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Research Article

A Study on Molecular characterization of Crude oil degrading Bacteria under *In Vitro* conditions

Diptendu Sarkar[1]*, Goutam Paul[2], T.T.S Ramachandra Murthy[1], Parvathy Menon[3], Nagarjuna V[3], Geetanjali P[3] and Yamini A[3]

[1]Brindavan College, PGDept. of Biotechnology, Vinayaka layout, Bhoopsandra, RMV 2nd Stage, Bangalore, India - 560094.

[2]Dept. of Physiology, University of Kalyani, Environmental PhysiologyDivision, Microbiology Unit, Kalyani, Nadia, West Bengal, India - 741235.

[3]New Horizon College of Engineering, Dept. of Biotechnology, Ring Rd, Bellandur Post, Near Marathalli, Bangalore, India - 560103.

ABSTRACT

Extensive hydrocarbons exploration activities often result in the pollution of the environment. The present work was undertaken to assess, isolate and identify the hydrocarbon degrading bacteria associated with environmental samples collected from soil near petrol, diesel pumps in Bengaluru. The samples were enriched to isolate crude oil degrading organisms using standard microbiological techniques and selective media. Two of the isolate Par.a and Par.b were screened and used for further study. They were identified by Gram's staining (both are Gram positive) and biochemical characterization according to Bergy's manual. After 16 S rRNA sequencing it was found that isolate Par.a was *Exiguobacterium aurantiacum* and isolate Par.b was *Exiguobacterium sp.* Both the isolates were further studied to determine their biodegrading activities on hydrocarbons (diesel and petrol) as the sole carbon source using enrichment medium in closed system for 9 days as well as studied to determine their biodegrading activities on hydrocarbons diesel and petrol in presence of carbohydrate source dextrose in same condition for 9 days. The microbial growth was determined using colorimeter blanked at 660 nm. It was found that both isolate Par.a and Par.b in presence of 2.5% (W/V) petrol and diesel along with 0.9 gm dextrose had maximum growth compare to only dextrose containing minimal media. Our studies concluded that both isolate Par.a and Par.bwere potent crude oil degrading bacteria and their capacity may enhance in presence of dextrose along with petrol and diesel in minimal media. This study clearly demonstrates that our isolate microorganisms can be one of the potent bioremediating agent in near future.

Keywords: Hydrocarbon degrading bacteria, Mineral salt medium, Isolation, Identification, Biodegradation, Soil, Waste water, Diesel and Petrol.

INTRODUCTION

Oil contamination is one of the most dangerous pollution factors known today. It can cause a threat to the environment. It is very feared by environmentalists and it's very hard to control if it gets out of hand[1]. Oil spill have become a global problem in industrialized and developing countries. Attention has been focused on the marine environment, because of the largest and most dramatic spills[2]. Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Diesel oil spills contaminated soils on agricultural land generally reduce soil fertility and plant growth. Many indigenous microorganisms in water and soil are

capable of degrading hydrocarbon contaminants[3]. The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants[4]. The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry. Contamination of the soil by oil causes it to lose its useful properties such as soil fertility, water-holding capacity, permeability and binding capacity. The contamination of groundwater is also a potential problem, which receives a lot of untreated effluent from service stations containing oil and grease. To overcome these environmental problems, microbial bioremediation is only way to preserve our nature[3-5]. The purpose of this study was to isolate and identify the bacteria from oil contaminated environment and access their crude oil biodegradation potential under *in vitro* conditions.

MATERIALS AND METHODS

Sample Collection: The study includes three types of samples to isolate the hydrocarbon degrading bacteria. Soil sample extending from the ground surface to a depth of 10–20 cm were collected from petroleum-contaminated areas near petrol station in Bangaluru, Karnataka. Samples were then transported to laboratory under sterile conditions[3].

Growth and Isolation of Bacterial Cultures: The bacteria were isolated from the collected samples by spreading the sample on nutrient agar medium. From the numerous colonies obtained on the NAM plates, they were screened for the hydrocarbon degrading bacteria[6].

