



Biodegradation of toxic effluent using immobilized beads of Rhizobium

Sheela S.

sheelasloyola@gmail.com

Loyola College, Chennai, Tamil Nadu

Jenisha J. J.

jenishajj287@gmail.com

Loyola College, Chennai, Tamil Nadu

ABSTRACT

Rhizobium was isolated from the root nodules of *Arachis hypogea*. Biochemical and molecular characterization of the isolate was studied. The isolate was immobilized using 3% sodium alginate. Characteristics of the industrial effluent were analysed. The immobilized beads of the *Rhizobium* have the capacity to degrade the toxic chemicals present in the effluent.

Keywords— *Rhizobium*, Effluent, Degrade

1. INTRODUCTION

Water pollution is a major problem in the global context and has even been suggested to be the leading cause of death and disease worldwide, especially in developing countries. High production and use of dyes generate coloured wastewaters that pollute rivers: their presence in surface water blocks solar radiation from reaching aquatic organisms, thus affecting negatively the balance of ecosystems (Kindala et al., 2015)

The dyes like azo dye are toxic and highly carcinogenic. These toxic dyes are unfortunately derived to be resistant to many degradation processes, and those are highly toxic and may lead to cancer. (Dutta H et al., 2014).

Rhizobium is a well-known bacteria, which act as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants leading to the formation of lumps or nodules where the nitrogen fixation takes place. Set of genes in the bacteria control different aspects of the modulation process. One *Rhizobium* strain can infect certain species of legumes but not others. When legume plants encounter low nitrogen conditions and want to form a symbiotic relationship with *Rhizobia* they release flavonoids into the soil. *Rhizobia* responds by releasing nodulation factors which stimulate the formation of nodules in plant roots which in turn helps in a symbiotic relationship.

Various physicochemical methods are used to detoxify toxic effluent to achieve degradation. These include filtration, coagulation, carbon activated and chemical flocculation. These methods are effective but they are expensive and involve the formation of a concentrated sludge that creates a secondary

disposal problem. Microbial degradation is an environmentally friendly and a cost competitive effective to chemical decomposition process (Telke A et al., 2008).

Annually more than 50% of azo dyes are used. Around 2000 of them are used in the textile industry, leather, plastics, paper, cosmetics and food industries. Azo dyes are characterized by the presence of one or more azo groups substituted with aromatic amines. A substituent usually found in the azo dyes is the sulfonic acid. The azo dyes have this substituent is commonly known as sulfonated azo dyes. These azo dyes are used in various industries. The fixation rate of these reactive dyes in dyeing is as low as 50%, which results in the release of 10-15% water-soluble azo dyes into the environment through waste water discharge. Sulfonated and unsulfonated azo dyes have a negative bad effect on wastewater and some of these compounds and biodegraded substituents are toxic, carcinogenic, and mutagenic. These dyes have a number of structural diversity and they are made to resist physical, chemical and microbial attack. (Selva Raj et al., 2012).

2. MATERIALS AND METHODS

2.1 Media

Yeast Extract Mannitol Agar (YEMA/Congo red) is a selective media commonly used for the cultivation of soil microorganisms like *Rhizobium* species and studying root nodulation. It contains Mannitol, yeast extract, Dipotassium phosphate, magnesium sulphate, sodium chloride, agar and congo red. Congo red acts as an indicator.

2.2 Isolation of a bacterial strain

Root nodules were collected from *Arachis hypogea*, Loyola College. The root nodules were washed with 95% alcohol for 30 seconds to remove other microorganisms and again washed with sterile water. Using sterile forceps the root nodules were transferred into sterile mortar and pestle and gently it was grinded. The root nodule suspension was diluted by adding sterile water and serially diluted. A loopful of culture from 10^{-3} dilution was taken and spread plate technique was performed on Yeast Extract Mannitol Agar plate. The inoculated Petri plates were incubated for 24 hours at 37°C. After 24 hours the plates were observed.

2.3 Biochemical and molecular characterization

Series of biochemical tests were done. Which includes Gram’s staining, Oxidase test, Catalase test, methyl red, Voges-Proskauer, Urease test, nitrate reduction test, citrate utilization test, Indole acetic acid test and Triple sugar iron test. The isolated *Rhizobium* was inoculated in 100 ml freshly prepared Yeast Extract Mannitol Broth. The inoculated broth was incubated at 37°C for 24hrs. The bacterial genomic DNA was isolated using a Hyper Genomic DNA isolation kit method. 1Complete reagent recipe for polymerase chain reaction includes PCR grade water 19µl, 5 primers, forward primer 1.5µl, reverse primer 1.5µl, template DNA 3µl, PCR master mix 25µl. All the components of the reaction mixture were mixed well except the sample DNA solution. The mixture was loaded into PCR tubes. 8F forward primer and 1490 Reverse primer was used.

