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Biodegradation of Toxic Effluent Using Immobilized Beads of *Rhizobium*

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Abstract:- The effluent industries is a major source of water pollution. So their treatment before discharge is very crucial and biodegradation where microorganisms break down the azo bonds to form its nontoxic basic element is the most effective way. Toxic effluent present in the industrial effluent has highly carcinogenic substances like aromatic amines. The study involved isolation of *Rhizobium*, molecular and biochemical characterization, immobilization of *Rhizobium* in sodium alginate capsules and the examination of biodegradation of toxic chemicals present in the industrial effluent using the encapsulated *Rhizobium*, decolourization of methylene dye using immobilized beads. The spectrophotometric assessment of dye degradation was by measuring at optical density 530nm. Biodegradation of toxic effluent by immobilized *Rhizobium* is the novel technology that may be applied for the treatment of waste water containing a mixture of different dyes.

Keywords:- *Rhizobium; Immobilization; Biodegradation; Decolourization.*

I. INTRODUCTION

Water pollution is 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 colored waste waters that pollute rivers: their presence in surface water blocks solar radiation from reaching aquatic organisms, thus affecting negatively tea balance of ecosystems [1]. The dyes like azo dye are toxic are highly carcinogenic. These toxic dyes are unfortunately derived to be resistant to many degradation process, and those are highly toxic and may lead to cancer [2].

Rhizobium is a well-known bacteria that 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 nodulation 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 stimulates the formation of nodules in plant roots which in turn helps in symbiotic relationship.Various physico chemical 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 [3].

Annually more than 50% of azo dyes are used [9]. Around 2000 of them are used in textile industry, leather, plastics, paper, cosmetics and food industries [7]. Azo dyes are characterized by the presence of one or more azo groups substituted with aromatic amines [7]. A substituent usually found in the azo dyes is the sulfonic acid. The azo dyes has this substituent are commonly known as sulfonated azo dyes. These azo dyes are used in various industries [8]. The fixation rate of these reactive dyes in dying is as low as 50%, which results the release of 10-15% water soluble azo dyes into the environment through waste water discharge. Sulfonated and unsulfonated azo dyes has negative bad effect on waste water and some of these compounds and biodegraded substituents are toxic, carcinogenic, and mutagenic. These dyes have more number of structural diversity and they are made to resist physical, chemical and microbial attack [4].

II. MATERIALS AND METHODS

- *Media preparation*

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.

- *Isolation of bacterial strain*

Root nodules of *Arachis hypogea* were collected from Loyola College, Chennai and Tamil Nadu. The collected root nodules were washed with 95% alcohol for 30 secondsto remove other microorganisms and again washed with sterile

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water. Using sterile forceps the root nodules were transferred into sterile mortar and pestle and gently it was grounded. 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 37ºC. After 24 hours the plates were observed.

- *Biochemical and molecular characterization*

Series of biochemical tests were done which includes Gram's staining, Oxidase test, Catalase test, methyl red, Voges-Proskeur, 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 37ºc for 24hrs. The bacterial genomic DNA was isolated using Hyper Genomic DNA isolation kit method. Complete reagent recipe for polymerase chain reaction includes PCR grade water 19µl, 1.5µl forward and reverse primer, template DNA 3µl and PCR master mix 25µl. All the components of reaction mixture were mixed well except the sample DNA solution. The mixture was loaded into PCR tubes 27forward primer and 1490 Reverse primer.

- *Immobilization of Rhizobium*

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

- *Biodegradation of toxic effluent*

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

- *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.

III. RESULTS

The Rhizobial cells were isolated from the root nodules of *Arachis hypogea*(Fig 1: A). The growth of Rhizobium was clearly visible in Yeast Mannitol Agar containing Congo red. The Rhizobial colonies were observed as mucoid, translucent and slightly pink colonies(Fig1: B). Gram staining showed Gram negative rods (Fig1: C). The isolated microorganism was identified as Rhizobium by the biochemical characters from Bergey's Manual (Fig 1: D to H and Table 1). 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 (Fig 2).The presence and amount of carbon containing chemicals were assessed by GC-MS (Fig 3). 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 (Table 2).Rhizobium was immobilized in 3% sodium alginate. The capsules appeared spherical in shape and uniform diameter.After treating the effluent with the immobilized isolate GC-MS analysis was done (Fig 5). The number of chemicals had reduced from 15 to 2 (Table 3). Ocatanoic acid and hepatanoic acid-oxo-trimethylsilyl ester were the two chemicals found in the sample after degradation.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

