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A STUDY ON TOLUENE DEGRADING BACTERIA

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Abstract:

Petroleum hydrocarbon are classified into four; (1) the saturates, (2) the aromatics, (3) the asphaltenes (phenol, fatty acids, ketones, esters and porphyrins) and (4) the resins (pyridines, quinolines, carbazoles, sulfoxides and amides). The aromatic hydrocarbons are the class of chemicals that deal with benzene and its derivatives. BTEX or BTX is a mixture of benzene, toluene, ethyl benzene and xylene. This group of volatile organic compounds (VOCs) is found in petroleum hydrocarbons and other environmental contaminants. They are aromatic hydrocarbons containing one unsubstituted or methyl-substituted benzene ring. BTEX compounds are classified as major pollutants with high frequencies of occurrence on the EPA (Environmental protection agency) list of priority pollutants. Toluene is alkyl benzene having one methyl group added to the benzene ring.It's IUPAC name is Methylbenzene. Toluene has a serious health effect on the nervous system (brain and nerves). Acute and chronic effects are also observed when humans are exposed to toluene. Humans are primarily exposed to toluene and it is moderately toxic when ingested or inhaled and slightly hazardous when absorbed through skin. Toluene can enter human body from the air, water or soil. When toluene is inhaled, it is directly taken into the blood from the lungs. Toluene leaves the body unchanged during respiration and excretion or it is converted into a less harmful chemical such as hippuric acid. The present study is aimed at isolating toluene degrading from polluted environment which can be used as an eco – friendly and efficient Bioremediation tool

Keywords **— Petroleum Hydrocarbon, Toluene, Health Effect, Eco-friendly and Bioremediation.**

INTRODUCTION

Toluene is an aromatic hydrocarbon and its structure is shown in Figure 1. Its natural source includes tolu tree. Toluene is alkyl benzene having one methyl group added to the benzene ring.It's IUPAC name is Methylbenzene (Angela Woods *et. al.,* 2011).

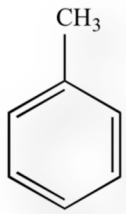

Figure 1: Toluene

If toluene is inhaled, the vapours irritate eyes and upper respiratory tract; cause anaesthesia and respiratory arrest (Beller *et. al.,* 1996). If inhaled, at 100 ppm concentration, psychological effects and transient irritation is seen. At 200 ppm, central nervous system is affected. At 400 ppm, mild eye irritation, lacrimation and hilarity are seen. At 600 ppm, lassitude, slight nausea are observed. At 800 ppm, metallic taste, headache, lassitude are experienced (Gericke *et. al.,* 2001). At approximately 1000 ppm it produces symptoms of CNS depression. It is followed by tremor, muscle cramps, behavioural changes, loss of judgement and weakness. At exposures above 1000 ppm in the air, it results in loss of consciousness and coma (Jacob H. Jacob and Fawzi I. Irshaid, 2015).

High levels of toluene exposure during pregnancy may lead to retardation of growth and mental abilities in children (M. Gopinath and R. Dhanasekar, 2012). Other health effects include damage to liver, kidney and respiratory system. According to EPA, exposure to high levels of toluene in occupational settings was found to have an increase in leukemia (Jessica R. Hanson *et.al.,* 1999). Continuous exposure to toluene can exert mutagenic effects on human cells due to covalent binding to DNA. It is also a human neurotoxin and may cause leukoencephalopathy at the long exposure time. Toluene causes death by interfering with the respiration pattern and heart beat (Lee *et.al.,* 1994).

Toluene is of lesser risk to non-human species such as fish and wildlife (Tury *et.al.*, 2003). This is because toluene is volatile and evaporates into the atmosphere. When there is a concentrated spill in land or water, the plants, fishes and birds are affected (Mukherjee AK and Bordoloi NK , 2012). At higher concentrations, toluene is dangerous to aquatic life and fouling to shoreline (Smith *et.al.,* 1945). The acute and chronic effects due to toluene exposure have been explained in Table 2 and Table 3.

