

Isolation of Microbes from Polluted Soil and Water Samples to Evaluate the Bioremediation Activity on Chromium

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ABSTRACT: Microorganisms and plants are usually used for the removal of heavy metals. The process of involvement of microorganisms to reduce pollutant concentration is known as bioremediation which is a natural process and its importance of biodiversity is increasingly considered for clean-up of metal contaminated and polluted ecosystem. A variety of microorganisms have been known for their ability to degrade these heavy metals. The current study aimed to isolate chromium-reducing microorganisms from industrial effluents and polluted soil. Nine organisms having chromium-reducing activity were isolated and screened for metal reduction activity. Among the isolates, one isolate (CRRB-1) was found to have very high metal-reducing activity. Chromium metal-reducing activity was studied using the Diphenyl Carbazide method in a visible spectrophotometer at a wavelength of 520 nm. The CRRB-1 isolate was found to reduce 92.5% chromium in aqueous solution in a short time of 12 hours and hence the organism was found to have very high chromium-reducing activity. Thus, our results show that the use of the isolated bacteria is useful in the bioremediation of Chromium heavy metal from the contaminated environment. Preliminary techniques confirm that the bacteria were found to be gram-positive cocci bacteria.

Keywords: Heavy Metals, Spectrophotometry, Transmittance, Standard Calibration Curve, Monochromator

I. INTRODUCTION

Ultraviolet-visible spectroscopy or **ultraviolet-visible spectrophotometry** (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared [NIR]) ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, atoms and molecules undergo electronic transitions. Absorption spectroscopy is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

Ultraviolet-visible spectrophotometer

The instrument used in ultraviolet-visible spectroscopy is called a UV/Vis **spectrophotometer**. It measures the intensity of light passing through a sample, and compares it to the intensity of light before it passes through the sample. The ratio is called the *transmittance*, and is usually expressed as a percentage (%T). The absorbance, is based on the transmittance:

The UV-visible spectrophotometer can also be configured to measure reflectance. In this case, the spectrophotometer measures the intensity of light reflected from a sample and compares it to the intensity of light reflected from a reference material (such as a white tile). The ratio is called the *reflectance*, and is usually expressed as a percentage (%R).

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Vol. 7, Issue 9, September 2018

The basic parts of a spectrophotometer are a light source, a holder for the sample, a diffraction grating in a monochromator or a prism to separate the different wavelengths of light, and a detector. The radiation source is often a Tungsten filament (300-2500 nm), a deuterium arc lamp, which is continuous over the ultraviolet region (190-400 nm), Xenon arc lamp, which is continuous from 160-2,000 nm; or more recently, light emitting diodes (LED)^[1] for the visible wavelengths. The detector is typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). Single photodiode detectors and photomultiplier tubes are used with scanning monochromators, which filter the light so that only light of a single wavelength reaches the detector at one time. The scanning monochromator moves the diffraction grating to "step-through" each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different pixels or groups of pixels simultaneously.

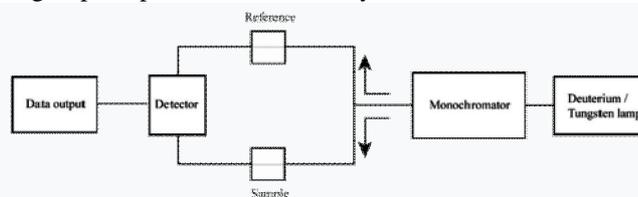


Figure Simplified schematic of a double beam UV- visible spectrophotometer.

A spectrophotometer can be either *single beam* or *double beam*. In a single beam instrument (such as the Spectronic 20), all of the light passes through the sample cell. Must be measured by removing the sample. This was the earliest design and is still in common use in both teaching and industrial labs.

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample. The reference beam intensity is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities. Some double-beam instruments have two detectors (photodiodes), and the sample and reference beam are measured at the same time. In other instruments, the two beams pass through a beam chopper, which blocks one beam at a time. The detector alternates between measuring the sample beam and the reference beam in synchronism with the chopper. There may also be one or more dark intervals in the chopper cycle. In this case, the measured beam intensities may be corrected by subtracting the intensity measured in the dark interval before the ratio is taken.

Samples for UV/Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Samples are typically placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1 cm. (This width becomes the path length, in the Beer-Lambert law.) Test tubes can also be used as cuvettes in some instruments. The type of sample container used must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths.

