolated from the sample collected.

The separation of DNA strands on agarose gel slab was visualized under UV light and the image with separated and glowing DNA bands is shown in the result section below.

Spectrophotometry: Another technique for DNA quantification is spectrophtometry. In this process, an instrument employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector. A spectrophotometer consists of two instruments; a spectrometerfor producing light of any selected color (wavelength); a photometer for measuring the intensity of light. It operates on Beer's law. For absorbance at 260 nm**:** Nucleic acids absorb UV light at 260 nm due to the aromatic base moietieswithin their structure. Purines (thymine, cytosine and uracil) and pyrimidines (Adenine and guanine) both have peak absorbances at 260 nm, thus making it the standard for quantitating nucleic acid samples. For absorbance at 280 nm: The 280 nm absorbance is measured where proteins and phenolic compounds have a strong absorbance. Similarly, the aromaticity of phenol groups of organic compounds absorbs strongly near 280 nm. Absorbance at 230 nm: Many organic compounds have strong absorbances at around 225 nm. In addition to phenol, TRIzol, and chaotropic salts, the peptide bonds in proteins absorb light between 200 and 230 nm. The A260/280ratio shows the purity of the sample analyzed. Pure DNA sample gives a 260:280 ratio of 1.8 and for pure RNA the ratio is 2. A260/230 ratio indicates the presence of organic contaminants, such as TRIzol, chaotropic salts and other aromatic compounds. Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants that will interfere with downstream applications. If the sample is contaminated with proteins or other organic compounds the ratios will vary from the above mentioned values. [27]

Compositions and calculations: Taken 10 µL of isolated DNA and added 990 µL of dH2O in it to make a total volume of 1000 µL that is 1 ml. Added this mixture in cuvette of 2 ml capacity. Since we have formula of "DNA concentration (µg/mL) = A260 x dilution factor x 50" and Dilution Factor is inversely proportional to dilution. Therefore, dilution factor is equal to 1/conc. of DNA sample/ total volume= 1000 µL/10 µL= 100 µL. Formula for "DNA concentration (µg/mL) = A260 x dilution factor x 50" and for nucleic acid purity is = A260/A280.
Protocol: Used dH2O or 1X TE as a solvent to suspend the nucleic acids, and placed each sample in a quartz cuvette. Zeroed the spectrophotometer with a sample of solvent. For more accurate readings of the nucleic acid sample of interest, diluted the sample to give readings between 0.1 and 1.0. For a 1-cm pathlength, the optical density at 260 nm (OD260) equals 1.0 for the following solutions: (1) a 50 µg/mL solution of dsDNA, (2) a 33 µg/mL solution of ssDNA, (3) a 20-30 µg/mL solution of oligonucleotide, (4) a 40 µg/mL solution of RNA. Contamination of nucleic acid solutions makes spectrophotometric quantification inaccurate. Calculated the OD260/OD280 ratio for an indication of nucleic acid purity. Pure DNA has an OD260/OD280 ratio of ~1.8; pure RNA has an OD260/OD280 ratio of ~2.0. Low ratios could be caused by protein or phenol contamination. [28]
Observation table for UV Spectrophotometric readings with DNA concentration, dilution factor and purity of the sample is given in the results.

Microbiological/Biochemical characterization:
IMViC Test: These series of microbiological tests are performed to differentiate Enterobacteria family. To obtain the results of these tests test tubes are inoculated with bacterial samples which are to be analyzed. Cultures of any members of enterobacteriaceae have to grow for 24 to 48 hours at 37°C and the respective tests can be performed:
Indole test: In Indole test, tryptone broth is used as media.
Tryptone broth composition in total volume of 100 ml:
(i) Peptone/Tryptone = 10/1000 x 100= 1 g
(ii) NaCl= 5/1000 x 100= 0.5 g
(iii) CaCl2= 1/1000 x 100 = 0.1 g
(iv) dH2O= 1000 ml.
(v) pH= 7.
Kovac's reagent is used as indicator.
Interpretation: After incubation, turbidity show positive test and red layer is formed over surface after addition of Kovac's reagent. Kovac's reagent reacts with Indole producing red colour and Indole is produced from tryptophan by the action of tryptophanase. Hence, red colour indicates the presence of tryptophanase producing bacteria.
MR-VP test: In both tests, MR-VP broth is used as media.
MR-VP broth composition in total volume of 100 ml:
(i) Peptone= 7/1000 x 100= 0.7 g.