IV. 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 bacteriods 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-napthylamine sulfonate) dye, which is included in culture media for isolating Rhizobia. Optimum temperature for the growth of Rhizobia is 25-30ºC. *Rhizobium* are bacteria capable of forming a nitrogen-fixing symbiosis with leguminous plants [5].

The Rhizobial isolate was identified on the basis of their morphological, cultural, microscopic and biochemical characteristics including size, shape form of the bacterial cells, presence or absence of spores, Gram reaction, Urease, Nitrate reduction, etc. the standard description given in " Bergey's Manual of Determinative Bacteriology" [6]. The molecular characterization was done after 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 *Rhizbium* hence it should be an unidentified *Rhizobium* species.

The effluent had 15 different types of chemicals. Which includes allyl methyl sulphide, Benzenedaiazepin, octadecamethyl, octaatomic sulphur. These are toxic chemicals which causes 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, octanoic acid mass was 117.10 m/z and hepatanoic

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acid-oxo-trimthylsyl ester, masss 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 *Rhizobiu*m 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 [11]. 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 the 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) [10]. 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 were 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 number of Rhizobial culture 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 beads the time required for the degradation can be reduced. May be 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 waste waters 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 formation of toxic and carcinogenic degradation products. Hence, the removal of dyes from industrial effluent is an important and necessary process [12]. Discoloration of water has become one of the major issues in waste water treatment as many industries use dyes to colour products such as textiles, rubber, paper, plastics, leather, cosmetics, food and minerals[13].

V. CONCLUSION

In the present study, the isolated microbe degraded toxic chemicals present in the effluent and also decolorized methylene blue. Thus this micro organism can be used to treat the effluent from the various industries. The microbe was immobilized within the sodium alginate. Thus the organisms will be trapped inside the matrix. Thus the beads can be re-used to treat the effluent. Direct culture of the isolated microbe can also be added to treat the effluent. This method of treatment is eco-friendly. This method of treatment is eco-friendly. Biological treatment method is highly efficient also. These microbes need suitable substrate to grow and multiply. Various carbon and nitrogen sources can be provided for the growth of the organism. Those organisms will take time to multiply and thus degradation time is also longer when compared to other methods. But the microbial biodegradation is much safer than the other methods of degradation.

ISOLATE	INDOLE TEST	CITRATE UTILIZATION	METHYL RED	VOGES PROSKEUR	UREASE TEST	NITRATE REDUCTION	OXIDASE TEST	CATALSE TEST
R_1	-	+	-	-	+	+	+	+

Table 1:- Biochemical Tests

S. No	Compound	RT (min)	Area (%)	Mass m/z	Super Impossibility
1	Sulphide, allyl methyl	7.415	3.47	73.20	53
2	5,6-Dihydro-5-methyluracil	8.745	8.75	42.20	72
3	2-Propylnoanoic acid	10.058	7.17	73.10	50
4	Piperidin-4ol,2,3,dimethyl trans	12.572	10.52	114.10	38
5	Cyclic octaatomic sulphur	13.738	2.05	63.90	56
6	Imidazole	20.108	4.82	42.20	78
7	1,4-Benzenedaiazepin-2-one	26.314	2.18	73.20	38
8	Cyclolnonsailoxane, octadecamethyl	28.168	2.85	207.0	43
9	Hexa (methoxymethyl) melamine	30.274	3.27	207.10	38
10	Benzeneaceticacide, alpha, 3, 34, tris Trimethysilyl ester	30.788	2.00	207	38
11	18,19-secolupan-3-ol,3beta	32.82	2.54	281.00	14
12	Benzamide,4-butyl-N-(2-ethylphenyl)	35.86	3.25	388.30	27
13	Cyclodecasiloxane, eicosamethyl	37.507	2.46	207.20	25
14	Bianthrone	38.021	2.19	384.50	43
15	Octasiloxane-hexadecamethyl	38.219	1.94	207.20	38

