**Research & Reviews in Biotechnology & Biosciences** **ISSN No: 2321-8681**
**Website: www.biotechjournal.in** **Research Article**
**Volume: 8, Issue: 2, Year: 2021** **PP: 126-138**
**DOI: https://doi.org/10.5281/zenodo.5775459** **Peer Reviewed Journal**

# Removal of metals using Isolated *Achromobacter denitrificans* from heavy metals contaminated soils

**Gnanasekaran R*, Yuvaraj D, Iyyappan J, Gopinath M,**

**Dhanushashree G, Reshma Pammi Harinath,**

***Department of Biotechnology, Vel Tech High Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Avadi, Chennai-600 062, Tamil Nadu, India***

*Article History*

Received: 10/10/2021
Accepted: 21/11/2021

Article ID: RRBB/112

*Corresponding Author:*
E-Mail:
***yuvarajdinakarkumar@gmail.com***

**Abstract**

Industry is backbone to global economic development. Due to the improvement of standards in industries and technologies in recent years, they have also caused pollution by releasing toxic, carcinogenic and mutagenic heavy metals like chromium, cadmium, lead and zinc especially from the tannery industries. In this study we have collected samples from the industrial wastes like tannery effluents from different potential habitat forisolating the bacterial strain. The bacterial strain was isolated and cultured on Nutrient Agar media. These traces are concentrated on the premise in their basic and physiological research together with length and shape of the organisms were also studied with the aid of visual statement of colony, microscopic observation and biochemical assessments and the sequencing exams recognized the particular bacterial species is obviously occurring microscopic organisms fit for diminishing and detoxification of toxic substances like Cadmium, Potassium and Zinc from tannery effluent. Its metal tolerance tests and enzymatic production tests were conducted. By using Atomic Absorption Spectrophotometer degrading potential is shown in various outcomes for the heavy metals. Accordingly, identification of this microorganism for their substantial metal opposition and biodegradation limit is most likely promising potential biosorption and which it helps to removes the toxic substances in the tannery industries waste effluent. This sort of study is huge for more extensive examination to acquire information about metal tolerant microscopic organisms and its interpretation studies.

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**Key words:** Industrial waste; Isolation and characterization; Bacteria; Metal tolerance.

## 1. Introduction

Raw wastewater contains huge amount of substantial metals that are not deteriorate by the regular procedure of sewage treatment. Nearness of high concentration of poisonous substantial metals in wastewater logically prompts both tainting of getting water bodies and harmful effect on amphibian life. Utilization of such contaminated water for utilization and different purposes can convey extreme issues to human wellbeing. At higher concentration, the heavy metals mixes in the cell that is unreasonably risky for any natural capacities. In terms, the overwhelming metal is connected to a gathering of components having nuclear thickness estimation of in excess of 6 gm/cm (Connell et al., 2008). Considerable heavy metals like Chromium (Cr), Manganese (Mn), Nickel (Ni), Copper (Cu), Zinc (Zn), Arsenic (As), Cadmium (Cd), Mercury (Hg) and Lead (Pb) which are contaminate the freshwater bodies (Babarinde et al., 2006) in light of their harmful, the biological waste which cannot be degradable and diligent nature.

The fact is most of the heavy metals are intensely toxic to most microbes, there are heavy metal tolerant bacteria; long term exposure to metals supports proliferation of microbes that are tolerant to metals. This has been researched by examining living species presented to anthropogenic or normal metal pollution over expanded period. Substantial metals are known to cause impact on soil microbial populace and their related exercises, which may directly impact the soil fertility (Ezaka et al., 2011). The expanding modern development is the significant wellspring of toxic substance appearance into various sections of the earth including Air, Biosphere, Soil and Water. These modern sources incorporate mining, purifying, surface completing the process of electric machines, electric sheets/circuits, electrolysis and electroplating producing enterprises just as farming division including composts, pesticides (Wang et al., 2009).Copper has a basic micronutrient which is dangerous substantial metal formost living cells. It is the primary segment of a few catalysts primarily taking part in electron stream and redox, and other significant responses. The job of copper in redox responses is because of its capacity to experience Cu(I) + Cu(II) advances which are reliant on the general condition. An overabundance of copper in the water effectively affects amphibian life, with harm to freshwater creatures, for example, fish. Copper harms the kidneys, sensory systems, and livers of most water animals.

