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RESEARCH ARTICLE

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF TOLUENE DEGRADING BACTERIA FROM CAR SPRAY PAINTING WORKSHOPS

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Abstract

Toluene is an important constituent of various commercial products including aints,dyes,cosmetics,pharmaceuticals, various chemicals and plastic articles. It is a natural component of crude petroleum and petroleum products such as gasoline and diesel fuel. The aim of present study is to isolate and characterize toluene-degrading bacteria from contaminated soil surrounding Car spray painting workshops. Four soil samples were collected from car spray painting workshops in Kolenchery. Totally 12 different bacterial isolates were obtained from the samples. Three were (TDB 3, TDB 9, TDB 12) finally selected through secondary screening by agar well diffusion method using Toluene as carbon source. These isolates were selected based on highest zone of degradation on agar well diffusion method. Biochemical tests and GC-MS of these strains were performed. Based on the GC MS result one strain(TDB 3 is selected for PCR analysis. The nucleotide sequence of 16S r RNA gene shown the isolated strain , TDB 3 belongs to Bacillus sp. were the most effective in degrading toluene and this may be used for environmental bioremediation.

Introduction:-

Globally, there is a growing concern about the risks of environmental contamination from the exploration, transport and storage of petroleum[1] .Environmental pollution caused by petroleum is of great concern because petroleum hydrocarbons are toxic to all forms of life[2]. Hydrocarbons enter into the environment through waste disposal, accidental spills, as pesticides and via losses during transport, storage, and use and their accumulation in the environment causes serious problems[3]. Benzene, Toluene, Ethylbenzene and Xylene (BTEX) are hazardous aromatic compounds contained in gasoline and other petroleum products. Collectively they are among the most commonly reported contaminants of the ground water in United States. Of these, toluene which widely exists in petroleum and related products is a serious cause for concern due to its adverse health effects and carcinogenic potential[4].

Toluene is a hydrophobic and carcinogenic compound. Even at low concentrations, toluene has been proven to damage human liver and kidney and paralyze the central nervous system[5] . Large amounts of toluene released into the atmosphere every year would impair the air quality and are a threat to public health[6]. It contaminates surface and ground water[7] .Toluene is daily released in large quantities in the environment due to crude oil spills following oil tanker accidents and pipeline disruption[8]. Toluene degradation is sensitive to many factors such as temperature, pH,

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incubation periods, carbon and nitrogen sources, and aeration rate[9] . Toluene degrading bacteria use different pathways for toluene consumption .The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols. Toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol 2,3-dioxygenase are enzymes involved in the degradation of toluene and are organized in two different pathways. The upper pathway codes enzymes for the conversion of aromatic alcohol to acid, while the lower pathway enzymes involved in the aromatic acid metabolism via an ortho and meta pathway[10]. The second pathway involves ring hydroxylation, yielding methyl catechols as the metabolic intermediate. The key enzyme involved in this pathway is toluene dioxygenase[11].

Compared to physiochemical methods, bioremediation offers a very feasible alternative for the decontamination of oil spills. Bioremediation is an effective, economical and environmentally friendly treatment method in which microbes are used to degrade hydrocarbons[12]. It had found that many species had capability to degrade toluene as it contain specific catabolic gene for this purpose. The aim of this study was to isolates and characterizes potent Toluene degraders which would be helpful in bioremediation of environmental pollutant toluene[13].

Materials and Methods:-

Sample Collection:

Soil samples were collected from Car spray painting workshops in Kolenchery. The samples were collected in a sterile container and they were stored air tight. The soil samples were cleaned by removing large stones and pebbles.

Isolation of Bacteria From Samples:

One gram of soil sample from each source was suspended in 10 ml of sterile normal saline and vortexed. The suspension were allowed to settle down and 2.5 ml of supernatant were used as an inoculum in 50 ml MS broth containing 1% toluene and incubated for 72 h at 37°C on a rotary shaker at 150 rpm.

Spread Plate Method:

After incubation, 0.1 ml and 0.2 ml of culture were spread plated on MS agar plates supplemented with 1 % Toluene. And the plates were incubated at 37°C for one week.

Morphological Characteristics:

The morphological identification of isolates was conducted. Colonies were compared for their diameters, overall colors, texture, size, cell arrangement, elevation, pigmentation and optical property.

