



ISSN NO. 2320-5407

*Journal homepage: <http://www.journalijar.com>***INTERNATIONAL JOURNAL  
OF ADVANCED RESEARCH****RESEARCH ARTICLE****Biodegradation of hexavalent chromium, oil and grease in paper mill effluent using  
Micrococcus luteus and Bacillus aquimaris isolated from soil****Leena.K<sup>1</sup>, Anusha Balasingh<sup>2</sup>\*****1.** Department of Microbiology, Ethiraj College for Women, Chennai 600 006, India**2.** Assistant Professor, Department of Microbiology, Ethiraj College for Women, Chennai India**Manuscript Info****Manuscript History:**

Received: 12 October 2015

Final Accepted: 22 November 2015

Published Online: December 2015

**Key words:**Effluent, Biodegradation,  
hexavalent chromium**\*Corresponding Author****Leena.K****Abstract**

Waste water released from various industries is a major source of pollution in day to day activities. Among the various industries present, paper mill industry is categorized as one of the major cause of pollution to the environment. The problems associated with pulp and paper mill effluent are of giving out waste water with a variable pH value, high level of BOD, COD, suspended solids, absorbable halides, heavy metals etc. Among the different toxic compounds present in the paper mill effluent, chromium is present in high levels. Hexavalent chromium (Cr (VI)) is considered to be highly toxic, carcinogenic, and mutagenic to living organisms. Oil and grease present in paper mill effluents are producing off foul odours in the surrounding environment, blocking sewer lines, causing ill effects to the soil fauna, flora and microorganisms, soil physico-chemical properties etc. Physico-chemical methods do not provide effective solutions in elimination of heavy metals from the effluent. Hence in this study efforts were made to degrade the heavy metals, oil and grease using bacteria isolated from soil. *Micrococcus luteus* and *Bacillus aquimaris* effectively removed the hexavalent chromium, oil and grease after 96 hours by degradation activity.

*Copy Right, IJAR, 2015., All rights reserved***INTRODUCTION**

Environmental pollution is one of the major problems of the world and it is increasing day by day due to urbanization and industrialization. An enormous industrial growth has taken place throughout the world in the past few decades. It has become so vast that, the environment has totally changed from what it was earlier. Pollution today is found in each and every thing that we need in our day to day life (i.e.) air, water, soil etc. Among the various industries, paper and pulp industry has been categorized as one of the most polluting industry due to the discharge of huge volumes of highly colored and toxic waste water (effluent) in the environment causing pollution of land, water and air.

The problems associated with pulp and paper mill effluent are pH, colour, high levels of BOD, COD, suspended solids, absorbable halides, heavy metals etc. Hexavalent chromium compounds can lead to mutation and cancer, inhibit enzymes and nucleic acid synthesis. Cr (VI) is not only highly toxic to all forms of living organisms, mutagenic to bacteria, mutagenic and carcinogenic to humans and animals (Losi et al., 1994), but is also involved in causing birth defects and the decrease of reproductive health (Kanojia et al., 1998). Other health problems that are caused by chromium (VI) are skin rashes, stomach upset, ulcers, respiratory problems, weakened immune systems, kidney and liver damage, alteration of genetic material, lung cancer, and death.

High concentrations of chromium in the water environment during disposal of the metal products in surface water can enter the gills of fish that swim near the point of disposal. In animals chromium can cause respiratory problems, a lower ability to fight disease, birth defects, infertility and tumor formation.

Fats, oils and greases (FOGs) in wastewater create problems including the production of foul odours, the blockage of sewer lines and may interfere with the proper operation of sewage treatment works. In natural environments, FOGs result in coating of animals and plants with oil, reduction of oxygen transfer rate and a high chemical oxygen demand (COD) in wastewater.

Physical and chemical methods are on the track of treatment, but they are not on par with biological treatment because of cost ineffectiveness and residual effects. The biological treatment is known to be effective in reducing the organic load and toxic effects of effluent. Thus there is a need to search or develop a suitable low cost biologically or environmental friendly methods for bioremediation of pulp and paper mill effluent which have the capacity of efficient biodegradation of organic and inorganic pollutants of mill effluents.

## **Materials and methods**

### **Effluent collection**

Industrial effluent was collected from paper mill industry situated in Karur district, Tamil nadu .The collected samples were transported and stored at 4<sup>0</sup>.C for further analysis and microbial isolation.