Ecological toxins from copper industry have numerous aggressive impacts on earth. A definitive impact is felt in the wellbeing and prosperity of men, ladies, kids and the absolute nature involving the creature, aquatic life, bugs and plants. Copper was accounted for to be one of the across the board overwhelming metal contaminations in characteristic and wastewaters in China coming about because of horticulture and modern exercises, for example, colors, mining, refining and electroplating, and so on. There are numerous strategies to treat various sorts of mechanical wastewater that are debased with substantial metals, such as adsorption, layer filtration, cementation and electrodialysis (Li et al., 2004). Chemical

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hydroxide precipitation is the most economic and the most regularly utilized procedure for the treatment of heavy metal bearing industrial effluents but after this treatment the wastewater stream can still contain up to 5 ppm heavy metals which is an inadmissible concentration for release to the environment (Konstantinos et al., 2011).

Many numbers of physico-chemical protocols are being used world-wide to purify the water contaminated with heavy metals. However, these methods are inherently difficult in their uses and are not economically feasible (Suryan et al., 2012). In the process of organic treatment microorganisms assume a work for settling solids in the arrangement. Actuated well up, streaming channels, adjustment lakes are generally utilized for treating modern wastewater. Bio-ingestion is another natural system and low in cost which is used for most prominent removal of toxic substance from wastewater. Bio-osmosis methodology is also eco-friendly and potential best responses for the disposal of toxic substance like substantialmetals from sewage water rather than physicochemical strategies (Zolgharnein et al., 2010). A variety of chemical and natural parameters are utilized to depict the phase of natural issue adjustment during various organic medications. Chemicals information alone are not adequate to assess the organic impacts, since it is difficult to examine all the mixes and synergistic impacts adding to danger. Natural and biochemical properties including the pace of CO2 discharge or oxygen utilization, microbial biomass and addition of enzymes are viewed as important factors of remediation progress due of their pertinence in cycling of organic matter (Aparna et al., 2010).

Various sorbents utilized so far in copper evacuation, for example, promegranate strip, degreased coffee beans, dried animal bones, nut body pellets, fly debris, agricultural products and so on. The power of adsorption is exceptionally less in the greater part of these above investigations. The so far utilized commercial sorbents utilizes precursors like wood, bamboo, low position Turkish coal, coconut shell, saw dust and rice husk and so on the vast majority of these require high working expense, are restricted in supply and expensive (Surya Narayan Dash et al,. 2010). Hence, in this present study, we carried out the isolation and identification of copper resistance bacteria from polluted discharge sample of industrial effluent and to study their capacity in copper bio-sorption.

## 2. Materials and Methods

### 2.1 Sample Collection

Aliquot samples of soil were composed in pre-sterilized bottles from the places near chemical industrial habitats around Chennai, Tamil Nadu, India. Further, it was permitted to shade dried at room temperature for 48 h. The collected sample was stored at refrigeration for further analysis (Naveenkumar et al., 2010).

### 2.2 Isolation of bacteria

1 gm of collected soil sample was serially diluted from 10-1 to 10-10 with distilled water. From this serial dilution, the 10-4 and 10-5 dilution sample was taken for further studies. 0.1mL of samples were inoculated on sterilized nutrient agar medium (1.5% agar, 0.5% NaCl, 0.5% peptone, 0.3% yeast extract, 100 ml distilled water and pH 7 maintained) and incubated at 34$^{o}$C for 48 h (Payel et al., 2017, ). The growth of bacterial

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colonies was observed after the incubation period. In order to obtain pure culture, the subculture was performed by streak plate method and also in liquid culture method. The isolated colony of bacterial culture was taken and it was covered with silicone tape for long term storage in the refrigerator.

### 2.3 Identification of isolated bacteria

Genomic DNA of bacterial isolate was identified by using standard protocol (Edward et al., 1989). Further more,the isolated DNA was amplified using PCR with help of universal primers as 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3') primers. The 16s rRNA gene was sequenced using ABI 3730xl sequencer (Applied Biosystems) at Yaazh Xenomics (India) Pvt. Ltd., Madurai. The neighbouring recognised linkage of the families of the new isolates was determined by performing a nucleotide database search (BLAST).

### 2.4 Phylogenetic tree construction

The phylogenetic tree construction were previously described by Zheng et al., 2000 was followed for BLAST the Nucleotide to Nucleotide using BLASTn search tool. The neighbour joining standard linkage of the families with the new isolates was searched in nucleotide database search for constructing evolutionary relationship with the other comparable similar sequences with the new isolates. The alignment of new isolated strain sequence was done with help of CLUSTAL omega tool based on Hidden Markov Model (HMM) profile – profile technique with jukes cantor model by multiple sequence alignment (MSA). The ClustalW2 phylogeny program was used to construct the phylogenetic tree. The percentage identity of other strains with new isolate strain was listed in Table 1.

