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ISOLATION OF TOLUENE DEGRADING BACTERIA FROM POLLUTED ENVIRONMENT

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ABSTRACT

Over the past few decades, several types of pollutants have been released into the environment due to human activities. Oil spill is one of the major causes for the release of oil/liquid petroleum hydrocarbon into the sea, land or any part of the environment. Since crude oil is lighter than water, it poses the threat for swift fire accidents on the sea surface. Thus Oil spills poses a great threat for environmental pollution. Toluene is an aromatic hydrocarbon. It is produced during the process of making gasoline and other fuels from crude oil and also as a by-product in the manufacture of styrene. Worldwide annual production of toluene is estimated to be 10 million. Thus Soil and land pollution continue to happen all around the world due to various causes. Cleaning up the environment using commercially available methods requires the removal of polluted area from the site and may lead to significant alteration in the nature of contaminated soil or water. Bioremediation serves as a solution for such problems where the cost is low, nature of the contaminated site remains unchanged and bioremediation of the contaminated area can be carried out *in-situ*. The present study is aimed at degrading toluene using bacteria which serves as a powerful bioremediation tool. The isolated efficient bacterium can be cultivated on a large scale to carry out *in-situ* bioremediation of polluted sites.

KEYWORDS: Environment, Oil Spill, Pollution, Toluene and Bioremediation.

INTRODUCTION

Toluene is an aromatic hydrocarbon (**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 **(Beller *et.al.*, 1996).**

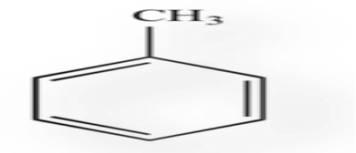


Figure. 1: Toluene.

Toluene is extensively used in industrial activities such as in the production of thinners, pesticides, cigarettes, polymers, gums, oils, resins and as a non-clinical thermometer liquid (**M. Gopinath** and **R. Dhanasekar, 2012)**. It is used as a solvent in carbon nanotubes and used as cement for polystyrene kits. In biochemistry experiments, it is used in the disruption of RBC in order to extract haemoglobin **(Gericke *et. al.*, 2001)**. Toluene can be released in the environment where it is produced or used. It is also released in the environment because of oil spills and pipeline disruption. It is found in air when there is a heavy vehicular traffic (**Jacob H. Jacob** and **Fawzi I. Irshaid, 2015)**. Higher levels of toluene in indoor air can be found where paint thinners, solvents or tobacco products are used. Toluene enters surface waters and ground water from solvent, petroleum product spills, underground storage tanks. It can enter a soil and water when toluene-containing products are placed in landfills or waste disposal sites **(B. Tury, 2003)**.

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 **(Jessica *et al.*, 1999)**. When toluene is inhaled, it is directly taken into the blood from the lungs. When toluene is ingested, it is absorbed from the GI tract into the bloodstream. Small amount of toluene accumulates in fat tissue with daily exposure and majority of toluene is removed from the body within a day **(Lee *et al.*, 2002)**. Toluene leaves the body unchanged during respiration and excretion or it is converted into a less harmful chemical such as hippuric acid **(W. Smith *et al.*, 1945).** High levels of toluene exposure during pregnancy may lead to retardation of growth and mental abilities in children. Other health effects include damage to liver, kidney and respiratory system (**Gericke *et al.*, 2001)**. According to EPA, exposure to high levels of toluene in occupational settings was found to have an increase in leukemia (**Marcelo *et al.*, 2005)**. 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 **(Jeyaratnam *et al.*, 1990)**. The process of using microbes to degrade environmental contaminants to non harmful or less harmful products is termed as microbial biodegradation which can occur aerobically or anaerobically **(Pugalenthi, 2002)**. The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols (**Mukherjee AK** and **Bordoloi NK**, **2012)**.