### **Effluent analysis**

The sample was subjected to analysis for various physiochemical and biological parameters such as BOD, COD, pH, total suspended solids, total dissolved solids, chlorides, copper, zinc hexavalent chromium, oil and grease etc.

### **Isolation of organisms**

Soil sample and effluent sample were serially diluted (10 folds) with sterile saline. The diluted sample was plated on nutrient agar by spread plate technique. The plates were incubated at 37°C for 24 hours. A number of morphologically different colonies were randomly selected and sequentially cultivated in nutrient agar for selection.

### **Inoculum preparation**

The mother inoculum was prepared by inoculating one loopful of the four selected strains of individual bacterial isolates separately in 5 ml sterilized nutrient broth. The inoculated broths were incubated in an orbital shaker at 37°C for 16–24 hours.

### **Preliminary study**

250 ml conical flasks were sterilized by autoclaving. 100ml of paper mill effluent was measured and taken in sterile conical flask. 5ml of overnight incubated culture was added to respective flask. 5ml of sterile distilled water was added to the control flask. All the conical flasks were incubated at 37°C for 4 days ( Ameer Basha S., et al., 2014).

### **Effluent analysis**

After 4 days of degradation the effluent was subjected for physiochemical and biological parameters such as BOD, COD, pH, total suspended solids, chlorides, copper, hexavalent chromium, oil and grease etc.

### **Identification of organisms**

The bacteria were identified by colony morphology, gram staining, and biochemical analysis and confirmed by 16srRNA study.

### **DNA Extraction:**

1. Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit – As per the kit instruction below procedure followed.
2. An isolated bacterial colony was picked and suspended in 1ml of sterile water in a microfuge tube.
3. Centrifuge it for 1 minute at 10,000–12,000 rpm to remove the supernatant.
4. Add 200 µl of Insta Gene matrix to the pellet and incubate at 56 °C for 15 minutes.
5. Vortex at high speed for 10 seconds and place the tube in a 100 °C in heat block or boiling water bath for 8 minutes.
6. Finally, vortex the content at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes.

7. In result, 20µl of the supernatant was used per 50 µl PCR reaction

#### **PCR Protocol:**

Using below 16S rRNA Universal primers ,gene fragment was amplified using MJ Research Peltier Thermal Cycler

#### **Primer**

<b>Primer Name</b>	<b>Sequence Details</b>	<b>Number of Base</b>
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTACGACTT	22

Add 1µL of template DNA in 20 µL of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions :

Initial Denaturation 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. Final Extension at 72°C for 10 min. DNA fragments are amplified about 1,400bp in the case of bacteria.

#### **Purification of PCR products**

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

#### **Sequencing protocol**

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

#### **Sequencing Primer :**

<b>Primer Name</b>	<b>Sequence Details</b>	<b>Number of Base</b>
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

Sequence data was aligned and analyzed for identifying the Sample.

#### **Bioinformatics protocol:**

1.The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper et al., 2008).

#### **Results**

The paper mill effluent collected from Karur district indicates the presence of high level of hexavalent chromium, oil and grease compared with the standard of pollution control board. The pH of the effluent was measured using potentiometric method and was found to be 8.35. This indicates that the effluent from the textile industry is slightly

alkaline in nature. The amount of oil and grease were found to be 30 ppm. The hexavalent chromium was determined by iodometric titration and found to be 14 ppm.

#### Physio – chemical and biological parameters of raw effluent

PARAMETERS	RESULT
<b>PH</b>	<b>8.35</b>
<b>TOTAL DISSOLVED SOLIDS (PPM)</b>	<b>1540</b>
<b>TOTAL SUSPENDED SOLIDS (PPM)</b>	<b>96</b>
<b>BIOLOGICAL OXYGEN DEMAND (PPM)</b>	<b>42</b>
<b>CHEMICAL OXYGEN DEMAND (PPM)</b>	<b>200</b>
<b>OIL AND GREASE (PPM)</b>	<b>30</b>
<b>ZINC (PPM)</b>	<b>63</b>
<b>HEXAVALENT CHROMIUM (PPM)</b>	<b>14</b>

