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Isolation of diesel degrading bacteria, identification of Catechol gene and its biogas production

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Abstract: *Microorganisms are the important factors in the degradation of the toxic substances in our environment. Diesel oil is one of the complex mixtures which cannot be easily degraded. Many microorganisms are involved in the degradation of diesel like complex toxic substances into non toxic. In our study we have identified nine different isolates from the diesel polluted. The identified isolates were morphologically and biochemically characterized. The identified isolates were morphologically and biochemically characterized. Then the colonies were grown in the Bushnell Haas medium to identify the degradation capacity of an organism by zone formation. The isolates with a High zone of clearance were 16s rRNA sequenced by using specific primers and identified has Pseudomonas stutzeri. The ability of these isolates to degrade hydrocarbons is clear evidence that their genome harbors the relevant degrading gene. These bacteria were screened for the presence of hydrocarbon degrading gene catechol 2, 3 dioxygenase gene, PCR amplification of a gene using specific primers. High diesel degrading potential organism was treated for biogas production with cow dung as substrate. In which the Pseudomonas stutzeri treated biogas digester showed better production of methane comparing to the other organism.*

Keywords: *Diesel, degradation, Pseudomonas*

I. Introduction

Oil spillages pollute our environment. Most of the oil spillage occurs in the United states and Arab countries. Oil spillage affects the marine environment which induces stress and change in the health of the aquatic environment condition. (Agbogidi et al 2005; Kori-Siakpere, 2000). It also affects the activities of soil microbes (Wang et al 2007). The effect of crude oil contamination in the soil has been studied by the Adesina and Adelasoye, 2014 stated that the growth of Maize and Cow pea in the polluted soil are poor performer in all the activity from germination itself compared to their growth in normal soil.

Diesel is one of the sources of pollution in the environment. It pollutes the environment by different factors through, Motor vehicles, oil tanks, Ship carrying diesel oil spills in the environment (Hill et al 1980). The spillages in the agricultural land affect the soil and growth of the plants (Baker 1982). Spillage in the marine condition affects the aquatic living environment (Dede 2001). The cleanup of diesel oil contamination is one of the important to protect our environment. The method using for cleaning should be easy, simple handling, cost effective and should not affect other living environment. Biodegradation using the microorganism to clean the oil spillage from the environment. In our present study we investigated to isolate the diesel degrading bacteria and their sequence analysis.

II. Materials And Methods

Sample Collection

The diesel polluted soil samples were aseptically collected from Namakkal, Tamil nadu,India.

Physiochemical Characterization

The diesel polluted soil samples were analyzed for pH (potentiometric method), electrical conductivity (EC meter), nitrogen (Alkaline Permanganate method, (Subbiah and Asija, 1956)), phosphrous (Olsen's method (Olsen et al., 1954)) and potassium (boiling nitric acid method using flame photometer). **Micronutrients** were analysed by atomic absorption spectrophotometer.

Isolation of microorganisms from soil sample

1 gm of diesel polluted soil sample were taken and serially diluted from 10^{-1} to 10^{-8} dilutions. The colonies were obtained by spread plate method. The microbial population was calculated as follows.

Population of microorganisms present in 1 gm of soil sample = Average no. of colonies X plate detection factor.

Identification of microorganism
The cultures were morphologically and biochemically identified by staining and biochemical tests like Indole test, Methyl red test, Voges-proskauer test, Citrate utilization test, Triple sugar ion test, Catalase test, Oxidase test, Nitrate reduction test, Litmus milk reaction test, Carbohydrate fermentation test, Starch hydrolysis test and Gelatin hydrolysis test.

Hydrocarbon drgradation
Isolation of hydrocarbon degrading bacteria
The bacteria were isolated by inoculating the soil samples on enrichment medium that contains the autoclaved Bushnell – Hass agar supplemented with single hydrocarbon compound as sole carbon source (1% diesel). The plates were incubated for 7-10 days and observed by zone formation.