Isolation of Hydrocarbon Degrading Bacteria: The bacteria were isolated by inoculating the soil and water samples on enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% liquid petrol and diesel). The medium contains K_2HPO_4 (1.8 g/L); NH_4Cl (4 g/L); $MgSO_4.7H_2O$ (0.2 g/L); NaCl (0.1 g/L); $Na_2SO_4.7H_2O$ (0.01 g/L); agar (20 g/L); carbon source (1% petrol , diesel); and distilled water (1L) with pH 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) filter sterilized hydrocarbons (petrol, diesel) to serve as the only source of carbon and energy. The medium was incubated at 37 C for 5-10 days. After the incubation period the bacterial colonies that were grown on the medium were identified by Gram's staining and biochemical characterization according to Bergy's manual as well as 16S rRNA sequencing and phylogenetic analysis[2, 7, 12, 16].

Isolation of genomic DNA from bacteria: DNA was extracted from 1ml of bacterial culture. the culture was pelleted by centrifuging at 12,000rpm for 2 min. the pellet was treated with lysis solution and proteinase k and incubated at 600C for 30min. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpm for 10 min, washed with 1 ml of a 70% (v/v) ethanol solution and dissolved in 0.1 ml of a TE buffer. The purity and quantity of DNA were examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis[7-10].

Sequence determination of 16s rDNA: The DNA isolated was amplified using 16s rDNA universal primers and sequenced for the identification of bacterial strain at molecular level. Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rDNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (ABI). The gene sequences of each isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database[2, 9, 13, 18].

Determination of Bacterial Biodegradative Activity by Turbidometry: Turbidometry is to determine the bacterial growth by utilizing the hydrocarbons (1% petrol and diesel) given as carbon source in MSM broth. This shows whether the bacterium possess the degrading activity of hydrocarbons like phenol, petrol and diesel. The degrading activities of each isolates were obtained by using Mineral salt broth (MSB) in which 1% of each hydrocarbon (petrol and diesel) was added and incubated at room temperature for 9 days. The effect of dextrose supplement in crude oil was studied by adding dextrose in the above media. The growth of the bacterium was measured by taking the O.D readings at 660 nm from 0hrs- 9 days at regular intervals against mineral salt medium as blank[10,15,17].

RESULTS AND DISCUSSION

The bacteria were isolated from two different types of samples on nutrient agar medium. Further the samples were screened for the presence of

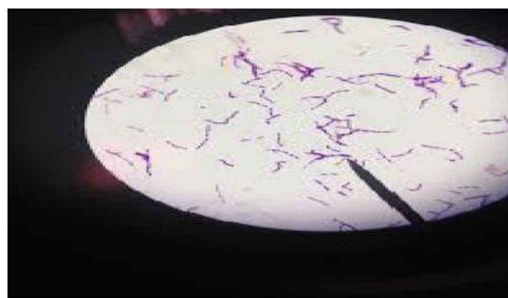
hydrocarbon degrading bacteria on mineral salt medium with 1% of the hydrocarbons as the sole carbon source namely petrol and diesel individually. Hydrocarbons which are needed as a carbon source, can be toxic to microorganisms due to the solvent effects of diesel and petrol, which may destroy bacterial cell membrane. Many biodegradation studies were reported on diesel are carried out using lesser diesel concentrations ranging from 0.5 to 1.5%. But Kaplan*et al*., (2004) reported degradation of diesel by microorganisms at 3.5% and 6% diesel by Mandri*et al.,* (2007). It has been found that degradation is generally unfavorable at concentrations higher than 1 or 1.5% [7, 15].

This result showed that the bacteria grown on enriched medium were able to degrade the hydrocarbon source. The two best screened organisms were labelled as Par.a and Par.b respectively. After Gram staining both of them were found as Gram positive and tinny rod shape (Fig 1 and 2).

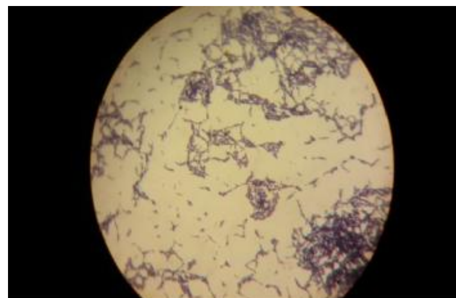
(A)

(B)