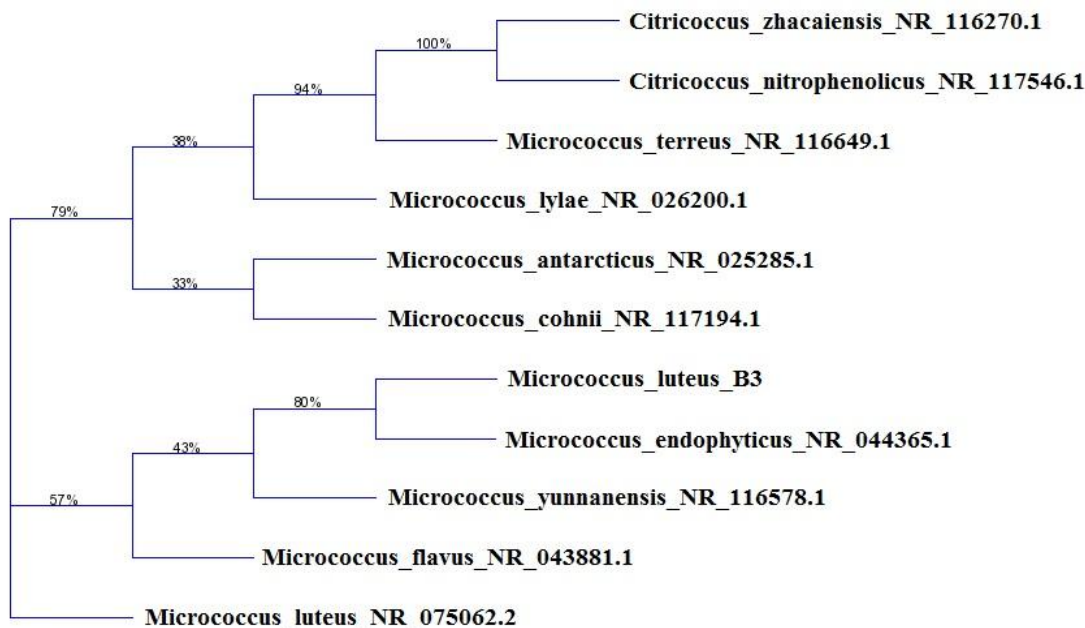
#### Isolation and characterization of microorganisms

The bacterial strains were isolated from soil and effluent sample. Based on colony morphology, motility, gram staining and biochemical analysis the bacteria were identified and confirmed by 16srRNA study as B3 – *Micrococcus luteus* and B4 – *Bacillus aquimaris*.

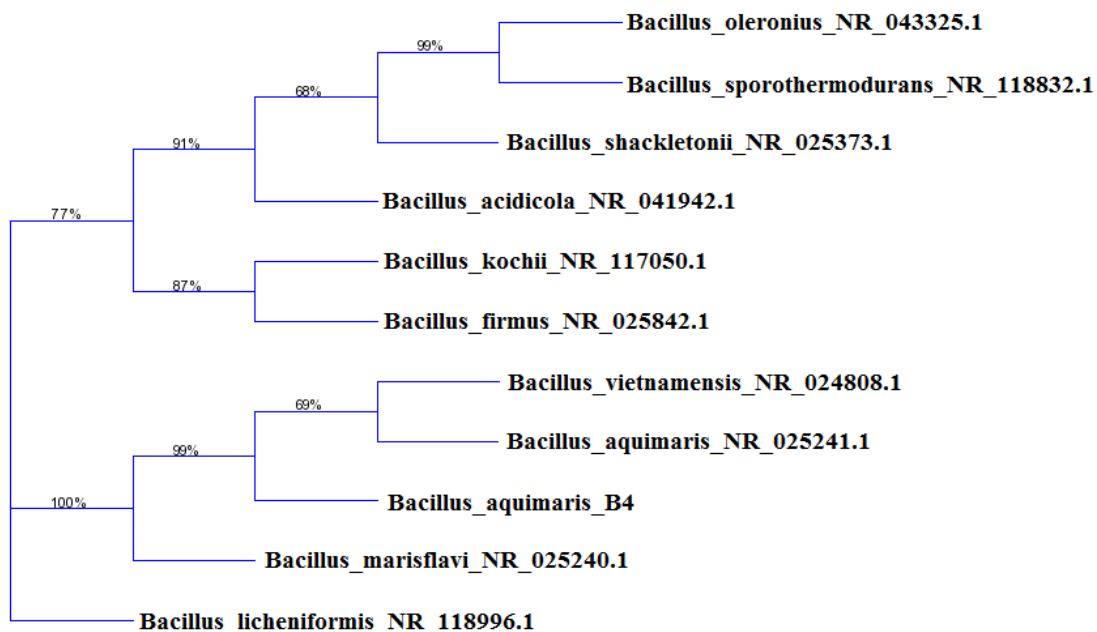
**Preliminary test: Gram staining and motility** Among the two isolates, B3 was found to be gram positive cocci and B4 was found to be gram variable rods. B3 is non motile where as B4 was found to be actively motile.