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(A High Impact Factor, Monthly, Peer Reviewed Journal)

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II. MATERIALS AND METHODS

List of Instrumentation:

S.NO	INSTRUMENT/ APPARATUS	SPECIFICATIONS
1	Spectrophotometer	Double Beam UV visible spectrophotometer UV 2301
2	Software	Hitachi UV solutions version 2.0
3	Cuvetts	Quartz
4	Analytical balance	Denver electronic analytical balance (SI-234)
5	Incubator	Thermo Scientific Heratherm Compact Microbiological Incubators
6	Hot air oven	Bionics BST/HAO-1124 model hot air oven
7	Laminar air flow	Bionics BST/HAO-1124 HEPA Filter laminar air flow
8	Orbital Shaker	BTI
9	Centrifuge	BMI

Table : List of instruments/apparatus used

List of chemicals:

S. No	Chemicals	Grade	Make
1	Potassium dichromate	LR	Merck Chemicals, Mumbai
2	Diphenyl carbazide	SQ	Fisher sci. Mumbai.
3	Peptone	Bacteriological	Himedia Laboratories, Mumbai.
4	Beef extract	Bacteriological	SDFCL, Mumbai.
5	Yeast extract	Microbiology	MERCK Specialities
6	Agar	Bacteriological	Fisher sci. Mumbai.
7	Sodium chloride	LR	SDFCL, Mumbai.
8	Sulphuric acid	LR	Fisher sci. Mumbai.
9	Water	---	Fisher sci. Mumbai.
10	Crystal Violet	LR	Fine chem. industries, Mumbai
11	Grams Iodine	LR	Fine chem. industries, Mumbai
12	Ethyl Alcohol	LR	Merck Chemicals, Mumbai
13	Safranin	LR	Fine chem. industries, Mumbai

Table 4.2: List of chemicals used

Collection of samples:

Chromium reducing bacteria were isolated from soil and water samples collected from polluted areas. One soil sample and one water sample were collected in auto nagar, Guntur, Andhra Pradesh and one soil sample collected in auto nagar, Vijayawada, Andhra Pradesh where pollution was high. These soil samples were suspended in water by vigorous vortexing, serial dilutions were made up to 10^{-6} and 10^{-7} in sterile water. Water samples were directly used for isolation of bacteria. Nutrient agar medium was used for isolation of bacteria.

Nutrient agar medium composition:

S No	Composition	Quantity
1	Peptone	0.5%
2	beef extract	0.3%
3	Sodium Chloride	0.5%
4	Agar	1.5%
5	distilled water	1000mL
6	pH	6.8
7	Temperature	25 °C

Table 4.3: Composition of Nutrient agar medium

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III. PREPARATION OF SOLUTIONS

Standard potassium dichromate solution:

282.87mg of potassium dichromate was weighed accurately and was dissolved in 100ml water, resulting a standard Cr stock solution concentration of 1000 μ g/ml was obtained. From the standard 1000 μ g/ml chromium stock solution, working standard solution containing 100 μ g/ml chromium solution was prepared by diluting in the ratio of 1:9 (v/v). From working the standard 100 μ g/ml chromium stock solution, working standard solution containing 10 μ g/ml chromium solution was prepared by diluting in the ratio of 2:8 (v/v).

Di phenyl carbazide solution:

Diphenylcarbazine solution was widely used for estimating the chromium level by spectrophotometer. 0.10g of di phenyl carbazine was weighed accurately and was dissolved in 25ml methanol and 75 ml of H₂O. Sonicate the content well till reagent dissolves completely in methanol.

0.1N Hydrochloric Acid solution:

8.6ml of concentrated HCl was measured accurately and was make up to 1000ml with distilled water.

Isolation of bacteria:

Isolation of bacteria was achieved by using directly collected water samples. Soil suspensions were carried out by dissolving sample in distilled water and serial dilutions were prepared from the suspension. Nutrient agar medium plates were prepared for the isolation of chromium and different aliquots dilution are plated on Agar medium and incubated at different incubation temperatures for several days. Colonies obtained were picked and purified by many rounds of re-streaking (subculture) on agar plates until single homogeneous colonies were obtained. From these, colonies showing resistance to chromium was isolated and employed for further studies.

Screening of chromium (VI) resistant bacteria:

The screening test to evaluate chromium (VI) reduction or biosorption was carried out to select most promising bacterial strain.

Screening of Bacteria at 100 μ g/ml chromium concentration:

For the screening test, 100 μ g/ml of chromium solution were prepared it was added to nutrient agar medium by pour plate method the medium is suspended in the Petri plates. Various isolates were streaked on the medium.