Table 2:- Characteristics of the Effluent Before

S.NO	Compound	RT (min)	Area (%)	Mass m/z	Super Impossibility
1	Octanoic acid	17.180	61.96	117.10	30
2	Hepatanoic acid-oxo-trimethylsilyl ester	17.500	38.04	75.10	25

Table 3:- Characteristics of the Effluent after Biodegradation

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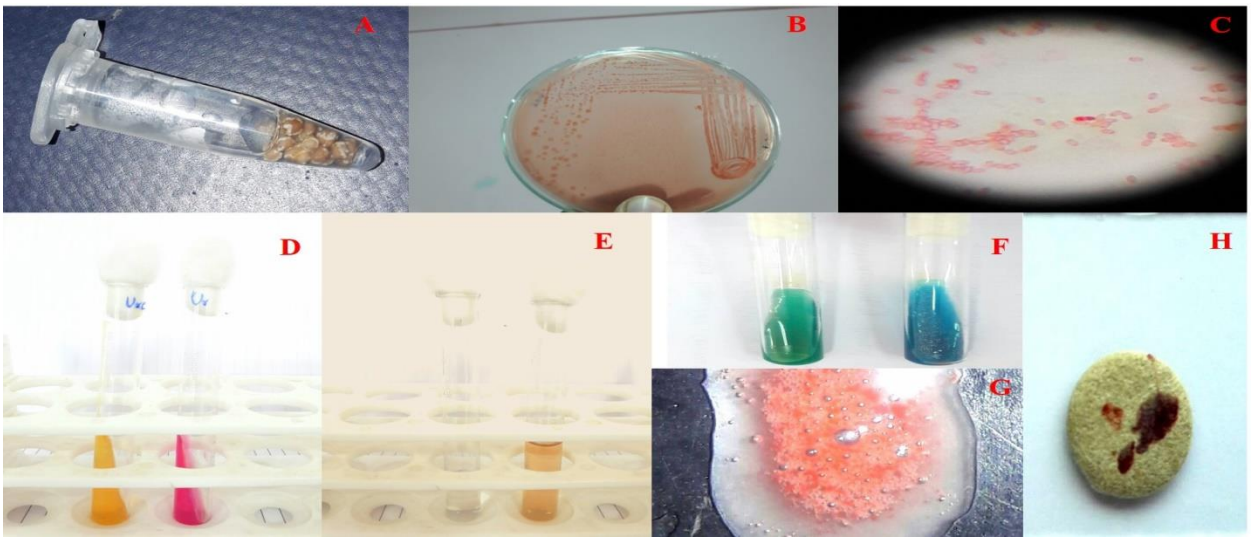

Fig 1:- A) Root nodules collected from *Arachis hypogea*;B) isolated colonies of *Rhizobium* C) Gram stainingD – H) Biochemical Tests such as, D) urease test, E) nitrate reduction, F) citrate utilization, G) catalase test and H) oxidase test.

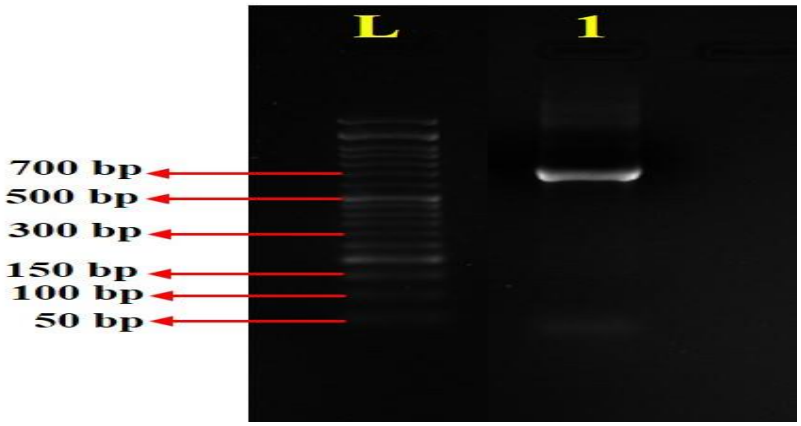

Fig 2:- PCR amplification of Genomic DNA from isolates - Lane L – 100 bp marker; Lane 1 – R_1