Table1: PCR Primer

PCR Assay	Target Gene	Primer name	Primer sequence	Product size
Rhizobium	16SrRNA	8F-Forward 1490-Reverse	AGAGTTTGTATCCT GACTTACCAGGGT	700 bp

2.4 Immobilization of Rhizobium

The isolated Rhizobial species was immobilized in 3% sodium alginate.

2.5 Biodegradation of toxic effluent

The effluent along with the media was incubated with immobilized beads of Rhizobium for about 48 hrs at 37°C.

2.6 Decolorization

The immobilized bacterial culture was inoculated in the conical flask containing 100 mL yeast mannitol broth and was grown in static or shaking conditions (120 rpm) and incubated at 30°C for 48 hrs. The optical density of the dye was taken before and after incubation by using UV- Visible Spectrophotometer.

3. RESULTS

The Rhizobial cells were isolated from the root nodules of *Arachis hypogea*. The growth of Rhizobium was clearly visible in Yeast Extract Mannitol Agar (YEMA) containing Congo red.



Fig. 1: Rhizobium on YEMA medium

The Rhizobial colonies were observed as mucoid, translucent and slightly pink colonies. Gram staining showed Gram negative rods. The isolated genomic DNA was electrophoresed on 0.8 % Agarose gel which was stained with Ethidium Bromide. After running the gel, it was observed under UV transilluminator. Clear bands were observed. The PCR reaction product was analyzed by Agarose gel electrophoresis and the DNA of the expected size was purified and sequenced.

An amplicon of 700 bp was obtained on PCR amplification of the DNA with specific forward and reverse primers. The blasted result was MG2708921:2-667Unidentified microorganism

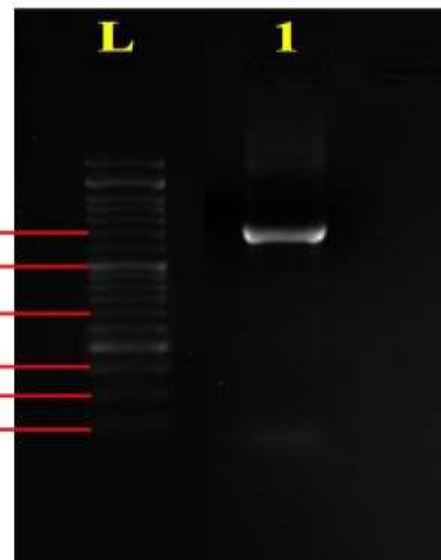


Fig. 2: Agarose Gel Electrophoresis showing DNA band at 700bp

Table 2: BLAST result of the isolated organism

Score	Expect	Identities	Gaps	Strand
1142 bits(618)	0.0	653/669(98%)	5/669(0%)	Plus/Plus

The presence and amount of carbon-containing chemicals were assessed by GC-MS. The mass of certain chemicals shows high. The mass of Benzamide,4-butyl-N-(2-ethylphenyl) was the highest of 388.30 m/z and the mass of 5,6-Dihydro-5-methyluracil was the least of 42.20 m/z. Totally 15 different chemicals were identified in the sample.

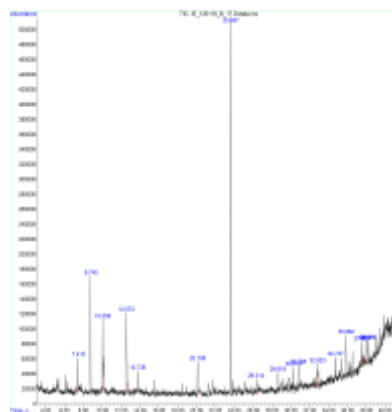


Fig. 3: GC- MS graph before treatment

Rhizobium was immobilized in 3% sodium alginate.



Fig. 4: Immobilised beads with Rhizobium

The capsules appeared spherical in shape and uniform diameter. After treating the effluent with the immobilized isolate GC-MS was done.

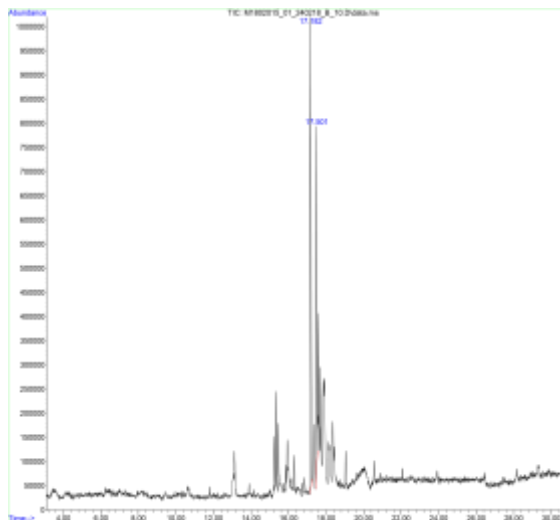


Fig. 5: GC- MS graph after treatment

The number of chemicals had reduced from 15 to 2. octanoic acid and heptanoic acid-oxo-trimethylsilyl ester were the two chemicals found in the sample after degradation.