ACUTE EFFECTS

ROUTE OF EXPOSURE	ORGAN AFFECTED	EFFECTS
Inhalation	Central nervous system (CNS)	Headache, dizziness, blurred vision, nausea, coma and death.
Inhalation	Respiratory tract	RADS (Reactive Airway Dysfunction Syndrome) and hydrocarbon pneumonitis.
Inhalation	Heart	Irregular heart rhythm and cardiac arrest.
Inhalation	Kidney	Renal tubular acidosis, myoglobinuria, glomerulonephritis and renal failure.
Contact	Skin	Irritation, redness and blisters.
Contact	Eyes	Inflammation, eye irritation, conjunctivitis, keratinitis and blepharospasm.
Ingestion	Gastrointestinal	Nausea, vomiting and diarrhoea.

Table 2: Acute effects due to Toluene Exposure

CHRONIC EFFECTS

ROUTE	EFFECTS	RESULTS
Inhalation	Disorders of muscles, permanent neuropsychiatric effects, renal tubular damage.	Sudden death.
Inhalation or ingestion	Nausea and vomiting	No carcinogenic effects
Ingestion	Excreted in breast milk, microcephaly, CNS dysfunction and limb anomalies.	Not teratogenic in nature. Causes reproductive and developmental defects (fetotoxic)

Table 3: Chronic effects due to Toluene Exposure

In the present study, bacteria which are capable of degrading toluene are isolated and their efficiencies are tested by growing them at various concentrations. The strain capable of degrading higher concentrations can be used as a tool for toluene bioremediation.

MATERIALS AND METHODOLOGY

COLLECTION OF SAMPLES

Soil samples were collected from mechanic shed in Medavakkam (**Figure 2**). The samples were collected in a sterile container and they were stored air tight. The soil samples were taken from a depth of 10-15cms and the samples were cleaned by removing large stones and pebbles.

Figure 2: Medavakkam - Sample Collection Site

The soil and marine microbes were enriched in nutrient broth medium. Following this it was further enriched with Minimal Salt Medium and Minimal Salt Agar which contains toluene as carbon source.

SERIAL DILUTION

The incubated tubes were taken for serial dilution. 9ml of saline was added to the 10 sterilized test tubes. 1ml from the incubated test tubes was added to the first test tube which gives 1:10 dilution. The tube was mixed well and 1ml from the 1:10 dilution was transferred to the second tube which gives 1:100 dilutions. This was continued till the 8^{th} tube and 1ml from the 8^{th} tube was discarded. Dilutions such as 10^5, 10^6 and 10^7 were chosen for Streak plating and the selected colonies were subjected to Gram Staining.

BIOCHEMICAL CHARACTERIZATION

Biochemical tests for identification of microbes are a set of biochemical tests which allows preliminary identification of microorganism. Biochemical tests are more specific than staining techniques. These tests involve chemicals and each genus of microbes have specific results with these chemicals. Catalase Test, Indole Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Triple Sugar Iron Test and Urease Test are the Biochemical Tests performed in the present study.

THIN LAYER CHROMATOGRAPHY

The solvent Petroleum ether : Acetone (9:1) was used as a mobile phase for the compound in the sample to be identified. Silica slurry coated TLC plate was used as a stationary phase. A line was drawn at the bottom of the TLC plate and the sample was placed using the capillary tube over the line marked. The TLC plate was placed in a beaker containing the mobile phase and was left undisturbed for the solvent to reach the top of the TLC plate. The TLC plate was removed and air dried. The pigment was identified by observing under UV trans-illuminator. The Retention Factor (**R_f**) of the compound was calculated using the formula

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

GAS CHROMATOGRAPHY - MASS SPECTROSCOPY (GC-MS) (PERFORMED AT YAZH XENOMICS)

The sample which contained 2% toluene was treated with the isolated bacteria. The treated samples were analyzed for chemical constituents using GC-MS. The measurements of samples were conducted on GCMS-QP 2010 plus. 1 µL of the sample was injected using the split less injection mode, which was held at 300°C. A Capillary column (capillary 30m length, 0.32mm dia) was used for analytical separation. Helium was used as a carrier gas at a flow rate of 1 mL.min-1. The oven was pre programmed from 60 to 130°C at a rate of 15°C.min^{-1} , then ramping from 130 to 315°C at a rate of 3°C.min^{-1} and finally held there for 15 minutes. The mass spectrometer operated in a full scan mode in the range of m/z 50-550 and by electron impact ionization energy of 70 eV.