Determination of bacterial biodegradation activity by turbidometry method
Turbidometry is to determine the bacterial growth by utilizing the hydrocarbons (1% diesel) given as carbon source in MSM broth. The medium contains K_2HPO_4 (1.8g/l); NH_4Cl (4g/l); $MgSo_4.7H_2O$ (0.2g/l); NaCl (0.1g/l); $Na_2So_4.7H_2O$ (0.01g/l); Carbon source (1% DIESEL); and distilled water (1L) with pH 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The degrading activities of each isolates were obtained by using mineral salt broth (MSB) in which 1% of hydrocarbon (diesel) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D readings at 595 nm from 0 hrs – 15 days at regular intervals of 2 days against mineral salt medium as blank.

16S rRNA sequencing
Genomic DNA of the isolate was extracted with a GenElute DNA extraction kit from Sigma. The 16S rRNA gene of isolate was amplified using the universal primers 8F (5′- AGAGTTTGATCCTGGCTCAG) and 1541R (50-AAGGAGGTGATCCAGCCGCA-3′).

Isolation of catechol 2,3 dioxygenase
The hydrocarbon degrading genes catechol genes were isolated through PCR amplification with respective primers.

Anaerobic Digestion
Fresh cow dung was collected from the Namakkal surroundings. The cow dung was diluted in the ratio of 1:1. In the biogas tank 17 litres of each substrate was mixed with 1.5 litres of pure culture of high diesel degrading bacteria. They were treated for 45 days and the gas sample was collected in 15 days interval and analyzed for presence of different gases by GC-MS

III. Results

Physiochemical Analysis
The physicochemical characteristics of the soil influenced by the impact of diesel as shown in Table 1.The pH value of control soil sample was 7.2 and the four different diesel polluted soil sample shows the pH range between 6.2 to 6.4. The electric conductivity of the Control sample was 30 mS/cm, DSS1, DSS3 and DSS4 was 10mS/cm, DSS2 was 20mS/cm.

Macronutrients of the Polluted Soil
The high amount of Nitrogen was present in the control Soil sample CSS with 59.23 kg/ac, which is Sandy Loam (SL) soil. The diesel contaminated DSS1-DSS4 soil sample ranges from 53.26 to 47.54 kg/ac. Phosphorus content of the Control Soil sample was 10.23kg/ac, diesel contaminated DSS1-DSS4 soil sample ranges from 8.34 to 4.34 kg/ac. Potassium content of the Control Soil sample was 86.23kg/ac, diesel contaminated DSS1-DSS4 soil sample ranges from 66.20 to 76.23 kg/ac (Table 1).

Micronutrients of the Polluted Soil
In diesel polluted Soil (CSS, DSS1, DSS2, DSS3 and DSS4) the ferrous content ranged from 4.34 to 7.20ppm and control has 4.23ppm. Manganese content of the contaminated soil sample ranged from 3.26 to 2.30ppm and control has 3.23ppm. Zinc content of the contaminated soil sample ranged from 1.24 to 1.16ppm and control has 1.86ppm. Copper content of the contaminated soil sample ranged from 1.12 to 0.76ppm and control has 0.98ppm (Table 1).

Isolation of microorganisms

The result of the bacterial count show that diesel polluted soil has the $264x10^{-4}$, $112x10^{-5}$ and $98x10^{-6}$ CFU/ml. (TABLE 2)

Morphological characterization of microorganisms

The Morphological characterization of the KG1 showed gram positive cocci shape, KG9 showed gram positive rod shaped bacteria and all the other isolates KG2-KG8 showed gram negative rod shaped bacteria (TABLE 3).

Identification of isolated microorganisms

Isolated microorganisms are DG1 Staphylococcus sp, DG2, DG3 Pseudomonas sp, DG 4 Citrobacter sp, DG5 Bacillus sp.

Identification of the isolated species

According to Bergey's manual of determinative of bacteriology, 90% of results showed the similarity in characteristics with Staphylococus, Pseudomonas, Citrobacter Sp. and Bacillus Sp. (Table. 4)

Hydrocarbon degradation by Bushnell hass medium

Soil contaminated by diesel are the most potential source to isolate high performed diesel degrading microorganisms using Bushnell-Haas agar medium the inoculated sample to form an clear zone formation surrounding the hydrocarbon (Diesel) degrading microorganisms. DG1, DG2 and DG5 showed zone formation around the organisms.