### 2.5 Metal Sorption Studies

#### 2.5.1 Optical Densitymethod

To determine the metal biosorption isotherms, batch biosorption experiments wereconducted in conical flasks using Achromobactersp. The bacterial suspension (1mL) was added to 100 ml of metal solutions such as copper, potassium dichromate (Leung et al. 2000). A set of flasks of metal solutions were kept as control. The prepared heavy metal substance based solution pH was balanced to 7 by adding 0.1 N of NaOH or 0.1 N of H2SO4solutions, just before commencement of experiments. The flasks were kept in shaker for 250rpm at 37°C. After every 24hour incubation the metal solution were subjected to the spectrophotometric analysis and the OD value was taken periodically to study the absorption of heavy metal by the organisms.

#### 2.5.2 Absorption Studies

The energy of metal bio-sorption by a chose separate, Achromobactersp, was researched for two metals: Potassium and copper. Two heavy metals in their salt structure Copper sulphate (CuSo4.5H2O) and Potassium dichromate (K2Cr2O7) were utilized in ppm concentrations. Starting screening with generally high concentration of every one of the heavy metallic salts (1-5 ppm) was done to evaluate the broad range impact of the heavy metals and its resistance by the living beings with incubation at 37°C for 24, 48, 72, 96 and 120hrs time frame. After the hatching

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time the media was oppressed for turbidometric examination to consider the advancement of the microbes inside seeing the overwhelming metal under a particular concentration. A lot of flasks were in like manner kept up as control with no substantial metal for all the days. Supplement agar medium was also used to validate the impact of significant metal on the improvement instance of the living things.

After the hatching and turbidometric examination of the sample was centrifuged 10 minutes with 4000-5000 rpm for separation of cell mass and supernatant (aliquots). The heavy metal concentration was investigated using aliquots and the cell mass was discarded. The examination depended on the amount of heavy metal present in the unique fixation utilized was abandoned in the media after the rest being absorbed/adsorbed by the living being. This was finished by utilizing Atomic Absorption Spectroscopy (AAS) which was in a roundabout way the portrayal of percent take-up of heavy metal by the particular creature. The results of percent take-up of heavy metal was determined and calculated.

## 4. Results and Discussion

### 3.1 Isolation of bacteria

Figure 1a shows that the microbial identification of isolated strain. The strain was cultured in Nutrient agar medium and it was further permitted to grow for 48 h at 34°C in an incubator. Every 30 days once strain was sub cultured for maintain a stock for heavy metal removals process. Figure 1b shows that the morphology of strain by simple gram straining method. It is strictly aerobic, ubiquitous, motile bacterium and rod shaped. It usually forms circular shaped white colonies. This study confined that the isolated strain has been found to be *Achromobacter denitrificansis* non-spore former, gram negative bacterium.


**Figure 1**:

(a) Purified bacterial strain from heavy metal contaminated soil (b) Microscopic view of *Achromobacter denitrificans*

Furthermore, the isolated bacterial stain was taken into the molecular identification of 16 S rRNA gene sequence. The bacterial genomic DNA was extracted from 12 hr grown culture plate using DNA isolation kit. The extracted DNA was taken in to PCR for amplification. The amplified DNA was evaluated on 1% agarose gel for amplicons in 1xTBE buffer at 100 V. The gel segment with required band was deliberately extracted under UV light and exposed to extraction utilizing the QIA speedy gel extraction unit (Qiagen, Valencia, CA) from the agarose gel and sequenced utilizing programmed DNA sequencer (Model ABI 3730xl, Applied Biosystems). The Bootstrap examination was done dependent on 1000 replications. The sequences was tested utilizing the Basic Local Alignment Search Tool (BLAST) programming (http://www.ncbi.nlm.nih.gov/impact) against the nucleotide sequence database. The BLAST analysis result was presented in table 1 that the isolate had 95% similarity to

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genus is relatively consistent with Achromobacter sp. The partial 16S rRNA grouping of confine was submitted to the