Fig. 6: Effluent before treatment with immobilised beads



Fig. 7: Effluent after treatment with immobilised beads

The intention of this study was to check the ability of organism for decolourization of dye. The immobilized isolate decolourized the dye in the sample.

4. DISCUSSION

Rhizobia or bacteria isolated from the root nodules are rod-shaped and are Gram-negative. *Rhizobia* are Gram-negative and are mobile. Uneven Gram staining is frequently encountered with *Rhizobia*, depending on the age of the culture. Cells from a young culture and nodule bacteroids usually show even Gram staining while older and longer cells give a banded appearance with unstained areas. Most *Rhizobia* only weakly absorb Congo red (diphenyldiazo-bis- α -naphthylamine sulfonate) dye, which is included in culture media for isolating *Rhizobia*. The

optimum temperature for the growth of *Rhizobia* is 25-30°C. *Rhizobium* is bacteria capable of forming a nitrogen-fixing symbiosis with leguminous plants. (Keyser et al.)

The *Rhizobial* isolate was identified on the basis of their morphological, cultural, microscopic and biochemical characteristics. The standard description is given in “Bergey’s Manual of Determinative Bacteriology”. The molecular characterization was done after the Polymerase Chain Reaction. The e-value was found to be 0.0 and the similarity with bacillus species was 96%. Thus it has no 100 % similarity with any other organism. The biochemical test identifies this organism as *Rhizobium* hence it should be an unidentified *Rhizobium* species.

The effluent had 15 different types of chemicals. Which includes allyl methyl sulphide, Benzenediazepin, octadecamethyl, octaatomic sulphur. These are toxic chemicals which cause various health issues. Contact with these chemicals can cause skin irritation. Inhalation of these chemicals will cause health hazards. After contact with the skin, wash immediately with plenty of water. Very hazardous in case of ingestion, hazardous in case of skin contact, of eye contact, of inhalation. These are may be combustible at high temperature. After the degradation, the characteristics of the sample had changed. After degradation only 2 chemicals were found, an octanoic acid mass was 117.10 m/z and heptanoic acid-oxo-trimethylsilyl ester, mass was 75 m/z (Kabra et al, 2012)

The entrapment technology, immobilization had helped the *Rhizobium* to grow and multiply within the matrix. Thus the immobilized beads of *Rhizobium* were able to degrade the toxic chemicals found in the sample. The main advantage of the immobilization of *Rhizobium* using sodium alginate is that the beads can be re-used for the treatment of the effluent. (N Puvaneshwari et al., 2006)

Thus various types of chemicals discharged from various industries can be treated by *Rhizobium*. The effluent treated with the immobilized *Rhizobium* will not cause any environmental hazard. Hence this type of eco-friendly treatment is necessary for a healthy environment. This type of biological method can be used for any kind of effluent treatment.

Rhizobial beads are also used for the reduction of dye (1% methylene blue). Both shaking and static conditions showed dye degradation. In one set of degradation eight beads were used. In another one set of degradation 12 beads were used. Degradation of dye in both static and shaking condition was observed. Degradation in the shaking condition was faster when compared to the shaking condition. Degradation in the shaking condition with 12 beads showed faster than the degradation in the shaking condition with 8 beads. Thus this may be due to a number of *Rhizobial* cultures was higher in 12 beads than the 8 beads. Thus the degradation efficiency can be increased by adding 2 ml of culture. It took 48 hours to degrade 93% of methylene blue dye under shaking condition with 12 beads. By increasing the number of beads the time required for the degradation can be reduced. Maybe the dye was absorbed by sodium alginate which was used to entrap the *Rhizobium*. Control without sodium alginate fails to explain this. Sodium alginate is a gum derived from seaweed.

Methylene blue is a dye used in laboratories. High production and use of dyes generate coloured wastewaters that pollute rivers; their presence in surface water blocks solar radiation from reaching aquatic organisms, thus affecting the balance of aquatic ecosystems. In addition, the release of dyes into water

streams may result in the formation of toxic and carcinogenic degradation products. Hence, the removal of dyes from industrial effluent is an important and necessary process. Discolouration of water has become one of the major issues in waste water treatment as many industries use dyes to color products such as textiles, rubber, paper, plastics, leather, cosmetics, food and minerals. (Kindala et al., 2015)

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