Hydrocarbon degradation by turbidometry method

The hydrocarbon degradation of the entire organism utilize diesel as the sole source of carbon for their growth. The degradation concentration of the organism was observed at 595nm for 15 days. In which all the nine organisms showed increased level of the degradation concentration in 15^{th} day. DG2 showed highest degradation concentration next to that is observed in DG1 and DG5. (Table 5)

16S rRNA sequencing

The highly degrading microorganism isolated from diesel polluted soil were 16S rRNA sequenced. From the isolates DG2 showed the higher degradation potential. The organism was biochemically characterized, 16S rRNA sequenced and DG2 identified as Pseudomonas stutzeri gram negative bacteria with 274bp and the Catechol degrading gene with 354bp

Pseudomonas stutzeri 274bp

GACGGCTCCGCGATGGCGCTGAACGGCGAAGACGACAACGAGCTCGGCACCACCGCGGTGAAGAAGCTGG
TCGAAACCCTGGACAGCTACATCCCCGAGCCGGTTCGTGCCATCGACAAGCCGTTCCTGATGCCGATCGAAG
CGTGTTCTCGATCTCCGGTCGCGGCACCGTGGTAACCGGTCGCGTAGAGCGCGGCATCGTCAAGGTTCAGG
AAGAGATCGAGATCGTCGGTCTGCGTCCGACCACCAAGACTACCTGCACCGGCGTTTAGA

P. stutzeri - catechol 2, 3-dioxygenase gene 354bp

CCTGGCGCCTCGGAGGAGGCCTGTATATCGGTATCCGCTGCGGACGTTGCATAACCGCCACAGGCGCCAC
CACAGGATTTGCTCATGATACGACTCCACTTGAACAATGTTGTGGTACCATTTAAAACTATAAAGCTACT
ATAAGGTCAATAGAGTAAAGAATCCGTTGGGGAGGAGGCTGATGCGCATTGGTCAGTTGGCGCAGTTGGT
AGGGGTCGAAACACAGACGATCCGCGTCTATGAACAGCAGGGCTTGTTGCCGCCGCCTGATCGGCAGGAC
AACGGTTACCGTGTCTATACCGAGAAACACGTTAAGCGGCTGGCCTTTATTCGATGCCTGCCGCATCCTG
GATC

The biogas production potential of the Pseudomonas stutzeri showed high methane production in comparing to the control unit. However the increase in methane concentration in time decreased carbondioxide along with other gases (Table 6).

IV. Figures And Tables

SOIL SAMPLE		CSS	DSS1	DSS2	DSS3	DSS4
TEXTURE		SL	SL	SL	LS	LS
pH		7.2	6.2	6.3	6.4	6.3
ELECTRIC CONDUCTIVITY(mS/cm)		30	10	20	10	10
MACRO NUTRIENTS kg/ac	NITROGEN	59.23	49.45	52.67	47.34	53.26
	PHOSPHORUS	10.23	4.34	6.45	7.29	8.34
	POTASSIUM	86.23	76.23	74.34	78.34	66.20
MICRO NUTRIENTS CONTENT (PPM)	FERROUS	4.23	4.34	6.03	7.14	7.20
	MANGANESE	3.23	3.14	3.26	2.74	2.30
	ZINC	1.86	1.16	1.24	1.16	1.12
	COPPER	0.98	1.10	1.12	0.98	0.76

Table 1. Physicochemical property of soil samples

S.NO	DILUTION	COLONIES
1.	10^{-4}	66×4
2.	10^{-5}	28×4
3.	10^{-6}	17×4

Table 2. Bacterial colony count of diesel contaminated soil

ISOLATES		DG1	DG2	DG3	DG4	DG5
SIMPLE STAINING		cocci	Rod	Rod	Rod	Rod
GRAM STAINING		+	-	-	-	+
INDOLE		-	-	-	-	+
MR		-	+	+	+	+
VP		-	-	-	+	-
CITRATE		+	+	+	-	-
UREASE		+	+	+	+	+
CATALASE		+	+	+	+	+
OXIDASE		-	+	+	-	+
TSI			+	+	+	+
NO, REDUCTION TEST		+	-	-	-	-
LITMUS MILK REACTION		acid	acid	acid	Acid	Acid
GELATIN		-	-	-	-	-
STARCH HYDROLYSIS		-	-	-	-	-
CARBOHYDRATE FERMENTATION	GLUCOSE	+	-	-	-	-
	LACTOSE	+	-	-	-	+
	FRUCTOSE	+	-	-	+	+