Screening of Bacteria at 250 μ g/ml chromium concentration:

Chromium-resistant bacteria isolates which are resistant in 100 μ g/ml metal concentration were selected for screening at 250 μ g/ml. Again agar plates were prepared with 250 μ g/ml chromium concentration and fresh cultures were selected which are resistant for 100 μ g/ml chromium concentration were inoculated on the plates by streaking method. After incubation results were studied.

Screening of Bacteria at 500 μ g/ml chromium concentration:

Chromium-resistant bacteria isolates which are resistant in 250 μ g/ml metal concentration were selected for screening at 500 μ g/ml. Again agar plates were prepared with 500 μ g/ml chromium concentration and fresh cultures were selected which are resistant for 250 μ g/ml chromium concentration were inoculated on the plates by streaking method. After incubation results were studied.

Screening of Bacteria at 750 μ g/ml chromium concentration:

Chromium-resistant bacteria isolates which are resistant in 500 μ g/ml metal concentration were selected for screening at 750 μ g/ml. Again agar plates were prepared with 750 μ g/ml chromium concentration and fresh cultures were selected which are resistant for 500 μ g/ml chromium concentration were inoculated on the plates by streaking method. After incubation results were studied.

Screening of Bacteria at 1000 μ g/ml chromium concentration:

Chromium-resistant bacteria isolates which are resistant in 750 μ g/ml metal concentration were selected for screening at 1000 μ g/ml. Again agar plates were prepared with 1000 μ g/ml chromium concentration and fresh cultures were selected which are resistant for 750 μ g/ml chromium concentration were inoculated on the plates by streaking method. After incubation results were studied.

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Estimation of Chromium reducing activity by spectrophotometer:

Determination of Wavelength maxima:

From the prepared standard (10 μ g/ml) Cr stock solutions, 1ml was measured accurately, then 1ml HCl solution was added and mixed well for 2min. then 2ml diphenyl carbazide reagent was added and mix for 5min. purple color was developed in the standard solution. Similar reagent blank was prepared without Cr solution. The wavelength maximum was measured in the visible region i.e 800nm-400nm against reagent blank.

Construction of Standard calibration Curve:

For the prepared 10 μ g/ml working standard solution of Cr, in a series of calibrated volumetric flasks, aliquots of the solution (ranging from 0.5-2.5ml) was measured accurately. To this 1.0ml HCl solution was added, mixed well. Then 2ml diphenyl carbazide reagent was added. The content was mixed for 5min. the final volume of the solution was make up to 10ml with distilled water. The absorbance of the formed color was measured against reagent blank at suitable wavelength maxima.

Analysis of chromium reducing activity of isolated bacteria:

Nutrient agar broth containing 100 μ g/ml of chromium concentration was prepared and the isolated organism that grows at high metal concentration (1000 μ g/ml) was inoculated and was incubated on a rotator orbital shaker with 40rpm. After the incubation, the broth was centrifuged and 1ml supernatant was drawn from the reaction mixture and the samples were analyzed using dipheny carbazide method. From the results obtained the amount of Cr present in the broth and then the amount of Cr reduced by the isolated micro organism was calculated using standard calibration curve ($Y=mX+C$) equation.

Nutrient agar broth composition:

S No	Composition	Quantity
1	Peptone	0.5%
2	beef extract	0.3%
3	Sodium Chloride	0.5%
4	distilled water	1000mL
5	pH	6.8
6	Temperature	25 °C

Table : Composition of Nutrient agar broth

Optimization study:

Optimization of metal concentration:

Nutrient agar broth containing different concentration of chromium from 10-500 μ g/ml was prepared separately and the isolated organism that grows at high metal concentration (1000 μ g/ml) was inoculated and was incubated on a rotator orbital shaker with 40rpm. After the incubation, the broth was centrifuged and 1ml supernatant was drawn from each of the reaction mixture and the samples were analyzed using dipheny carbazide method. From the results obtained the amount of Cr present in the broth and then the amount of Cr reduced by the isolated micro organism was calculated using standard calibration curve ($Y=mX+C$) equation.

4.9.2 Optimization of incubation time:

Nutrient agar broth containing known concentration of chromium was prepared and the isolated organism that grows at high metal concentration (1000 μ g/ml) was inoculated and was incubated on a rotator orbital shaker with 40rpm. The broth was centrifuged at different time intervals and 1ml supernatant was drawn from each of the reaction mixture and the samples were analyzed using dipheny carbazide method. From the results obtained the amount of Cr present in the broth and then the amount of Cr reduced by the isolated micro organism was calculated using standard calibration curve ($Y=mX+C$) equation.