Agar Well Diffusion Method:

The best Toluene degrading bacterial strain was screened by agar well diffusion method. Isolated colonies were grown in LB broth and 200 µl of culture were transferred to wells created on MS agar plates supplemented with 1% toluene. The plates were incubated at 37°C for 72 h. Among all the colonies the colony exhibiting the highest zone of toluene degradation were selected for further works.

Gram Staining Technique:

Gram staining technique was used to differentiate between gram positive and gram negative bacteria.

Biochemical Tests:

Biochemical tests were performed for the identification of the isolated species.
ABIS Online Identification

The physical, colonial, morphological and biochemical characteristics of the isolate were used for ABIS online identification

Gas Chromatography- Mass Spectroscopy:

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 280°C. A Capillary column (capillary 30m length, 0.32mm diameter) was used for analytical separation. Helium was used as a carrier gas at a flow rate of 1 mL.min-1. The oven was preprogrammed from 60 to 130°C at a rate of 15°C min^{-1}then ramping from

130 to 280°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

Sequencing :
Automated sequencing of the samples were performed at AgriGenome Labs Pvt Ltd. Kochi.

In silico analysis of the sequences:
Search for homology:
The nucleotide sequence of the sample were compared the sequences available in the nucleotide databases using BLAST tool (www.ncbi.nlm.nih.gov/blast) provided by NCBI. Nucleotide –nucleotide sequence comparison was done using Blast X. The best sequence alignment results were noted. Phylogenetic tree construction using online tool phylogeny.fr

Results and Discussion:-

Table 1:- Morphological characteristics obtained for samples are indicated in table.

Morphology	Size of colony	Margin	Elevation	Pigmentation	Shape	Texture	Optical property
TDB 1	Pinpoint	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 2	Small	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 3	Small	Entire	Flat	Non pigmented	Round	Smooth	Opaque
TDB 4	Small	Entire	Raised	Non pigmented	Round	Smooth	Opaque
TDB 5	Moderate	Entire	Flat	Non pigmented	Round	Dry	Opaque
TDB 6	Small	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 7	Pinpoint	Entire	Flat	Non pigmented	Round	Dry	Opaque
TDB 8	Small	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 9	Pinpoint	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 10	Small	Entire	Raised	Non pigmented	Round	Dry	Opaque
TDB 11	Small	Entire	Flat	Non pigmented	Round	Dry	Opaque
TDB 12	Moderate	Entire	Flat	Non pigmented	Round	Dry	Opaque

Table 2:- Gram staining results.

Sample	Gram –ve or +ve	Color	Shape
TDB 3	+ve	Purple color	Rod
TDB 9	+ve	Purplecolor	Rod
TDB 12	+ve	Purplecolor	Rod

The three strains showed purple colored, rod shaped bacterial cells

Table 3:-The results of biochemical tests are summarized in table.

Test	TDB 3	TDB 9	TDB 12
Indole production	-ve	-ve	-ve
MR	+ve	+ve	-ve
VP	-ve	-ve	-ve
Citrate	+ve	+ve	+ve
Starch hydrolysis	+ve	-ve	+ve
Oxidase	+ve	+ve	+ve
Catalase	+ve	-ve	-ve
Urease	+ve	+ve	-ve
Carbohydrate fermentation	+ve	-ve	-ve

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 54.494 retention time shows the peak area 15.88% (Figure 1 and Table 4). The presence 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 1, 4-Diethyl-2-piperazinone, Bitolterol, Cyclohexanecarboxylic acid, dodec-9-ynyl ester, 1, 3-CYCLOUNDECANEDIONE, 6-NITRO .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.