Table 3. Morphological and biochemical characterization of bacterial strain isolated from diesel contaminated soil

S.NO	ISOLATED COLONIES	ORGANISMS
1	DG1	Staphylococcus sp
2	DG2, DG3	Pseudomonas sp
3	DG4,	Citrobacter sp
4	DG5	Bacillus sp

Table 4. List of Identified isolates

ORGANISMS	GROWTH CURVE READING AT 595 nm FOR 15 DAYS INCUBATION (O.D)							
	0th day	2nd day	4th day	6th day	8th day	11th day	13th day	15th day
Control	0.11	0.09	0.08	0.06	0.03	0.02	0.02	0.02
DG1	0.11	0.17	0.22	0.32	0.42	0.58	0.69	0.71
DG2	0.11	0.16	0.25	0.36	0.41	0.59	0.65	0.77
DG3	0.11	0.17	0.23	0.36	0.39	0.45	0.57	0.67
DG4	0.11	0.08	0.09	0.16	0.24	0.22	0.25	0.25
DG5	0.11	0.17	0.17	0.27	0.35	0.44	0.55	0.67

Table 5. Growth curve readings at 595nm for 15 days of incubation

Treatments	CH_4	CO_2	H_2S	Other
15th day				
DG2	12.17 ± 0.68	65.17 ± 1.22	20.30 ± 0.35	2.36 ± 0.59
Control	26.46±1.98	45.59±2.10	20.03±1.59	7.92±1.93
30th day				
DG2	42.80 ± 0.34	37.82 ± 0.21	15.44 ± 1.11	3.94 ± 0.74
Control	43.27±1.99	28.18±1.50	20.29±1.82	6.34±3.12
45th day				
DG2	82.02 ± 0.11	9.71 ± 0.13	5.52 ± 0.87	2.76 ± 0.66
Control	55.45±1.11	18.16±1.13	14.45±1.31	11.93±2.29

Table 6 Biogas production of the high diesel degrading organism (%).

V. Discussion

Carbon is most important for the growth of any living organism, it helps to stimulate the growth 50% of carbon is needed for the growth of microbial cell. The bacteria need macronutrient like nitrogen and phosphorous for effective degradation of oil.

The optimum nutrient balance required for hydrocarbon remediation is Carbon:Nitrogen:phosphorous equal 100:10:4. The nitrogen, phosphorous and potassium of the collected polluted soil sample ranges from 47.34 to 53.26kg/acre, Phosphorous of 4.34 to 8.34Kg/acre and the uncontaminated soil has 10.23kg/acre. The potassium ranges from 66.20 to 78.34Kg/acre and the uncontaminated soil has 86.23Kg/acre. The micronutrients of the soil need at least 0.5% of their concentration in the soil. (U S Congress). Hydrocarbon in soil reduces pH and conductivity. Several authors have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons especially on aromatics .

In our study we isolated nine different isolates from the diesel contaminated soil and three effective degrading bacteria. They were morphologically and biochemically analyzed and identified as Staphylococcus aureus, Pseudomonas stutzeri and Bacillus cereus by 16Sr RNA sequencing. They were effective in degrading the diesel which it takes diesel as the sole carbon source for their growth. From the three Pseudomonas stutzeri has the high degrading capacity of the diesel

Bacillus cereus was able to degrade 20 to 62% of hydrocarbons in oily waste water. One of the potential organisms for naturally degrading hydrocarbon contamination (Bujang et al., 2013). Some of the researchers have reported that biodegradation of soil bacteria ranges from 0.13 to 50% (Pinholt et al., 1979).Diesel degrading microorganism from the diesel polluted region of Iranian, the 16s rNA sequence strain has the close relationship Bacillus Cereus and Bacillus thurigenesis (Kebria et al., 2009).