Fig 1 A & B
Pure culture of isolate Par.a and Par.b

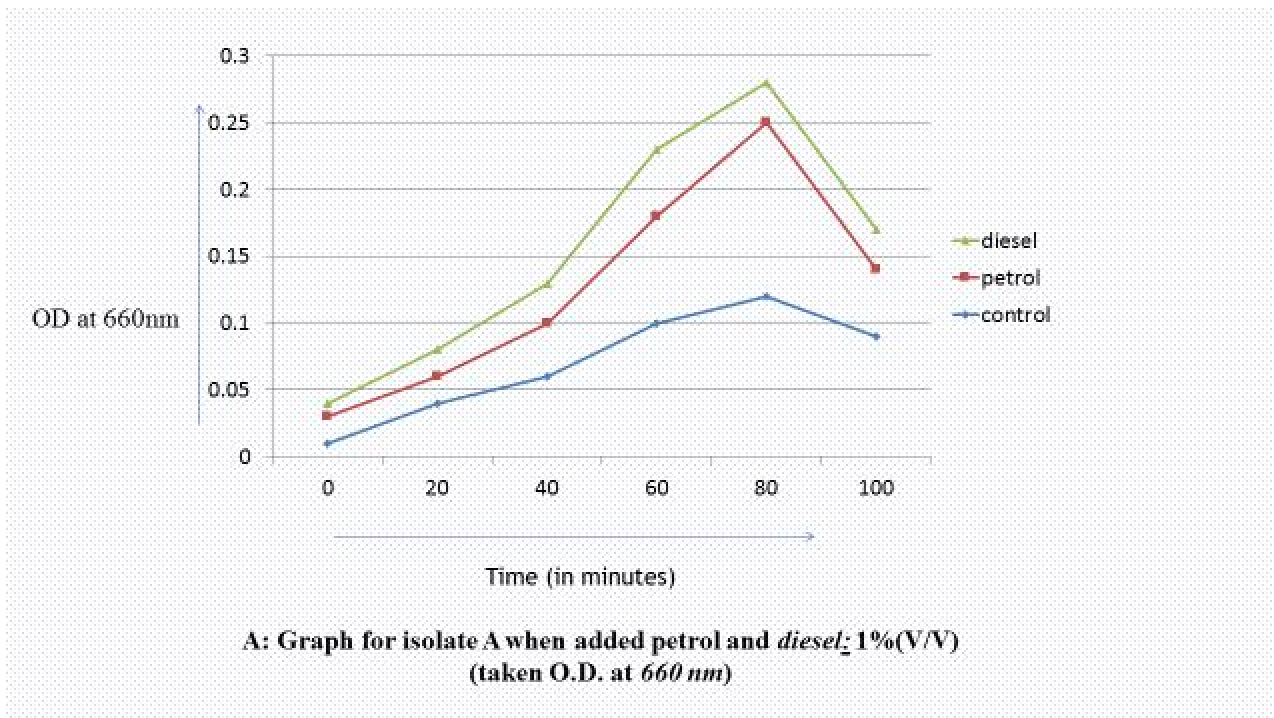


(A)