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 sheds in Kolathur (**Figure 2)**. The samples were collected in a sterile container and 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: Kolathur- 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 ($\mathbf{R_F}$) of the compound was calculated using the formula

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

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 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 the 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 S5 showed turbidity indicating the growth of bacteria (**Figure 4a & b)**. This sample was further used for spread plate method and degradation studies.

a **b**

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

Serial Dilution: Serial dilution 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)**.

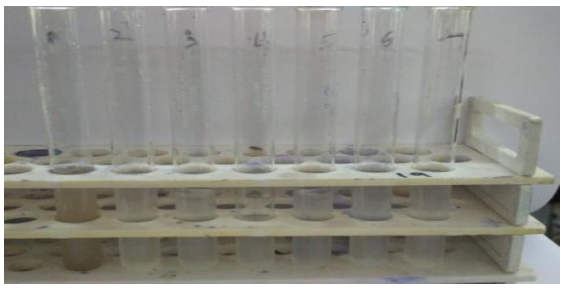


Figure. 5: Serial dilution using soil sample.

Minimal Salt Agar: Spread plate technique was done and incubated for 72 hours to observe growth of colonies. This technique yielded two different types of colonies on MSM agar plate **(Figure 6)**.

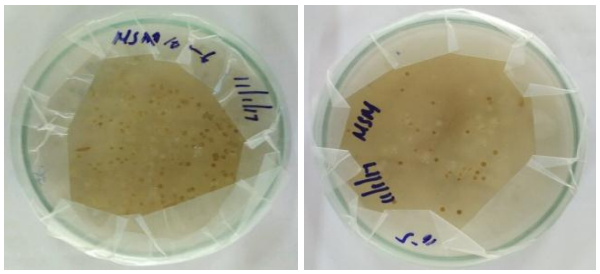


Figure. 6: Colonies obtained by spread plate technique.

Concentration and Degradation Studies

Various concentrations such as 0.5, 1, 2, 3, 4 and 5ml of toluene were used to identify the most efficient bacterial species isolated so far. Concentration studies were done using the four bacteria to test the maximum percentage of toluene degraded by the isolates. Only the brown colony producing bacteria was able to degrade 2% toluene. This bacteria was allowed to grow in the medium containing toluene for 10-15 days to obtain maximum degradation (**Figure 7**). The growth of bacteria was visually observed by the turbidity in the tubes and degradation of toluene to catechol was confirmed by TLC and GC – MS.

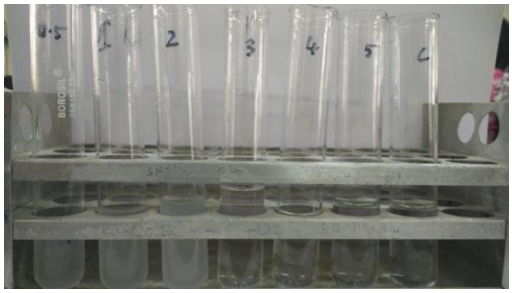
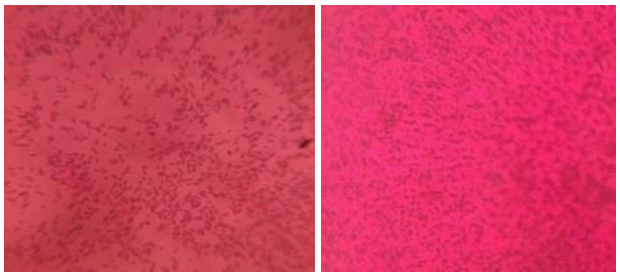


Figure. 7: Growth of bacteria in 2% toluene.

Gram Staining

Both the isolated bacteria were Gram negative and rod shaped **(Figure 8)**.



a **b**

Figure. 8: (a) Gram negative and rod shaped, (b) Gram negative and rod shaped.

BIOCHEMICAL TEST

Biochemical tests were 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 were performed to test whether the bacteria utilizes the particular growth medium and was identified based on the results. This majorly helps in identifying the Genus of the isolated bacteria.