RESULTS AND DISCUSSION

ENRICHMENT OF SAMPLE

The soil and water samples were enriched in nutrient broth. After 24 hours of incubation, turbidity was observed in the flask indicating the growth of bacteria (**Figure 3**). The growth of bacteria in nutrient medium aided in increasing in the number of bacteria present in the sample because addition of sample directly in a harsh medium containing toluene reduces the chances of growth of bacteria.

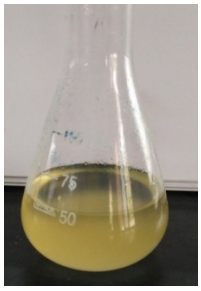

Figure 3: Sample enriched in nutrient broth

MINIMAL SALT MEDIUM

The samples from the incubated nutrient broth were used to screen the toluene degrading bacteria. Minimal salt medium was used to screen the toluene degrading isolates because minimal salt medium contains salts like magnesium, potassium which are used by the bacteria for the synthesis of nucleic acids and proteins. This is supplemented with 1% toluene so that only the ones which can utilize toluene as a source of carbon are grown. After 72 hours of incubation in minimal salt medium with 1% toluene, tube S4 showed turbidity indicating the growth of bacteria (**Figure 4**). This sample was further used for spread plate method and degradation studies.

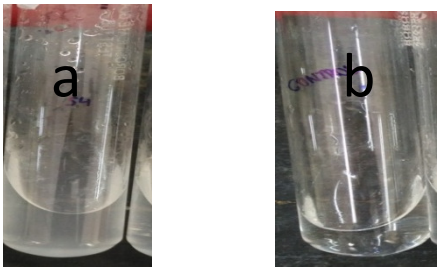

Figure 4: a) Growth in MSM supplemented with 1% Toluene
b) Control tube with no growth

SERIAL DILUTION

Serial dilution was performed using the tube which showed turbidity and the dilutions 10^{-5}, 10^{-6} and 10^{-7} were used for spread plate technique. These dilutions were selected as they would reduce the number of bacteria in the sample. This helped in obtaining isolated colonies by spread and streak plate technique (Figure 5).

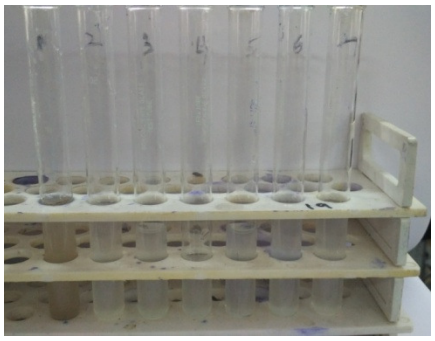

Figure 5: Serial dilution using soil sample

MINIMAL SALT AGAR

Spread plate technique was done and incubated for 72 hours to observe growth of colonies. This technique yielded two different colonies on MSM agar plate. Streak plate method was also performed to obtain isolated colonies. The colours of the colonies obtained were white and creamy white whose colony was pin pointed in nature while the white colony was mucoid in nature **(Figure 6)**.

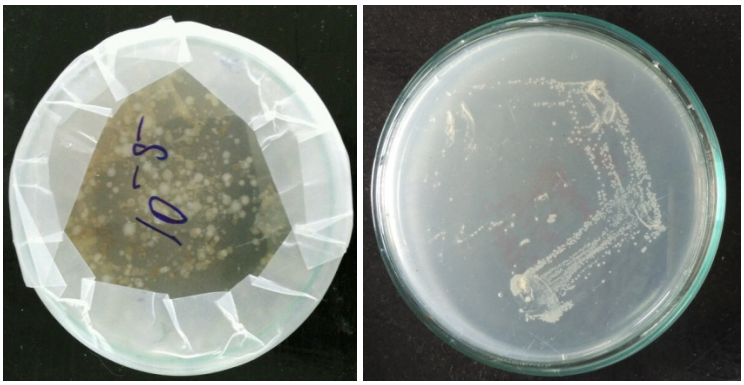

Figure 6: a) Colonies obtained by spread plate technique

b) Colonies obtained by streak plate technique

GRAM STAINING

Both the isolated bacteria were Gram negative and rod shaped **(Figure 7)**.