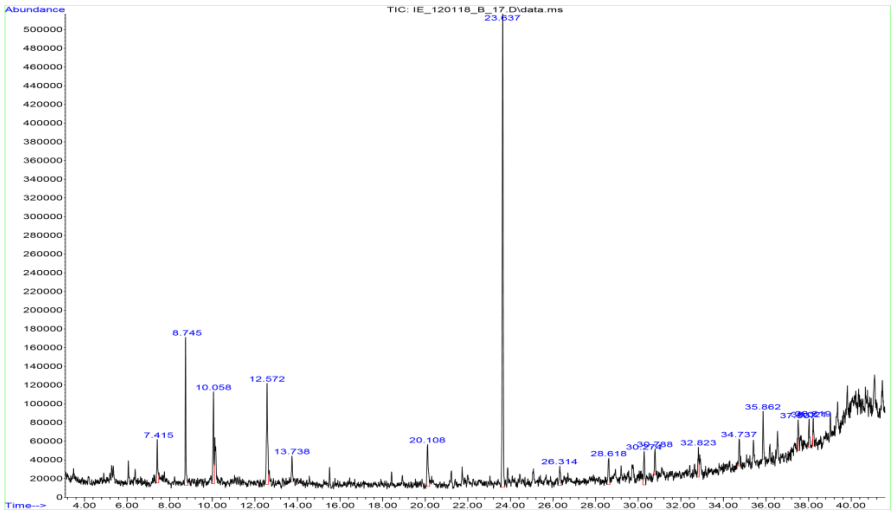

Fig 3:- Spectra indicating the characteristics of the effluent sample

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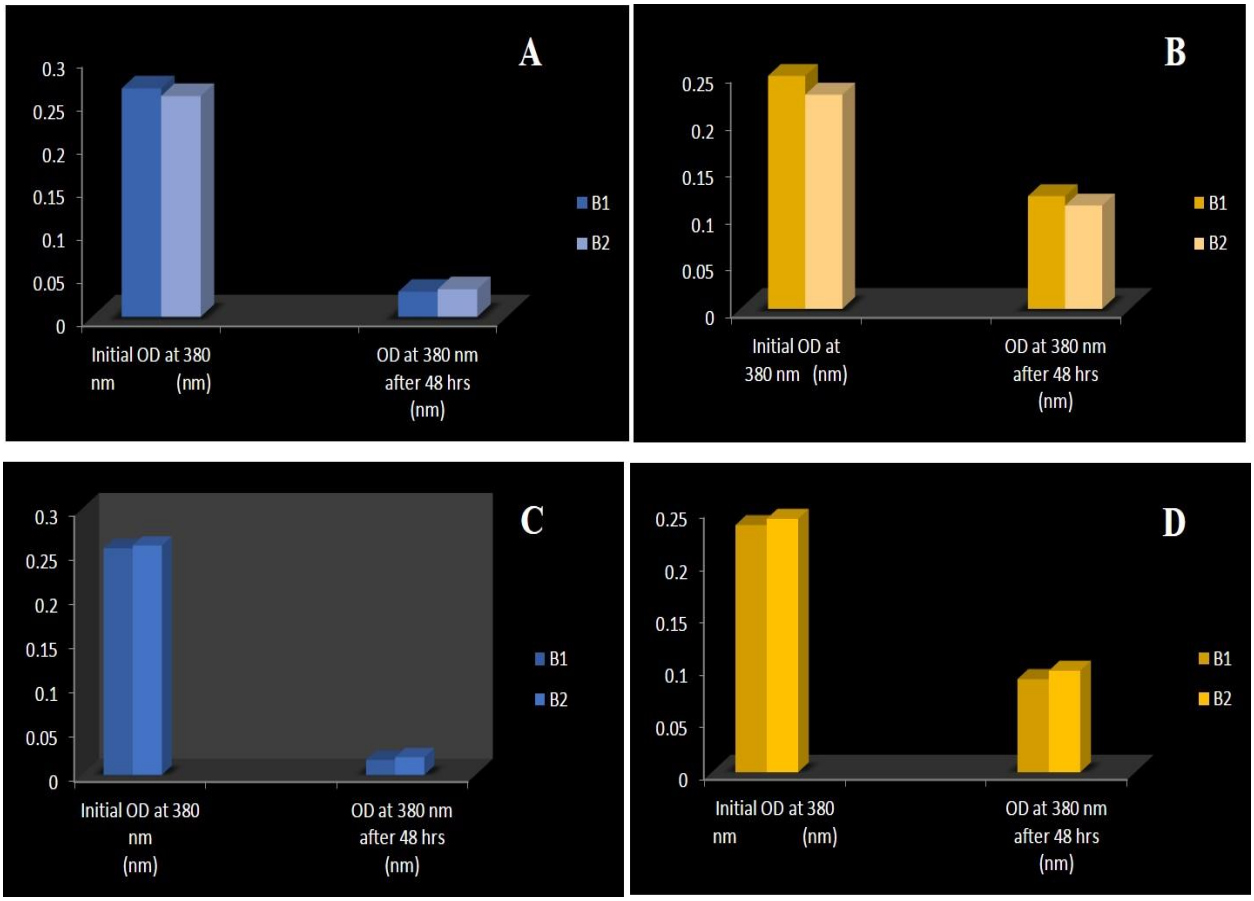