BACTERIUM 'a'

The bacterium 'a' showed positive results for Citrate utilization test, Oxidase test and Catalase test. Indole, MR, VP and Urease tests were negative for this bacterium. The TSI slant and butt were alkaline without gas production (**Figure 9 and Table 1**).

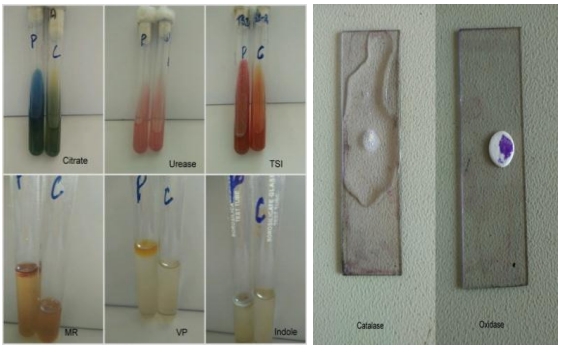


Figure. 9: Biochemical test results for bacterium 'a'.

Table. 1: Biochemical tests for bacterium 'a'.

S. NO.	Biochemical Test	Result
1.	Indole test	Negative
2.	Methyl red test	Negative
3.	Voges Proskauer test	Negative
4.	Citrate utilization test	Positive
5.	Urease test	Negative
6.	Triple sugar iron agar test	K/K, no gas
7.	Oxidase test	Positive
8.	Catalase test	Positive

Bacterium 'b': The bacterium 'b' showed positive results for MR, Citrate utilization test, Oxidase and Catalase tests. Indole, VP and Urease tests showed negative results. TSI slant and butt showed acid production as well as hydrogen sulphide gas production (**Figure 10 and Table 2**).

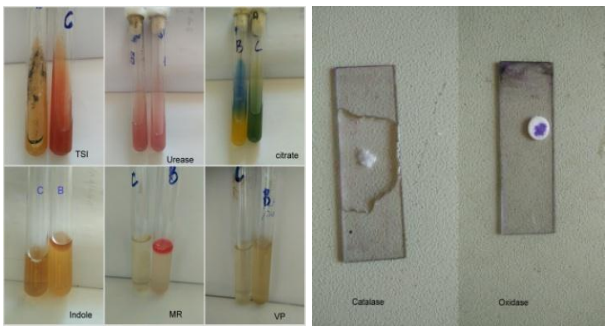


Figure. 10: Biochemical test results for bacterium 'b'.

Table 2: Biochemical tests for bacterium 'b'.

S. No.	Biochemical Test	RESULT
1.	Indole test	Negative
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, H_2S production
7.	Oxidase test	Positive
8.	Catalase test	Positive

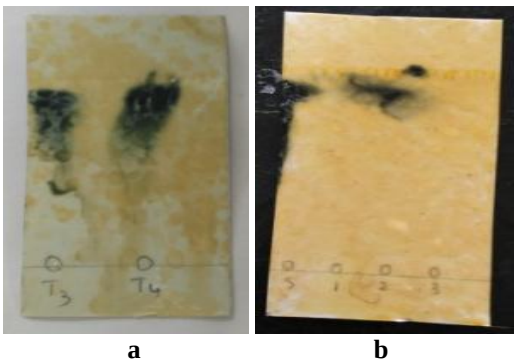
With the help of biochemical tests performed above, the isolated bacteria were identified as follows;

Table 3: Results of Biochemical tests.

S. NO.	Isolated Bacterium	Result
1.	Bacterium 'a'	*Pseudomonas spp.*
2.	Bacterium 'b'	*Pseudomonas spp.*

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 (**Figure 11 a**) S indicated standard catechol, T3 was degradation of 1% toluene using bacterium 'c' and T4 was degradation of 1% toluene using bacterium 'd'. In (**Figure 11 b**), S indicates standard catechol, 1, 2 and 3 were samples treated with bacterium 'b' in which only sample 2 showed degradation of toluene. Ferric chloride was used to develop the black spots. In (**Figure 11 a**) , all four samples developed black spots which indicated degradation of toluene to catechol while in (**Figure 11 b**) only one bacterium was able to degrade toluene. The black spot developed were similar to the standard catechol spot used for reference.



a **b**

Figure. 11: (a) TLC of 1% toluene treated with bacteria and (b) TLC of 2% toluene treated with bacteria.