Staphylococcus sp identified has hydrocarbon- degrading bacteria found in many hydrocarbon-polluted sites (Shukor et al., 2009) Pseudomonas sp, Micrococcus sp. and mixed consortium of this has been used fors bioremediation of diesel oil (Nikhil 2013). Pseudomonas aeruginosa had shown 49.93% of diesel oil degradation in 20days against 0.5% of diesel oil. So Pseudomonas aeruginosa is naturally occurring most potent oil degrading bacteria (Panda et al., 2013).

A study by Nisha et al 2013 evaluated the diesel degrading microorganism from the diesel contaminated soil. They have isolated three different organisms such as Staphylococcus sp, Pseudomonas sp and Bacillus sp.

Various species of Pseudomonas such as Pseudomonas stutzeri (Vazquez et al., 2009), Pseudomonas fluorescens (Olanipekun et al., 2012) Pseudomonas aeruginosa (Hong et al., 2004 and Olanipekun et al., 2012), Pseudomonas sp. strain DRYJ3 (Shukor et al., 2008) are reported for diesel degradation. The ability of bacteria to degrade soil pollutants such as hydrocarbons is useful for bioremediation purposes and their ability to emulsify hydrocarbons has been studied to this respect, making bacteria useful in the removal of these compounds from the environment (Ganesh and Lin, 2009).

Pseudomonas stutzeri showed high degradation potential on various hydrocarbons (Oboh et al., 2006).

Biogas is process of anaerobic degradation. Anaerobic degradation is the breakdown of organic substrates, it is used for the treatment of industrial wastes and stabilization of sludge (Noyola et al., 2006; Wei et al., 2011) The organic substrates, for examples; food processing waste: bakery waste or potato waste; restaurant kitchen waste: grease, oil or fats; animal manure: chicken, wine or cow manure, can be digested and produced useful energy for the world (Residua, 2007).

The gas production revealed increased concentration of methane up to 45 days in treated biogas unit with selective strains showed higher methane yield than the control However, P. stutzeri treated was the highest. Variety of Pseudomonas species have been reported to enhance the methane production under anaerobic degradation (Van Rijn et al., 1996). Enhancing of methane production in biogas unit is basically due to Pseudomonas denitrifying activity (Manuel et al., 1998). The anaerobic mechanisms of hydrocarbon biodegradation are not as well understood as the aerobic ones. Under anaerobic conditions, nitrate, manganese (IV), iron (III), sulphate or carbon dioxide serves as terminal electron acceptors (Anderson and Lovley 1997).

The n-alkane biodegradation mechanisms by anaerobic differ in alkaline metabolically and phylogenetically diverse microorganisms: In a denitrifying isolate, alkane activation is believed to occur through the addition of fumarate, whereas in a sulphate-reducing isolate the initial activation involves carboxylation (Rabus et al. 2001). Bacteria have different genetic and physiological mechanisms for adapting to the accumulation of hydrocarbons in the environment. Hydrocarbon-degradative genes often reside in mobile genetic elements, such as conjugative catabolic plasmids and catabolic transposons that can be transferred to other bacteria via horizontal gene transfer (Tan, 1999). Novel biodegradation mechanisms are also constantly evolving through genetic rearrangements, recombination and transposition, or by point mutations (Van Der Meer et al., 1992). In the present study catechol 2, 3-dioxygenase genes isolated and found their conserved region with the hydrocarbon degrading bacterial strains responsible for breaking down the complex organic molecule in anaerobic condition for enhanced biogas production.

VI. Conclusion

Biodegradation is one of the important process in the environmental degradation which was used for the cleaning the pollution. Because this is low cost and easily handles by the publics. Microorganism has the capable of degrading the hydrocarbons. From the experiments it was concluded that the Pseudomonas stutzeri has the capacity of high degrading diesel. Catechol is one of the hydrocarbon degrading gene which was also identified. This diesel degrading bacteria is identified from the diesel polluted soil. The bacteria also posses the hydrocarbon gene catechol which indicate that the bacteria has degradation potential.

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