(ii) Dextrose= 5/1000 x 100=0.5 g.
(iii) K2HPO4= 5/1000 x 100= 0.5 g.
(iv) dH2O= 1000 ml.
(v) pH= 6.9.
Methyl red as indicator for MR test and Barritt's reagent I and II as indicator for VP test.
Interpretation: MR test: It differentiates facultative enteric bacteria. These bacteria produce Formic acid. The addition of methyl red indicator show positive test if a red layer is formed and a negative test if a yellow layer is formed. VR test: To test acidic or neutral end product. (Acetyl methyl carbinol). Barritt's reagent I and II are used as an indicator. Rose pink colour shows a positive test and no colour shows the negative test.
Citrate test: In citrate test, Simon Citrate agar media is used.
Simon Citrate agar composition in total volume of 100 ml:
(i) (NH4)H2PO4= 1/1000 x 100= 0.1 g.
(ii) K2HPO4= 1/100 x 100= 0.1 g.
(iii) NaCl= 5/1000 x 100= 0.5 g.
(iv) Na3C6H5O7=2/1000 x 100= 0.2 g.
(v) MgSO4= 2/1000 x 100= 0.02 g.
(vi) Agar 15/1000 x 100= 15 g.
(vii) dH2O= 100 ml.
(viii) Bromothymol Blue= 0.08 g. (used as indicator)
Interpretation: The change in color from green to blue on slant slide indicates the presence of citrase producing bacteria which turns citrate to oxaloacetic acid, pyruvic acid and CO2.

The tabular form of observations for IMViC tests is given in the results section.

The cytological function assay of chromate exposure: The cytologically modifying effects of chromium in bacterial cells were investigated using a series of experiments by taking chromium (as K2Cr2O4) in different concentrations and adding it in vitro to bacterial cultures grown and isolated from the sample collected from various industrial locations. The different concentrations of K2Cr2O7 were introduced on bacterial cultures by growing them on phosphate buffer in nutrient agar and treating the centrifuged pellets with potassium phosphate buffer.
Potassium phosphate buffer saline (PBS) preparation in nutrient agar:
PBS composition in total volume of 300 ml
(i) NaCl= 2.4 g
(ii) KCl = .06 g
(iii) Na2HPO4= 0.426 g
(iv) KH2PO4= 0.072 g
(v) Agar= 4.5 g
Nutrient agar composition in total volume of 300 ml:
(i) Peptone= 1.5 g
(ii) Beef Extract = 0.9 g
(iii) Agar= 4.5 g
(iv) NaCl= 0.9 g

Potassium dichromate composition in PBS:
For 0.1 % K2Cr2O7, dH2O= 45 ml; K2Cr2O7= 5 ml.
For 0.2 % K2Cr2O7, dH2O= 40 ml; K2Cr2O7= 10 ml.
For 0.5 % K2Cr2O7, dH2O=25 ml; K2Cr2O7= 25ml.
Protocol: (i) Inoculated and grown the bacterial culture in PBS nutrient agar and added different concentrations of K2Cr2O7 (0.1%, 0.3% and 0.5%) to it and observed its growth.
Took 50 ml overnight broth. Centrifuged at 4000 rpm for 10 minutes at 4 degree Celsius. Washed pellets two times with 1 ml 100 mM potassium phosphate buffer, spiked with 0.1 % of K2Cr2O7 to 1 ml.Vortex or 2 minutes. Incubated at 30 degree Celsius for six hours at 4000 rpm for 5 minutes. Add 0.1 g DPC.
The **inferences** are discussed in the result section below.