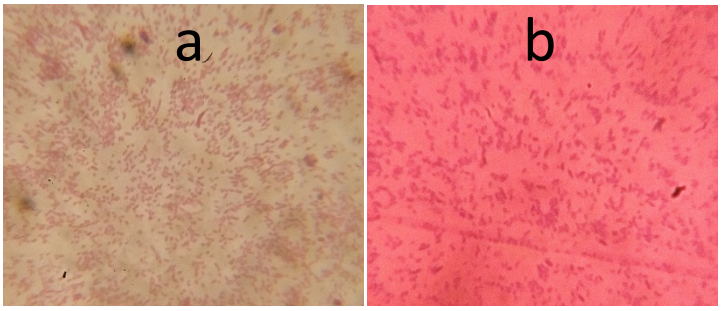

Figure 7: a) Gram negative and rod shaped b) Gram negative and rod shaped

BIOCHEMICAL TEST

Biochemical tests are performed to identify the isolated organism. Various tests such as indole, Methyl Red (MR), Voges Proskauer (VP), Citrate utilization, Urease, Catalase, Oxidase, Triple Sugar Iron (TSI) agar tests are performed to test whether the bacteria utilizes the particular growth medium and identify based on the results. This majorly helps in identifying the Genus of the isolated bacteria.

BACTERIUM 'a'

The bacterium 'a' exhibites positive results for Indole, MR, Citrate utilization test and Catalase tests. VP, Oxidase and Urease tests showed negative results for this bacterium. TSI slant and butt showed acid production with gas formation (Figure 8 and Table 1).

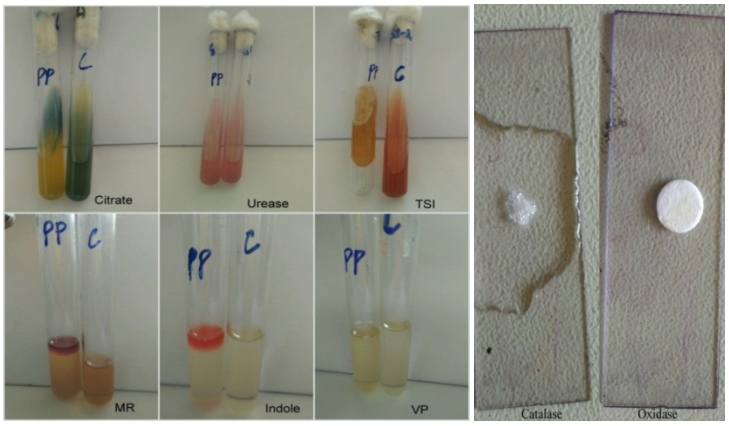

Figure 8: Biochemical test results for Bacterium 'a'

S.NO.	BIOCHEMICAL TEST	RESULT
1.	Indole test	Positive
2.	Methyl red test	Positive
3.	Voges Proskauer test	Negative
4.	Citrate utilization test	Positive
5.	Urease test	Negative
6.	Triple sugar iron agar test	A/A, gas production
7.	Oxidase test	Negative
8.	Catalase test	Positive

Table 1: Biochemical test results for Bacterium 'a'

BACTERIUM 'b'

The bacterium 'b' exhibited positive results for Indole, MR, Citrate utilization test and Catalase tests. It was negative for Oxidase, VP and Urease tests. TSI slant showed acid slant and butt with gas production (Figure 9 and Table 2).