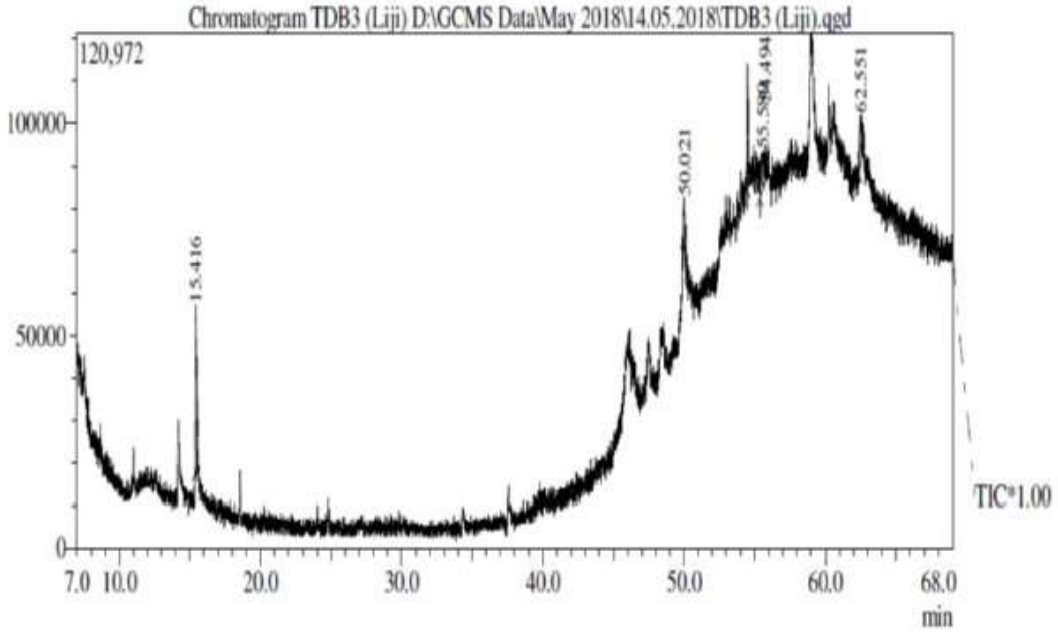

Figure1:- GC-MS OF SAMPLE TDB 3.

Table 4:- Peak Report TIC.

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	15.416	226849	33.63	40137	39.68	1,4-Diethyl-2-piperazinone	58.10
2	50.021	124162	18.41	13250	13.10	Bitolterol	86.10
3	54.494	107090	15.88	28577	28.25	Catechol	131.10
4	55.589	106307	15.76	8817	8.72	Cyclohexanecarboxylic acid, dodec-9-ynyl ester	129.05
5	62.551	110172	16.33	10374	10.26	1,3-CYCLOUNDECANEDIONE, 6-NITRO	86.10

DNA Isolation:

DNA was isolated as per the protocol mentioned in section 3.10from two pure cultures TDB 3 and TDB 9. The DNA was visualized using agarose gel electrophoresis. (Figure 2) In agarose gel electrophoresis, nucleic acid molecules will get separated according to their molecular weight. The DNA obtained was viewed as fluorescent bands under UV light and the image was recorded using the VilberLourmat gel Documentation unit and stored for further reference.

Polymerase Chain Reaction:

The samples were loaded in 1.5% agarose gel and visualized in UV light. Culture TDB 3 were amplified with forward and reverse primers specific for 16SrDNA and generated specific amplicon size of 1500 bp (Figure 3)

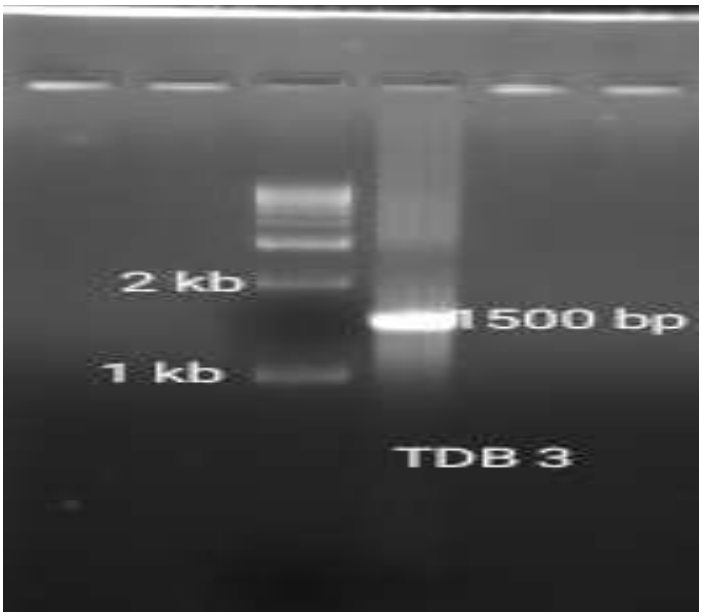

Figure 3:- Confirmation of sample TDB 3 by

1. PCR using 16s rRNA specific primers
2. Lane 1: DNA isolated from sample TDB 3
3. Lane 2: DNA isolated from sample TDB 9