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Gram staining of isolated bacteria:

Preparation of the smear and Heat Fixing:

With a sterile cooled loop, a drop of sterile water was placed on the slide. Sterilize and cool the loop again and pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion and then heat the smear gently to fix the bacteria on slide.

Gram Stain Procedure:

- Place slide with heat fixed smear on staining tray.
- Gently flood smear with crystal violet and let stand for 1 minute.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Gently flood the smear with Gram's iodine and let stand for 1 minute.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
- Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
- Immediately rinse with water.
- Gently flood with safranin to counter-stain and let stand for 45 seconds.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Blots dry the slide with bibulous paper.
- View the smear using a light-microscope under oil-immersion.
- Gram-positive bacteria shows purple color where as gram-negative bacteria shows red color

IV. RESULTS AND DISCUSSIONS

Estimation of Chromium reducing activity by spectrophotometer:

The organism that shows resistant in the 1000 µg/ml chromium coded CRRB-9 was proceeded to study the reducing activity by using spectrophotometer. Here the organisms were grown in the nutrient agar broth containing chromium. Diphenyl Carbazide method was used for the analysis of Cr using spectrophotometer in visible region. The addition of diphenyl carbazide to Cr in the acidic condition gives a characteristic purple color. Based on the color intensity obtained, calibration curve was constructed and samples were analyzed. The wavelength maxima found to be 520nm for standard Cr. The obtained standard wavelength 520nm was selected as suitable wavelength for analysis of samples. The wavelength scanning spectrum was given.

The calibration curve was obtained in the concentration range of 10-60 µg/ml. Accurate fit calibration curve was observed for standard Cr. Standard regression equation was found to be $y = y = 0.015x + 0.001$ with correlation coefficient of 0.999. The calibration curve results were given in table 5.6 and calibration curve was given in figure 5.9

S. No	Concentration in µg/ml	Absorbance
1	10	0.166
2	20	0.313
3	30	0.479
4	40	0.625
5	50	0.801
6	60	0.953

Table Calibration curve results for standard chromium

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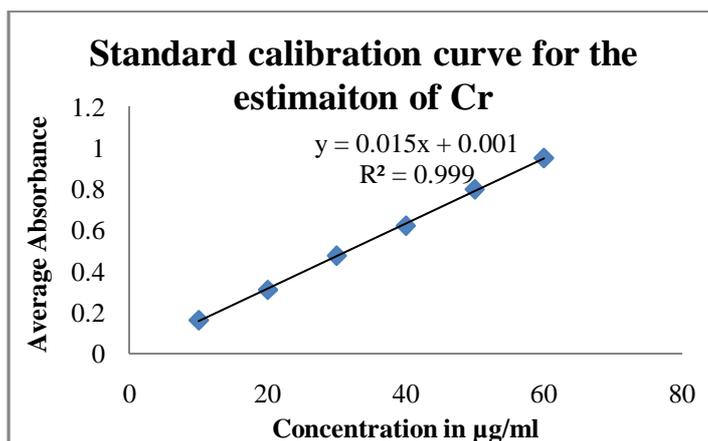


Figure Standard Calibration curve for standard chromium

Different concentrations of chromium metal solution in nutrient agar broth were prepared. the isolated organism that grow in high concentration of chromium i.e CRRB 9 was inoculated in the broth and the broth was incubated in a rotator orbital shaker for 24hours. the incubation working image was given

Among the concentrations 10, 50, 100, 250 and 500µg/ml studied for metal reduction activity, the organism can reduce all the concentrations in the study. High % chromium reduction was observed at a concentration of 500µg/ml. in this concentration, 93.26% of chromium was reduced by the isolated bacteria coded CRRB 9. At 250µg/ml concentration the organism reduce 80.93% of chromium metal. Hence the isolated organism (CRRB 9) was found to having chromium metal reduction activity. The result of chromium metal reduction activity at different concentration of metal was given and comparative graph was given

S No	Concentration of Chromium (µg/ml)	Absorbance	Average absorbance	concentration of chromium present (µg/ml)	% Cr present	% Cr reduced
1	10	0.721	0.727	---	---	---
		0.730				
		0.731				
2	50	0.238	0.246	23.16	46.34	53.66
		0.246				
		0.255				
3	100	0.332	0.332	31.27	31.28	68.72
		0.331				
		0.333				
4	250	0.520	0.505	47.68	19.07	80.93
		0.479				
		0.517				
5	500	0.352	0.357	33.70	6.74	93.26
		0.358				
		0.363				