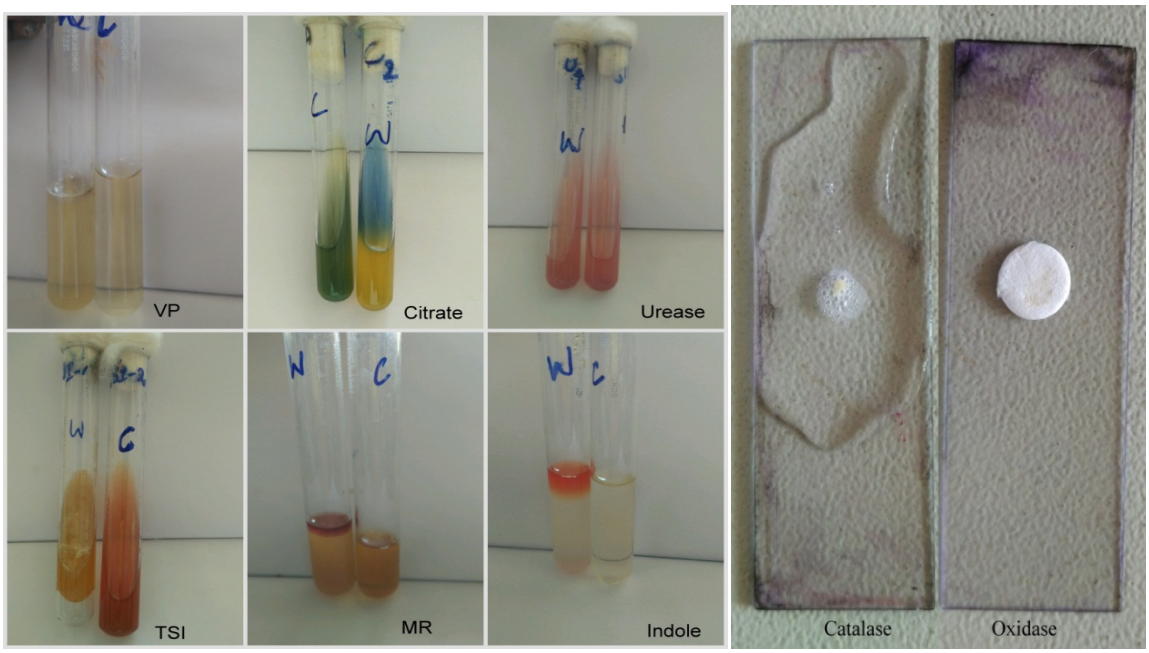

Figure 9: Biochemical test results for Bacterium 'b'

S.NO.	BIOCHEMICAL TEST	RESULT
1.	Indole test	Positive
2.	Methyl red test	Positive
3.	Voges Proskauer test	Negative
4.	Citrate utilization test	Positive
5.	Urease test	Negative
6.	Triple sugar iron agar test	A/A, gas production
7.	Oxidase test	Negative
8.	Catalase test	Positive

Table 2: Biochemical test results for Bacterium 'a'

With the help of biochemical tests performed above, the isolated bacteria were identified as follows (Table 3)

S.NO.	ISOLATED BACTERIUM	RESULT
1.	Bacterium 'a'	*Citrobacter spp.*
2.	Bacterium 'b'	*Citrobacter spp.*

Table 3: Results of Biochemical tests

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography of the samples was done to identify the degradation of toluene. Catechol was used as the standard and the unknown values were spotted. Here in S indicated standard catechol, T1 was degradation of 1% toluene using bacterium 'a', T2 was degradation of 1% toluene using bacterium 'b'. Ferric chloride was used to develop the black spots. The black spot developed were similar to the standard catechol spot used for reference **(Figure 10)**.

Figure 10: TLC of 1% toluene treated with bacteria

FOURIER TRANSFORM – INFRA RED SPECTROSCOPY

All the samples with different concentrations of toluene were degraded to similar compounds. The peak values and the corresponded compounds were present in the sample. This showed the presence of alcohol, alkyne, alkene, nitro, ether, alkoxy and arene groups in the degraded sample. When compared to the toluene FT – IR, the peak range as well as the compounds differs in all the three cases. A C - H bending at 1035 peak range in toluene has been converted to C-O stretching peak indicating presence of alkoxy. C-C bond has been converted to C-O, C=C and N-O bonds in the treated sample in 1100 – 1635 peak range. The C-H stretching bonds aft 1635 peak range have been converted to O-H and C≡C compounds (Figure 11, Figure 12 and Table 4).