Phylogenetic analysis:

Gene sequence of 16S rRNA was compared with sequences available in databases. Strain TDB 3 showed a highest homology of 99% with Bacillus sp strain BMP -1 ,Bacillus sp.BoGlc83, Bacillus sp. COOI3B ,Bacillus sp. SF-1,.Bacillus sp T-15Z, Bacillus sp CV53, Bacillus spYKJ-10, Bacillus sp 0911MAR22V3 and Bacillus sp LMG18435.Phylogenetic tree was generated (Figure 4).Phylogram of Bacillus sp. isolate TDB 3 based on 16S rRNA gene analysis and constructed byPhylogeny.fr software (online version) with respect to the closely related sequence available in Gene Bank.

Picture showing the Phylogenetic analysis (BLAST) of TDB 3

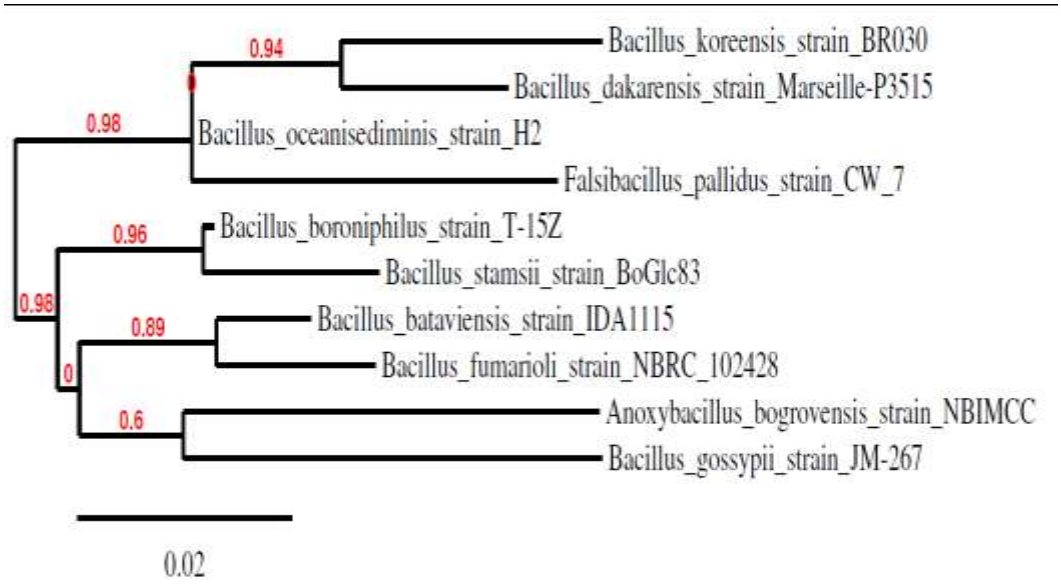

Figure 4:- Neighbour joining tree of the isolate TDB3 showing the phylogenetic relationship within the Bacillus :species. The number at the nodes indicates the percent levels of boost strap support based on the analysis of 1000 replicates. The scale bar indicates number of changes (0.02) per base position.

Discussion:-

Soil and water contamination with hydrocarbons caused extensive damage of the local system, this contamination are crisis to plants and animals. An efficient way of remediation the oil-contaminated sites could be employment of special microorganisms, such as bacteria, microalgae, and fungi[14].Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients[15] .The results of this work were compared with earlier research studies done by V. Varshini et al., (2017) in which they reported that Pseudomonas sp have the ability to degrade toluene.Out of the three samples ,sample TDB 3 shows peak of catechol,which is a degradative product of toluene[16].By observing thes results we can conclude that, Bacillus sp. posses greater potential to degrade toluene when compare with other bacteria.Isolation of the genes for the enzymes required for degradation of toluene could pave way for recombinant DNA technologies where recombinant strains which would degrade toluene in a shorter period can be developed.The nucleotide sequence of 16S r RNA gene shown the isolated strain , TDB 3 belongs to Bacillus sp. were the most effective in degrading toluene and this may be used for environmental bioremediation.

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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