Table Results of chromium reduction activity at different concentration of metal

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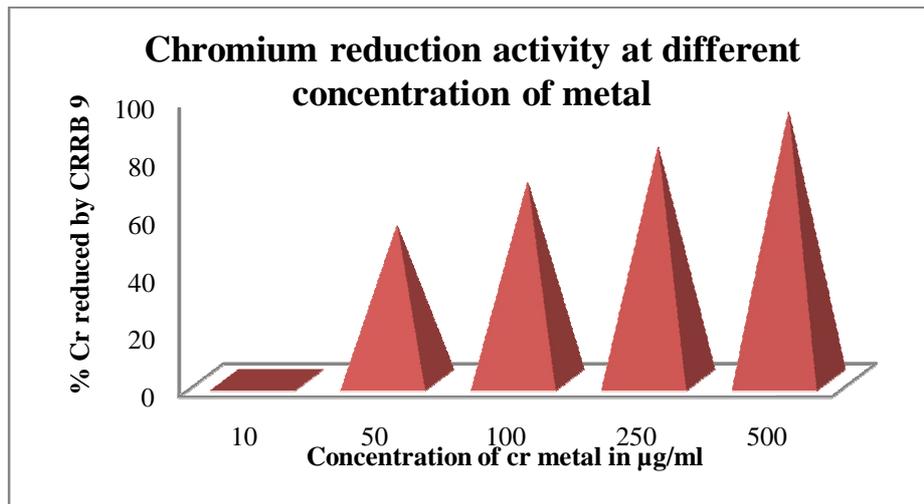


Figure :Comparative results of chromium reduction activity at different concentration of metal

Standard chromium concentration of 500µg/ml in nutrient broth was prepared and the organism was incubated and the % reduction of metal by the isolated bacteria was studied different time intervals and results confirmed that the high % metal reduction of 92.50% was observed with less time of 12hours. Results were given in table 5.8 and comparison graph was given.

S No	Incubation time in Hours	Absorbance	Average absorbance	concentration of chromium present (µg/ml)	% Cr present	% Cr reduced
1	1	0.758	0.757	476.98	95.40	4.60
		0.751				
		0.763				
2	2	0.611	0.617	388.19	77.64	22.36
		0.619				
		0.620				
3	4	0.349	0.316	217.55	43.51	56.49
		0.344				
		0.346				
4	6	0.881	0.882	55.59	11.12	88.88
		0.883				
		0.883				
5	8	0.793	0.793	49.95	9.99	90.01
		0.795				
		0.791				
6	12	0.590	0.596	37.51	7.50	92.50
		0.597				
		0.601				
7	24	0.342	0.345	21.69	4.34	95.66
		0.346				
		0.348				
8	48	0.221	0.224	14.01	2.80	97.20
		0.226				
		0.224				

Table Results of chromium reduction activity at different time intervals

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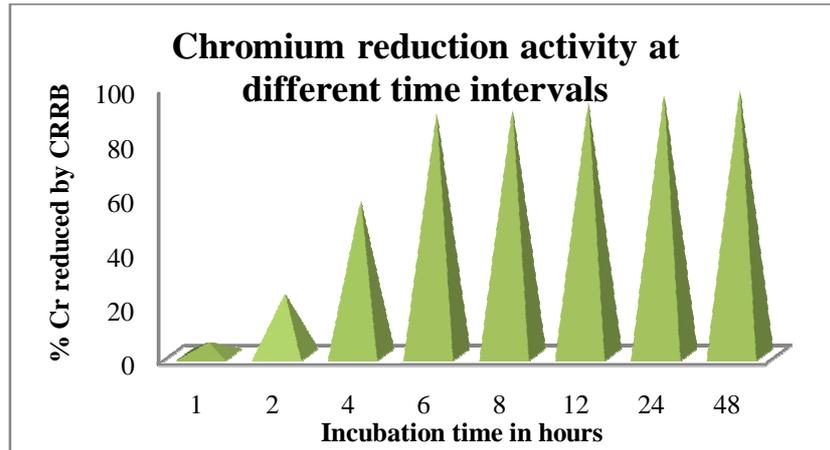


Table: Comparative results of results of chromium reduction activity at different time intervals

Characterization of bacteria:

The isolated bacterium having high metal reducing activity was characterized using the microscopic observation for surface morphology and shape. Gram staining was performed for identifying the isolated bacteria gram positive or gram negative. Results confirmed that the isolated bacteria were found to be round shape or spherical in shape hence are cocci bacteria and surface was found to be smooth. the un-stained CRRB – 9 strain slide under microscope.