16srRNA study revealed that B3 strain was found to be *Micrococcus luteus* and B4 was *Bacillus aquimaris*.

#### Phylogenetic tree of *Micrococcus luteus*



#### Phylogenetic tree of *Bacillus aquimaris*

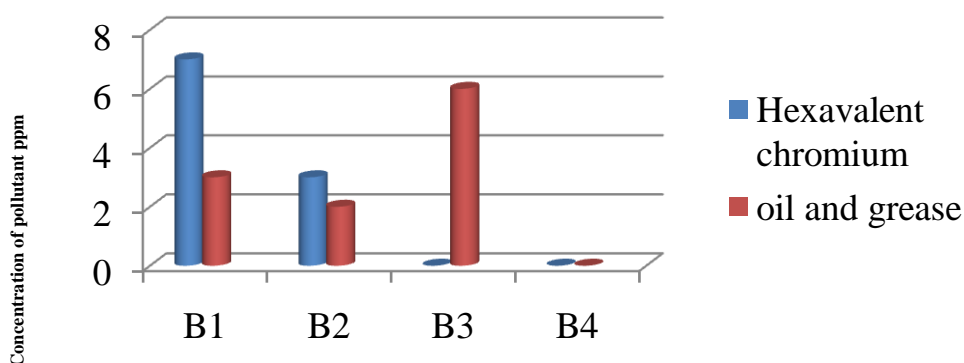


**Degradation activity**

Degradation of hexavalent chromium, oil and grease using isolated bacteria was performed. After degradation the effluent was analyzed for physio-chemical and biological parameters. Results revealed that B3 and B4 were effective in degradation of hexavalent chromium, oil and grease. Both the bacteria degraded the hexavalent chromium completely. B3 was found to degrade the oil and grease to reach 6ppm. B4 was found to degrade oil and grease completely.

PARAMETERS	B1	B2	B3	B4
Hexavalent chromium(ppm)	7	3.5	Nil	Nil
Oil and grease(ppm)	3	2	6	Nil

**Level of hexavalent chromium, oil and grease after biodegradation**



**Discussion** Bacterial isolates

The raw effluent collected from paper mill industry was subjected to various physio – chemical and biological parameters .The raw effluent was found to contain a pH of 8.35, TDS – 1540ppm, TSS – 96ppm, oil and grease – 30ppm, BOD – 42ppm, COD – 200ppm, zinc – 63ppm, and hexavalent chromium – 14ppm. From this analysis, it was

clear that oil and grease, hexavalent chromium and zinc are more than the effluent standard limit provided by the environmental pollution control board.

In a study, bacterial isolates *Micrococcus luteus*, *Pseudomonas putida*, *Serratia marcescens* and *Acinetobacter calcoaceticus* tolerated hexavalent chromium beyond 500 ppm. (V. Mishra et al., 2010). Researches also reveal that *Bacillus* sp was found to reduce the hexavalent chromium from chromite mine overburden soil (B. Dhal et al., 2012). A maximum of about 88% of hexavalent chromium was removed by *Pseudomonas putida* in 96 hours. (Harpreet kaur et al., 2014). In accordance to earlier reports with different organisms, an effort was made to degrade hexavalent chromium, oil and grease using bacterial isolates generated from soil samples and effluent sample. After isolating different bacterial isolates from the soil *Micrococcus luteus* and *Bacillus aquimaris* was found to have high degrading activity.

*Micrococcus luteus* have been already studied in biodegradation activity of different metals. On the other hand *Bacillus aquimaris* is a new organism found to have degradation activity against hexavalent chromium, oil and grease. *Micrococcus luteus* degraded the hexavalent chromium completely and oil and grease to reach 6 ppm. Whereas *Bacillus aquimaris* have degraded both the hexavalent chromium and oil and grease completely. From the result of this study, it may be concluded that the present study can be used in paper mill effluent treatment and also the results of study adds to the knowledge of tertiary treatment technique. The present study also calls for more studies employing microorganisms in the treatment of toxic waste in effluent.

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