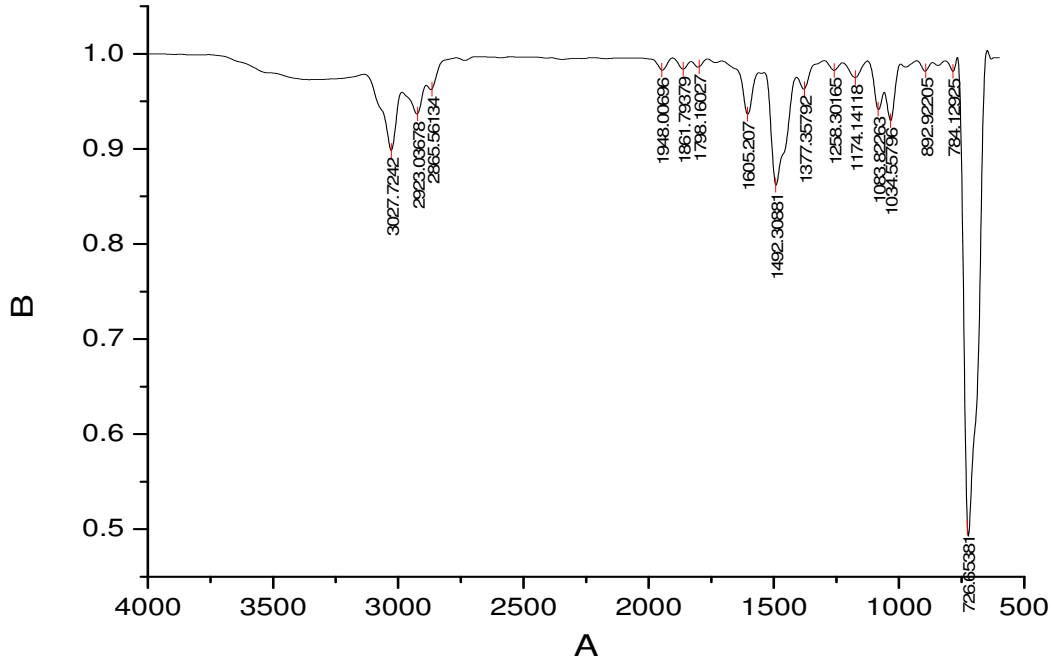

Figure 11: FT – IR of Control sample containing toluene

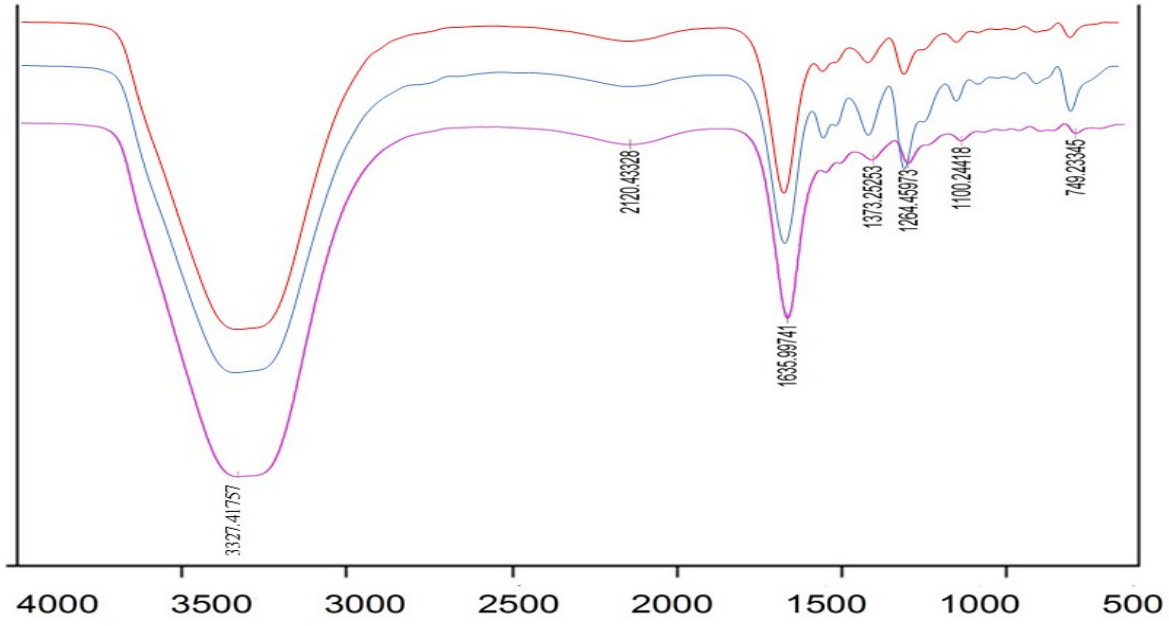

Figure 12: FT – IR analysis of 0.5%, 1% and 1% toluene degradation by the 2 *Citrobacter spp.*