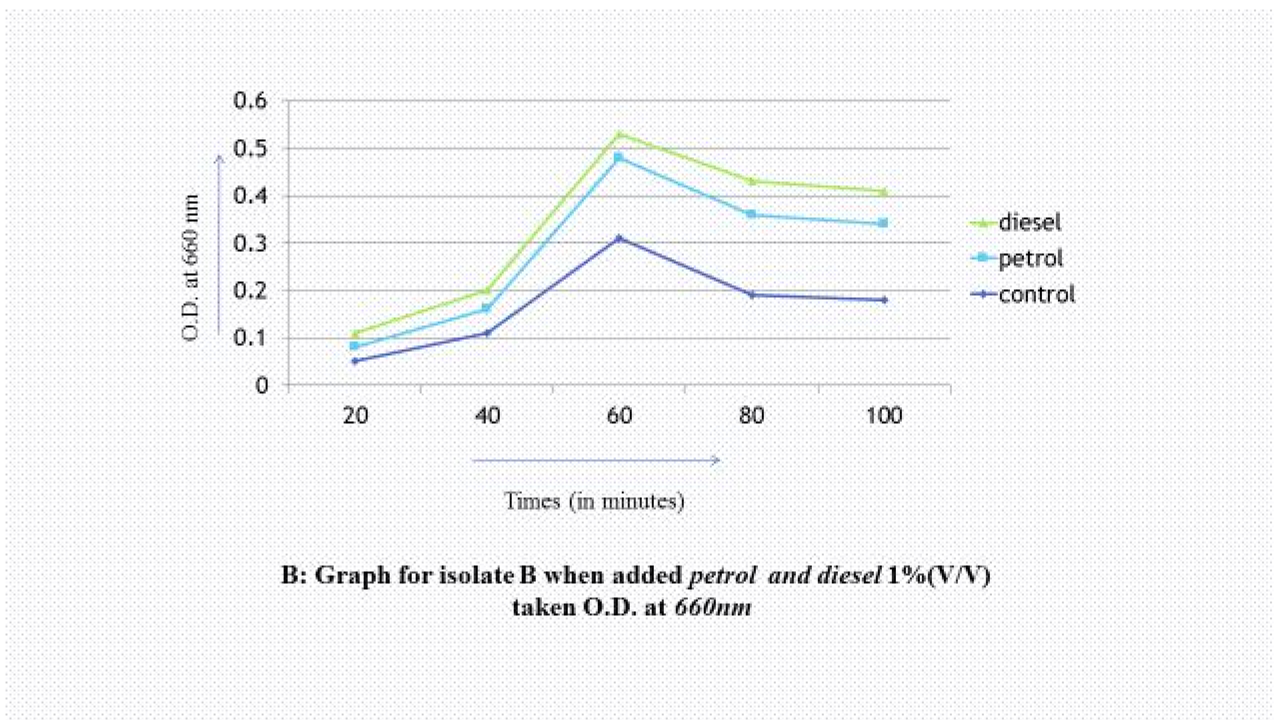


(B)

Fig 2 A & B
Gram staining of isolate Par.a and Par.b



A: Graph for isolate A when added petrol and *diesel*: 1%(V/V) (taken O.D. at *660 nm*)



B: Graph for isolate B when added *petrol and diesel* 1%(V/V) taken O.D. at *660nm*

Fig 3 A & B
Growth curve of Par.a and Par.b in presence of 1% V/V petrol and diesel

The isolates were further confirmed by 16s rDNA sequencing. Based on 16S rDNA which amplified by PCR using 35 cycles and primers 16sF and 16sR was got sequence result and listed in Fig 3, 4 and 6 respectively. The bacterial 16s rDNA sequences were aligned with Blast search of NCBI databases (Fig 5 and 7). The sequence aligned of Par.a gave 97% similarity with *Exiguobacterium aurantiacum and* Par.b also gave 97% similarity with*Exiguobacterium sp.* This is best of our knowledge that till now no body reported that *Exiguobacterium* sp involved in crude oil degradation.

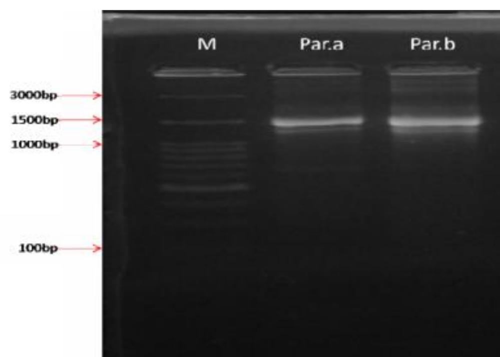


Fig 4
16S rRNA in agarose gel

CCGTAGCTCTTGTTCGACTTCCCCCAATCATCTGCCCCACCTTCGGCGGCTGGCTCCTTAAGGTTACCTC
ACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAACAGCCTGCAATCCGAAC
TGAGAACGGCTTTCTGGGATTGGCTCCACCTCGCGGCTTCGCTGCCCTTTGTAACCCGTCCAATTGTAG
GCACGTGTGTAGCCCAACTCATAARGGGCATGATGACTGAATGCTGGCAAACTAAGGACAAGGGTTG
CGCTCGTTGCGGGAACTTAACCCAAACATCTCACGACACGAGCTGACGACAAYCATGCAMCACCTTGT
CACCC