National Center for Biotechnology Information (NCBI) with an accession number of MH100662.1. So as to decide the connection between bacterial strain and the Achromobacter sp., the phylogenetic dependent on halfway 16S rRNA was constructed and it was shown in Figure 2. Furthermore, isolates was confined with connection of biochemical qualities, physiological and morphological studies with 16S rRNA succession examination, the bacterial segregate was distinguished as Achromobacterdenitrificans which is strictly aerobic in nature (Syed et al., 2014)


**Figure 2:** Phylogenetic tree of the bacterial isolate *Achromobacter denitrificans*, 970 bp (MH100662.1)

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| S.No | Description | Max Score | Total Score | Query Cover | E Value | % Identity | NCBI Accession Number |
|---|---|---|---|---|---|---|---|
| 1 | *Achromobacter denitrificans* | 1849 | 1849 | 100% | 0.0 | 100.00% | MH100662.1 (Test Strain) |
| 2 | *Achromobacter xylosoxidans* | 1369 | 1369 | 99% | 0.0 | 91.04% | FJ827751.1 |
| 3 | *Achromobacter xylosoxidans* | 1365 | 1365 | 99% | 0.0 | 91.00% | HG423436.1 |
| 4 | *Achromobacter insolitus* | 1363 | 1363 | 99% | 0.0 | 90.94% | HG423431.1 |
| 5 | *Achromobacter sp.* | 1354 | 1354 | 98% | 0.0 | 91.24% | KP974274.1 |
| 6 | *Achromobacter sp.* | 1367 | 1367 | 97% | 0.0 | 91.67% | MN747140.1 |
| 7 | *Achromobacter denitrificans* | 1369 | 1369 | 97% | 0.0 | 91.60% | KT799661.1 |
| 8 | *Achromobacter sp.* | 1365 | 1365 | 97% | 0.0 | 91.57% | MH773405.1 |
| 9 | *Achromobacter sp.* | 1365 | 1365 | 97% | 0.0 | 91.57% | MH773331.1 |
| 10 | *Achromobacter sp.* | 1365 | 1365 | 97% | 0.0 | 91.57% | MH773281.1 |
| 11 | *Achromobacter sp.* | 1362 | 1362 | 97% | 0.0 | 91.57% | MN636462.1 |
| 12 | *Achromobacter sp.* | 1362 | 1362 | 97% | 0.0 | 91.57% | MF993519.1 |
| 13 | *Achromobacter sp.* | 1362 | 1362 | 97% | 0.0 | 91.57% | KX348283.1 |
| 14 | *Alcaligenes sp.* | 1362 | 1362 | 97% | 0.0 | 91.57% | JN226402.1 |
| 15 | *Achromobacter denitrificans* | 1362 | 1362 | 97% | 0.0 | 91.57% | EU869274.1 |
| 16 | *Achromobacter cerru* | 1362 | 1362 | 97% | 0.0 | 91.57% | EU862292.1 |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *hlandii* | | | | | | |
| 17 | *Achromobacter sp.* | 1371 | 1371 | 97% | 0.0 | 91.54% | MH773412.1 |
| 18 | *Achromobacter sp.* | 1371 | 1371 | 97% | 0.0 | 91.54% | MH773336.1 |
| 19 | *Achromobacter sp.* | 1371 | 1371 | 97% | 0.0 | 91.54% | MH773286.1 |
| 20 | *Achromobacterdenitrificans* | 1369 | 5450 | 97% | 0.0 | 91.54% | CP020917.1 |
| 21 | *Achromobacterdenitrificans* | 1369 | 1369 | 97% | 0.0 | 91.54% | KT792724.1 |
| 22 | *Achromobacterxylosoxidans* | 1358 | 1358 | 97% | 0.0 | 91.54% | KP236299.1 |
| 23 | *Achromobacter sp.* | 1369 | 1369 | 97% | 0.0 | 91.53% | EU430054.1 |
| 24 | *Achromobacter sp.* | 1363 | 1363 | 97% | 0.0 | 91.50% | KJ124851.1 |
| 25 | *Achromobacter sp.* | 1358 | 1358 | 96% | 0.0 | 91.69% | LC097207.1 |
| 26 | *Achromobacteranxifer* | 1352 | 1352 | 96% | 0.0 | 91.60% | MN197585.1 |
| 27 | *Achromobacterxylosoxidans* | 1352 | 1352 | 96% | 0.0 | 91.51% | KY508299.1 |

**Table 1: Similarity result for bacterial isolate strains using BLASTn**

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**The following sequence data was submitted to the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/1368658705).**