Fig 4:- A) Decolourization of 1 % methylene blue in shaking condition within 48 hours (8beads); B) Decolourization of methylene blue within 48 hours in static condition (8 beads); C) Decolourization of 1% methylene blue within 48 hours under shaking condition (12 beads); D) Decolourization of 1% methylene blue within 48 hours under static condition (12 beads) 10-13.

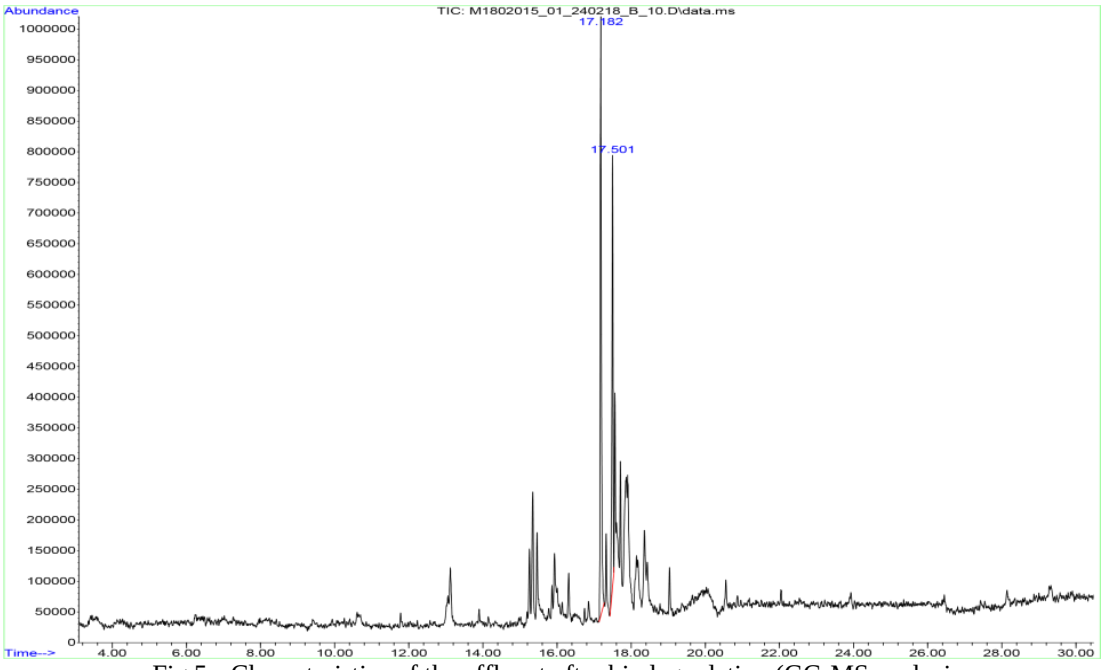

Fig 5:- Characteristics of the effluent after biodegradation (GC-MS analysi

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