S.NO.	PEAK VALUE	UNKNOWN COMPOUND
1.	3327.41757	O-H alcohol stretching
2.	2120.43328	C≡C alkyne stretching
3.	1635.99741	C=C alkene stretching
4.	1373.25253	N-O nitro stretching
5.	1264.45973	C-O ether stretching
6.	1100.24418	C-O alkoxy stretching
7.	749.23345	C-H arene bending

Table 4: FT – IR interpretation of degraded sample

CONCLUSION

The soil and water samples obtained from various harsh environments were used for the isolation of bacteria. The enrichment of samples in nutrient broth before exposing the bacteria to toluene enhanced the growth in MSM supplemented with toluene. The methods used for the isolation of toluene degrading bacteria were simple but accurate methods to obtain the efficient bacterium. The degradation of toluene to catechol was visualized conveniently using thin layer chromatography where black spots indicated presence of catechol. Biochemical tests such as Indole, MR, VP, Citrate utilization, TSI, Oxidase and Catalase tests were performed to identify the four toluene degrading bacteria. Two *Pseudomonas* spp. and two *Citrobacter* spp. were identified using the biochemical tests that were able to degrade 1% toluene. FT – IR spectroscopy was carried to identify the functional groups present in the degraded sample. GC – MS served as an important tool in confirming the presence of catechol in the degraded sample. 16s rRNA sequencing was performed to identify the most potential bacterium *Pseudomonas putida* which was able to degrade 2% of toluene. *Pseudomonas putida* was observed to be the most efficient bacterium among the four isolated bacteria.

BIBLIOGRAPHY

1. Angela Woods, Maribeth Watwood, and Egbert Schwartz (2011) Identification of a Toluene-Degrading Bacterium from a Soil Sample through $H_2{}^{18}O$ DNA Stable Isotope Probing. Applied and Environmental Microbiology, 77(17): 5995–5999.
2. Beller. H R, Spormann A M, Sharma P K, Cole J R and Reinhard M (1996) Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Applied and environmental microbiology, 62(4): 1188–1196.

3. Gericke C, Hanke B, Beckmann, Baltes G, Kühl M M, and Neubert, D. (2001) Multicenter field trial on possible health effects of toluene: III. Evaluation of effects after long-term exposure. Toxicology, 168(2), pp.185-209.
4. Jacob H. Jacob and Fawzi I. Irshaid (2015) Toluene Biodegradation by Novel Bacteria Isolated from Polluted Soil Surrounding Car Body Repair and Spray Painting Workshops. Journal of Environmental Protection, 6, 1417-1429.
5. M. Gopinath and R. Dhanasekar (2012) Microbial degradation of toluene. African Journal of Biotechnology Vol. 11(96), pp. 16210-16219.
6. Jessica R. Hanson, Jennifer L. Macalady, David Harris and Kate M. Scow (1999) Linking Toluene Degradation with Specific Microbial Populations in Soil. Applied and environmental microbiology, 65(12): 5403–5408.
7. Lee J.Y., Roh J.R. and Kim H.S. (1994) Metabolic engineering P. putida for the simulataneous biodegradation of benzene, toluene and p-xylene mixture. Biotechnol. Bioengg, Vol. 43, pp. 1146-1152.
8. Tury, B., Pemberton, D. and Bailey, R.E (2003) Fate and potential environmental effects of methylenediphenyl diisocyanate and toluene diisocyanate released into the atmosphere. Journal of the Air & Waste Management Association, 53(1), pp.61-66.
9. Mukherjee AK and Bordoloi NK (2012) Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by Bacillus subtilis and Pseudomonas aeruginosa strains and a formulated bacterial consortium. Environmental science and pollution research international, 19(8):3380-3388.
10. Smith, H.W., Finkelstein, N., Aliminosa, L., Crawford, B. and Graber, M (1945) The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. Journal of Clinical Investigation, 24(3), p.388.