**ATGACAACTCGAACGAGCAGCACGG
ACTTCGGTCTGGTGGCGAGTGGCGA
ACGGGTGAGTAATGTATCGGAACGT
GCCCTGTATCGGGGGATAACTACGCG
AAGGCGTAGGTGATACCGCATACGCC
CTACGGGGGAAAGCAGGGGATCGCA
AGACCTTGCACTATTGGAGCGGCCGA
TATCGGATTAGCTAGTTGGTGGGGTA
ACGGCTCACCAAGGCAACGATCCGTA
GCTGGTTTGAGAGGACGACCAGCCA
CACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGA
ATTTTGGACGATGGGGGAAACCCTGA
TCCCGCCATCCCGCGTGTGCGATGAA
GGCCTTCGGATTGTAAAGCACTTTTG
GCAGGAAAGAAACGTCGCGTGTTAG
TACCTCGCGGAACTGACGGTACCTGC
AGAATAAGCACCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGGT
GCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGTGCGCACGCGGTTCGTAA
AGATCGATGTGAAATCCCAGAGCTTA
GCTTTGGAACTGCGTTTTTAACTACC
GGGCTAGAGTGTGTCAGAGGGAGGT
GGAATTCCGCGTGTACAGTGAAATGC
GGAATCTGCGGAGGACACCGATGGC
GAAGGCAGCCTCCTGGGATATGCTGA
CGCTCATGCACGAAACGTGGGGAGC
AACCGGATTAATACCCTGGTAGTCAC
GCCCTAACGATGTCACTAGCCTTGGG
ACTTCGCACTTGATGCGCTCTAAGCG
TGACGTGACCCCCGGAGAGTCGGTC
GCAGATTAAACTCAAGGATTGACGG
GACCGCACAGCGGAGATGATGGGAT
TATTCATGCACGCGAAAACTTACTAC
CTTGACTGTCTGAATCCTGACAGATT
AGGAGGCTCGCAGAGACCGAACACA
GTGCTGCAGGATGTCTCACTCGTGCC
TGAATGT**

3.2 Spectrophotometry studies

After the incubation of isolated bacterial strain A.denitrificansinto the 250mL metal solutions in a conical flasks (Potassium dichromate and Copper sulphate) the OD values are taken periodically after every 24hours. It was observed that the metal solution which was inoculated with bacteria got decreased in its metal concentration after every 24hour. But the OD values of the controls are stayed constant at every evaluation. This experiment shows that the bacterial strain can absorb the copper than the other metals. The spectrophotometric method is efficinetly used in the estimation of concentration of SO42-, NO3-, F- in ground water (Rajkumar et al., 2010) and biosorption of lead and copper by using roger mushrooms biomass (ZachariaKariuki et al., 2017).

3.3 Biosorption studies

Fundamental evaluation of the gathered sample for heavy metal resistance capacity demonstrated that the sample was emphatically developed using substantial metal (Cu) and (K) in their way of life media. The point of this work was to choose and distinguish the fitting strains of microscopic organisms, which exhibit more noteworthy confrontation towards heavy metal poisonous quality. It was seen that Gram-negative bacterium Achromobacterdenitrificansstrain GSYDR2018 indicates ideal level removal of copper from the waste water. In this investigation at first we described the previously mentioned bacterial strain disengaged from soil of unequivocally dirtied by modern exercises. Marzan et al., (2017) detailed that the general effects of

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bacterial improvement in closeness of substantial metal(s) in different concentrations(100–1000 mg/mL) were analyzed and it was seen that bacterial advancement is focus subordinate, since it indicated diminishing optical density (at k = 465 nm) as per the expanding overwhelming metal fixation (Table 2). When the strain GSYDR2018 got inoculated in the Copper sulphate metal solution after the incubation 24 hours the OD values got decreased like 0.14, 0.13, 0.11 and so on. Besides it shows sensitivity to the other metals like Potassium. The removal efficacy of Potassium dichromate using Achromobacterdenitrificans and Copper sulphate using Achromobacterdenitrificans is shown in figure3.