Fig 5

Partialsequence of16S rRNAof Par.a

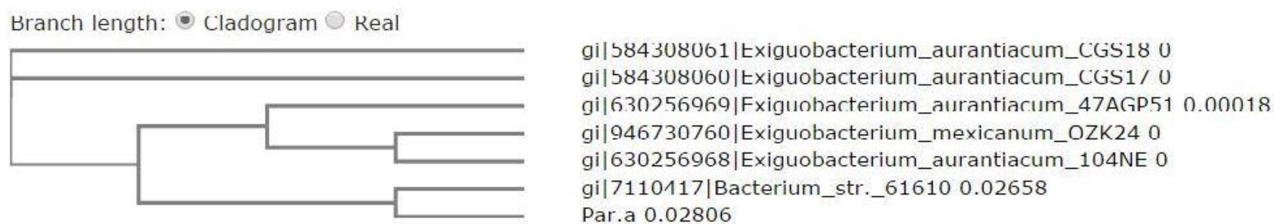


Fig 6
Phylogenetic tree of 16SrRNA sequence of Par.a

CCGTTAGCTCTTGCTCGACTTCACCCCAATCATCTGCCCCACCTTCGGCGGCTGGCTCCTTACGGTTAC
CTCACCGACTTCGGGTGTTGCAAACTCTCGTGGTGTGACGGGCGGTGTGTAAAGACCCGGGAACGTAT
TCACCGCAGTATGCTGACCTGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAA
TCCGAACTGAGAACGGCTTTCTGGGATTGGCTCCACAWCTCATAAGGGSCATGATGATTTGACGTCAT
CCCCACCTTCCTCCGRTTTGTCACCGGCAGTCTCCCTTAGAGTGCCCAACCAAATGCTGGCAACTAAGG
ACAMGGGTTGCGCTCGT

Fig 7
Partial sequence of 16S rRNAof Par.b

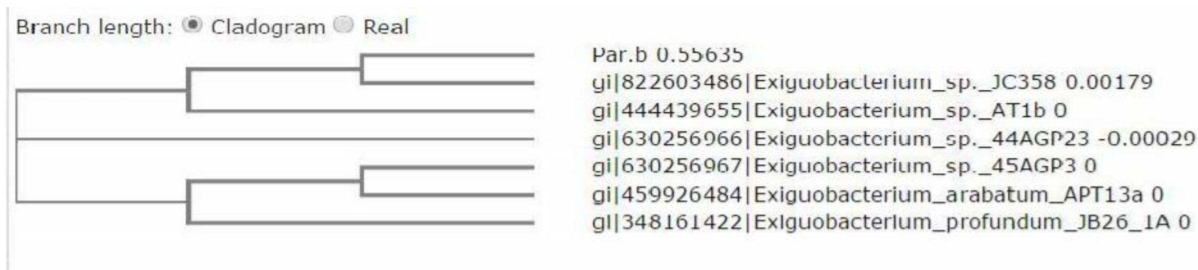


Fig 8
Phylogenetic tree of 16SrRNA sequence of Par.b

These results highlight the different group of bacterial genera involved in hydrocarbon degradation. Many scientists studied the petroleum degradation by various *Pseudomonas species* and by *Bacillus* species [2, 12]. Ficker*et al*., (1999)[9] reported the degradation of hydrocarbons form petroleum polluted areas by Staphylococcus *aureus, Micrococcus luteus, Lactobacillus acidophillus and Bacillus species.* LikewiseSingh*et al.,*(2008)[17] reported hydrocarbons-utilizers in his research such as *Bacillus megaterium, Pseudomonas putida, Micrococcus luteus, Bacillus brevis, Bacillus pumilis and Enterobacter aerogenes.*

It is evident from our study that the proportion of hydrocarbon- degrading microorganisms 'number increases rapidly, when the environment was contaminated with petroleum and diesel components. More numbers of certain hydrocarbon-degrading microorganisms from acrude oil environment stated that those organisms are the active degraders of crude oil[14, 16]. The metabolic activity of indigenous oil-degrading microbes were carrying out rapidly in presence of polluted soil and water and such activities could be responsible for the bioremediation of crude oil contaminated environment[12,17,20].

Fig 9 shows the biodegrading activity of each isolates on hydrocarbons (petrol and diesel). The O.D vs timing readings based on the turbidity of MSM broth at regular intervals give the degradative activity on hydrocarbons by bacteria. Our results showed that both the organisms maximally utilized all the hydrocarbon substrates (petrol and diesel) when supplied as the sole source of carbon along with dextrose although, the level of utilization differs from one microbe to another (due to differences in their growth. Both These degrading capabilities on different hydrocarbons proved that the microorganisms isolated from the soil samples were able to degrade hydrocarbons[19-20]. The cells were able to multiply within the days of study which is indicating that for their growth and development, they were able to degrade and utilize the oil, hence the proportionately increase in the O.D of broth. This gradual increase in the O.D of the broth suggested bacterial growth, which implies degradation of hydrocarbons, mostly between 5 and 9days and gradual decline in the O.D of the broth reveals decrease in the bacterial population and hence the decline of hydrocarbon degradation, mostly between days 8 and 9.