III. RESULT AND DISCUSSION

(A)(i)**The gram-staining results and microscopic observations of the bacterial colonies grown over the incubated LB-nutrient agar plates inoculated with the sample collected from the industry are as follows**:

Slide Title	Gram Positive/Negative	Shape of the bacteria
K1	Positive	Bacillus
K2	Positive	Cocci
K3	Positive	Bacillus
G2	Positive	Bacillus
G3	Positive	Bacillus

Table 1.4, Gram staining results based upon microbiological tests and microscopic observations.

(ii)**Microscopic images of the bacterial strains as observed after gram-stain analysis.**

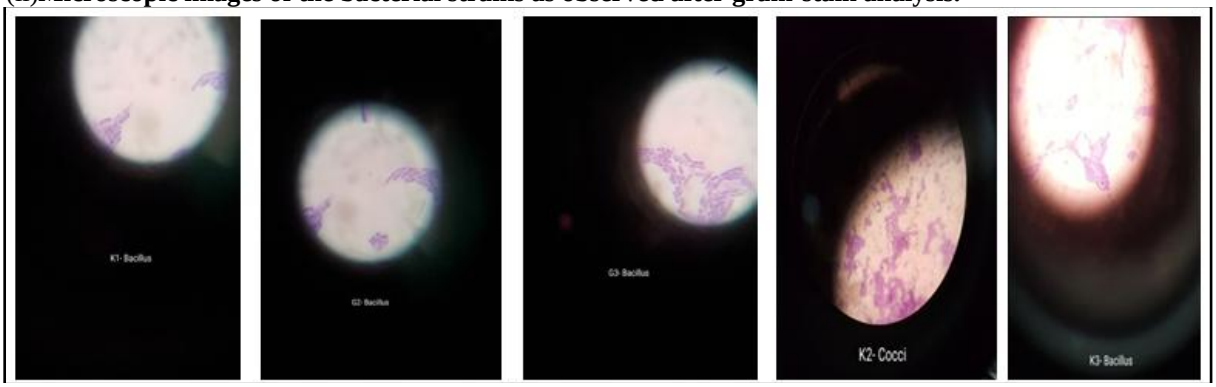

Fig.1.3.*K1*Fig.1.4.*G2*Fig.1.5.*K1*Fig.1.6.*K2*Fig.1.7.*K3*

(B) **Image given below was clicked through the UV transilluminator showing DNA quantification process of gel electrophoresis separated differently sized DNA bands on agarose gel when electric field was applied.**

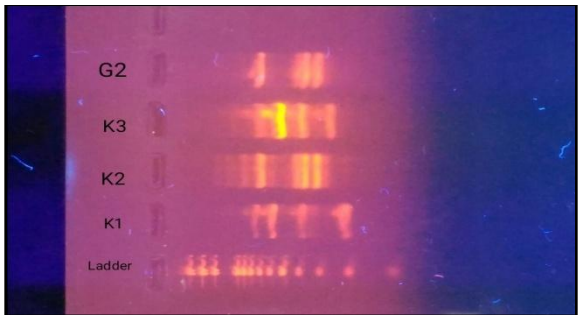

Fig. 1.8.Fluoroscent DNA bands on agarose gel slab visible through UV transilluminator.

(C) **The spectrophotometric readings for bacterial strains:**

Strain	A260 nm	A280 nm
K2	-0.082	-0.087
K3	-0.087	-0.129
G2	-0.036	-0.035

Table 1.5. The results for UV Spectrophotometry as recorded while performing quantification of DNA.