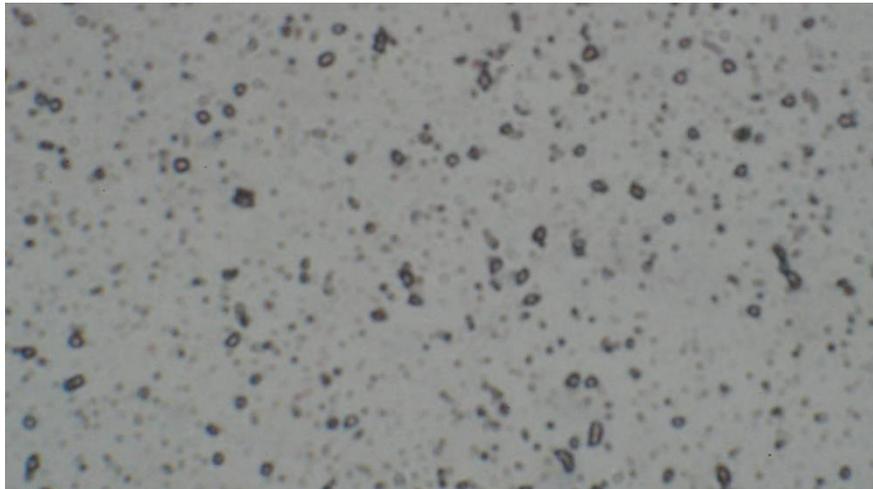


Figure : Microscopic observation of CRRB – 9 isolate before gram staining

he CRRB – 9 isolate was observed under microscope after gram staining. The slide clearly shows that the bacteria retain the crystal violet dye and thus are violet was observed confirms that the isolated bacteria were found to be gram-positive.

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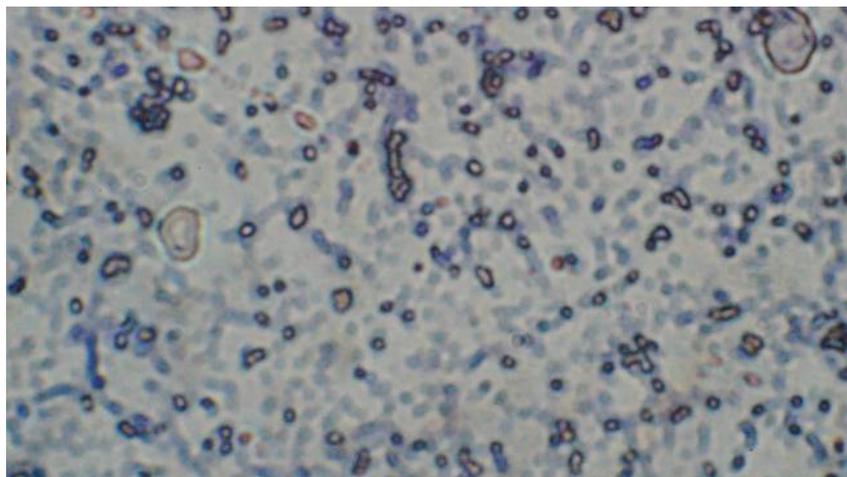


Figure: Microscopic observation of CRRB – 9 isolate after gram staining

V. CONCLUSION

Bioremediation is the most promising and cost effective technology widely used now a days to clean up both soils and wastewaters containing organic or inorganic contaminants. A variety of microorganisms have been known for their ability to degrade these heavy metals. The current study aimed to isolate Chromium reducing microorganism from industrial effluents and polluted soil. Nine organisms having chromium reducing activity was isolated and screened for metal reduction activity. Among the isolates one isolate (CRRB – 1) found to having very high metal reducing activity. The CRRB – 1 isolate found to reduce 92.5% chromium in aqueous solution in a short time of 12 hours and hence the organism was found to be having very high chromium reduction activity. Preliminary techniques confirm that the bacteria were found to be gram-positive cocci bacteria. Further studies need to be carried for the bio-chemical analysis and identification of bacteria and the mechanism involved in chromium metal reduction and applications of the bacteria.

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