Gas Chromatography – Mass Spectroscopy

The sample which contained 2% toluene and showed turbidity was given for GC – MS to identify the degraded compound. The compound catechol at 9.888 retention time shows the highest peak of area 99.11% (**Figure 12 and Table 4**). This high amount of catechol in the degraded sample was due to incubation of the organism with toluene in a shaker at 150rpm for 10-15 days to obtain the maximum degradation of toluene. This confirms the degradation of toluene to catechol which is a less toxic compound and a product of toluene degradation by bacteria. The other compounds present in the samples were vinylcyclohexyl ether, 1-decene, 1-hexanol, 4-methyl, decane, heptane, 2,5,5-trimethyl-, 2-undecene, 4,5-dimethyl, 3-dodecene, oxalic acid, isobutyl pentyl ester, 1,1-dioctyloxyoctane, dodecane, 1,1-difluoro, 1,2-benzenediol, brenzcatechin, benzamide, 2-hydroxy-, benzaldehyde, 2,6-dichloro groups. These were found in trace amounts in the degraded sample. The presence of these products could be due to the presence of MSM residues present in the sample after utilization of salts.

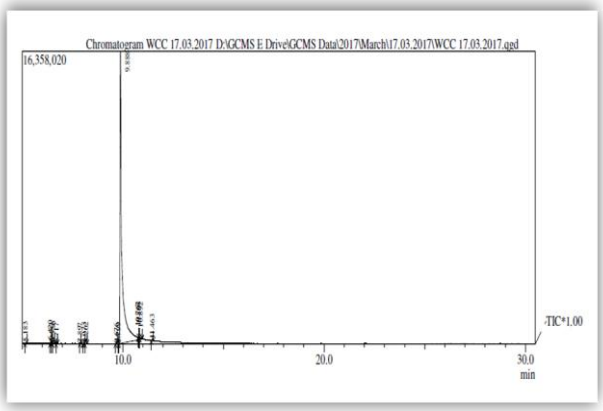


Figure. 12: GC – MS of 2% toluene degraded sample.

Table 4: Compound identification by GC – MS.

Peak No.	Retention Time in Minutes	Compounds	Peak Area %
1.	5.183	vinylcyclohexyl ether	0.04
2.	6.420	1-decene	0.22
3.	6.483	1-hexanol, 4-methyl	0.02
4.	6.556	Decane	0.11
5.	6.717	heptane, 2,5,5-trimethyl	0.02
6.	7.897	2-undecene, 4,5-dimethyl	0.03
7.	8.073	3-dodecene	0.12
8.	8.202	oxalic acid, isobutyl pentyl ester	0.05
9.	9.676	1,1-dioctyloxyoctane	00.4
10.	9.799	dodecane, 1,1-difluoro	0.02
11.	9.888	Catechol	99.11
12	10.767	1,2-benzenediol	0.00
13.	10.808	Brenzcatechin	0.01
14.	10.892	benzamide, 2-hydroxy	0.03
15.	11.463	benzaldehyde, 2,6-dichloro	0.18

CONCLUSION

To conclude, soil and land pollution continue to happen all around the world due to various causes. Cleaning up the environment using commercially available methods requires the removal of polluted area from the site and may lead to significant alteration in the nature of contaminated soil or water. Bioremediation serves as a best solution for such problems where the cost is low, nature of the contaminated site remains unchanged and bioremediation of the contaminated area can be carried out *in-situ.* Thus, the present study on degradation of toluene using bacteria serves as a powerful bioremediation tool wherein the isolated efficient bacterium can be cultivated on a large scale to carry out *in-situ* bioremediation of polluted sites.

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