**Table 2**: Results for batch biosorption of metals using *Achromobacterdenitrificans*

| Name of the metal | Incubation period in hrs | Absorbance ofControl(430nm) | Absorbance ofCulture(430nm) |
|---|---|---|---|
| Potassium dichromate | 24 | 0.2 | 0.15 |
| | 48 | 0.2 | 0.11 |
| | 72 | 0.2 | 0.08 |
| | 96 | 0.2 | 0.06 |
| | 120 | 0.2 | 0.02 |
| **Name of the metal** | **Incubation period in hrs** | **Absorbance ofControl(465 nm)** | **Absorbance ofCulture(465 nm)** |
| Copper sulphate | 24 | 0.19 | 0.14 |
| | 48 | 0.19 | 0.13 |
| | 72 | 0.19 | 0.11 |
| | 96 | 0.19 | 0.08 |
| | 120 | 0.19 | 0.06 |

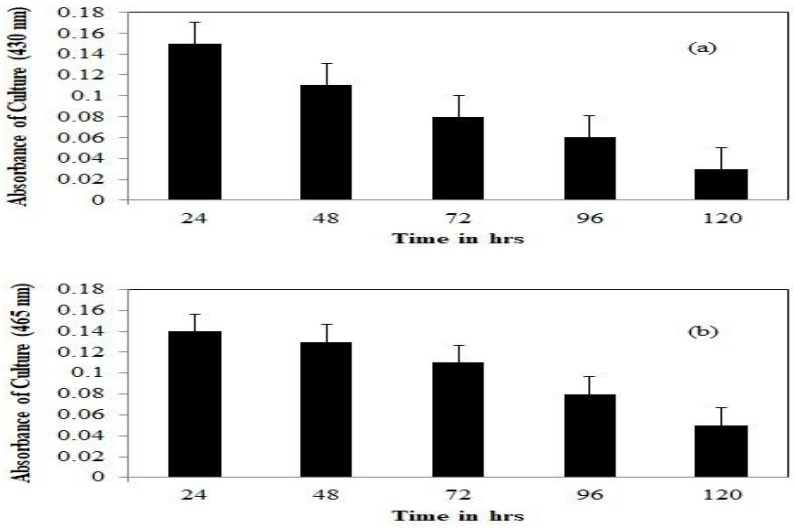


**Figure 3**: Graph showing the comparison between absorbance over uniform time intervals

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Bio-absorption is one of the accessible proficient procedures for heavy metals expulsion at low fixations and cheap material, quick metals recuperation and recovery of bio-adsorbents are some of the real highlights of this process (Osama el gialani elsaid et al., 2010). It is one of the developing advancements which use the dormant squander biomass to evacuate dangerous metal particles. It includes strong stage (sorbent) and a fluid stage (dissolvable) havingthe disintegrated species to be adsorbed. The living beings are those utilized for this treatment are called as bioabsorbents. There are reports about adsorption of copper and nickel ions by the cross linked chitosan-g-acrylonitrile copolymer of metal solution but compared with polymer the microbes are more reliable (Ramya et al., 2011). Be that as it may, pH is a standout amongst the most significant components influencing the metals absorption onto biomass. Hydrogen particle fixation extraordinarily influences the outsideof the adsorbents which thusly impact the metal expulsion from wastewater (Acar et al., 2006). Accessibility, inexpensiveness and adequacy are the better factors of biomasses which make them a good choice for the heavy metal treatment. Thus, the vital purpose of combination of this appraisal is to seclude and see the major bioremediating bacterial directors that help the standard recuperation of condition (green and general condition) and survey the near liberal metal remediation limit with respect to the pick segregates that can be a reaction for recovering future contamination by untreated generous metal containing release

## 4. Conclusion

Biological treatment, the utilization of microscopic organisms and different microorganisms to expel contaminants by assimilating them for quite some time been a pillar of wastewater treatment in the chemical process industries. Because they are effective and widely used, many biological treatment options are available today (Majid, 2010).In this study the sample was gathered from the surrounding places near to the chemical industrial habitats in Chennai. The collected sample was serial diluted and cultured in the agar plates. After the incubation it developed multiple colonies and they isolated for the identification. Depending on various biochemical characterization tests such as 16srRNA gene sequence and BLAST analysis, we identified the isolate as gram negative bacterium *Achromobacter denitirificans*. The bacterial strains of the isolate are subjected to the metal absorption studies at its optimum pH level and temperature. All the outcomes gave right now the idea that the *Achromobacter denitrificans* shows ideal degree of absorption to the copper from the surrounding environment. This kind of study is very helpful to develop significant bacterial strain with bioremediation potential for toxic metal removal process.

**Acknowledgement:**

The authors wish to express their gratitude to Vel Tech High Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Chennai, India for supporting this research work**.**

**Funding**

This is a non- funded research work

ISSN No: 2321-8681
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## Conflict of interest

Authors declare that, there is no conflict of interest

## Ethical statement

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