Putting these values in formula: "**DNA concentration (µg/mL) = A260 x dilution factor x 50**"
K2= -0.082 x 100 x 50 = -410
K3= -0.087 x 100 x 50= -435
G2= -0.036 x 100 x 50= -180
Since, **Purity= A260/A280**, therefore, K2= 0.082/0.087=0.942; K3= 0.087/0.129= 0.6744;
G2= 0.036/0.035=1.02851

(C) **The cytological function assay on chromate exposure: Biochemical characterization.**

The growth of chromium degrading bacterial strains was higher on PBS nutrient agar with potassium dichromate concentration confirming the presence of chromium tolerance and degradation property of bacterial culture grown from the sample collected from chromate releasing industries.

(D) **IMViC test results for microbiological characterization of bacterial samples are given below:**

Strain	MR test	VP test	Citrate test	Indole test
K1	Positive	Negative	Positive	Negative
K2	Positive	Negative	Positive	Negative
K3	Negative	Positive	Positive	Negative
G2	Negative	Positive	Positive	Negative
G3	Positive	Positive	Positive	Negative

Table 1.6. The results for Biochemical tests as recorded while performing IMViC test.

IV. CONCLUSION

The sample collected from the areas nearby paper and pulp industry where the wastewater effluents from the industries settle, contain numerous self defensive chromium degrading bacteria which have developed molecular mechanism of chromium degradation over time. To execute the idea of this research work, the successful DNA amplification process for template DNA or the gene of interest was achieved through the feasibility of the primers designed against BLAST nucleotide sequence results for *chr A* and *chr B* genes. The quantification of amplified DNA was done through gel electrophoresis and purity was analyzed through spectrophotometry. Also, various biochemical/microbiological characterization tests were performed to analyze properties of isolated bacteria. The gram staining results inferred all the bacterial samples to be gram positive; the IMViC test series had great variability in MR-VP tests for different strains but citrate and indole tests shown similar results among strain variety. Most importantly for the degradation of chromium, as we know that there are several genes involved in the process which makes the bacteria tolerant to toxic chromate ions present in the industrial discharge and hence increase their survival chances; The *"chr"* gene family which includes *chr A, chr B* genes play the most important role being the chromium resistant determinants. As a result, the chromium degrading bacteria have adopted various cytological and molecular mechanisms to fight the toxicity of bio-hazardous heavy metals like chromium. Microbes bearing *chr A* gene show high variability in chromium tolerance because *chr A* gene codes for the putative chromium efflux transporter driven by membrane potential and *chr B* gene in turn plays a positive regulatory role for *chr A* gene by coding for membrane bound protein necessary for chromate resistance. The chromate efflux, DNA repair, conversion of Cr (VI) to Cr (III) and the central metabolism are amongst major cytological processes which develop tolerance to chromium in microbes. *Pseudomonas CRB5, Brucella sp, Alishwanella sp, Bacillus sp.* are among those bacterial strains which have chromium degrading properties within them; the success of the designed primers against nucleotide sequence of *chr A* gene of *Bacillus sp. SJI* for PCR supports the former statement. The isolation of chromium degrading bacteria and the maintenance of their culture media to study their genetic makeup by applying various qualitative and quantitative analytical methods of molecular biology are useful in bioremediation processes and form the basis for developing preventive tools for environmental degradation. The chromium degrading property of peculiar microbes was confirmed by this research work by growing bacterial samples over potassium phosphate nutrient agar and treating the pellets isolated from nutrient broth culture with different concentrations of K2Cr2O7 (as chromium source) to carry out the **chromate reduction assay on K2Cr2O7 exposure** on isolated bacterial strains, the results show the notable growth of bacteria at different concentrations proving the presence of chromium tolerance and degradation capacity within their genetic makeup.

FUTURE WORK PLAN

The nanoparticles involved in the heavy metal ion degradation is beyond the theme of this research paper but holds an immense scope for research and development work. Recently, there had been a lot of research going on about the use of the highly recyclable nanoparticles for the wastewater treatment of industrial effluents containing heavy metal ions. The study of catalytic property of nanoparticles like (Pb, ZnO2 and Ti) could serve to be the area of intense future research emphasizing on their recyclable property.

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