Fava et al. (1995)[8] and Loh and Wang (1998)[21] showed that minerals or supplementary carbon substrates enhance the rate of biodegradation. The addition of nutrients supplements stimulates the degradative potential of the indigenous microorganisms thus allowing the microorganisms to break down the organic pollutants at a faster rate (Ausma et al., 2002)[22]. Okerentugba and Ezeronye (2003)[23] stated that microbiological communities exposed to crude oil will adapt to this exposure through selective enrichment and genetic changes, resulting in a high hydrocarbon-degradation efficiency. Thus previous exposure of microorganisms makes them better capacitor to degrade crude petrol and diesel by showing higher growth and cell division and more efficient in metabolism, thus maximizing the rate of crude oil removal from the culture media.

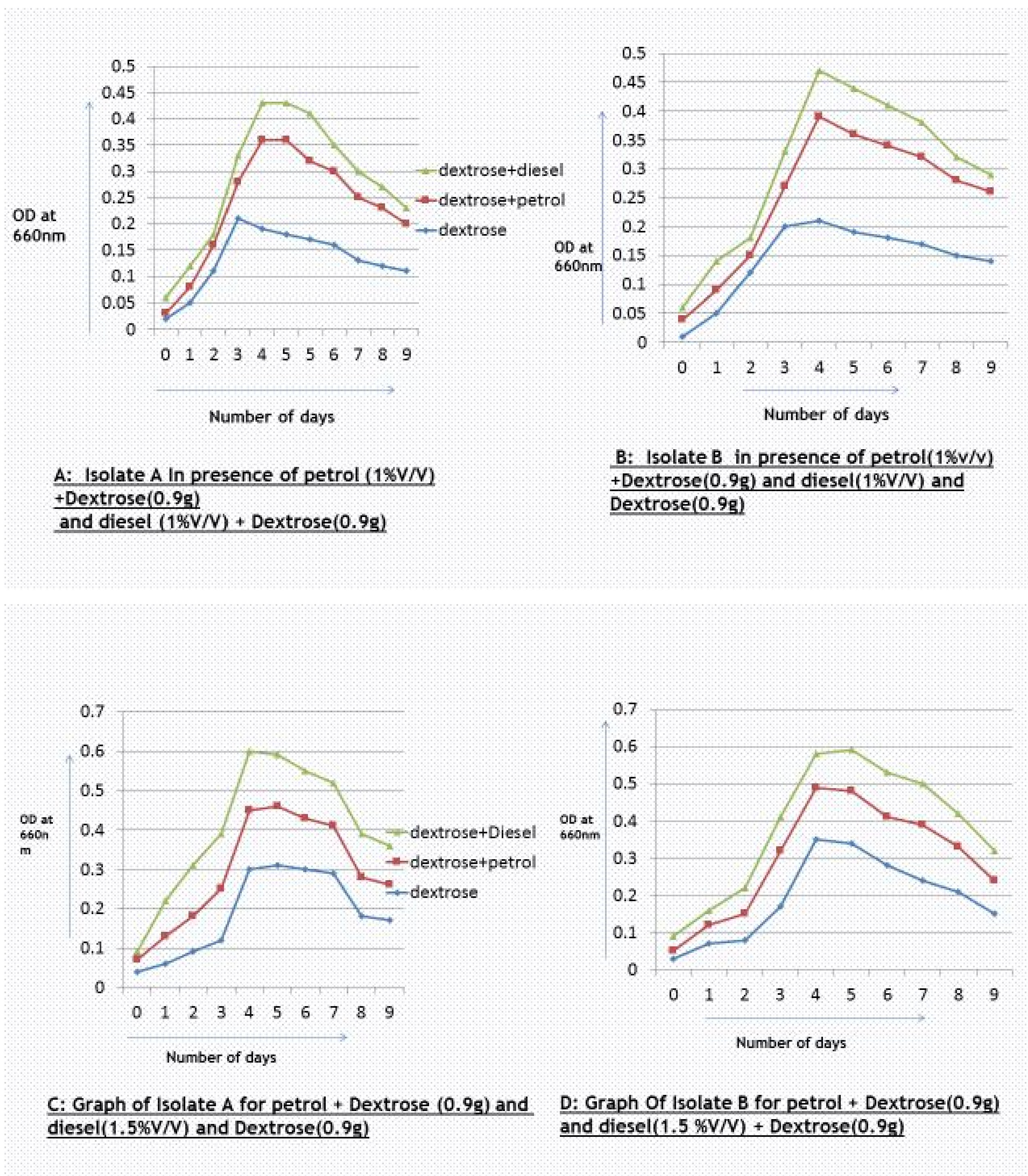
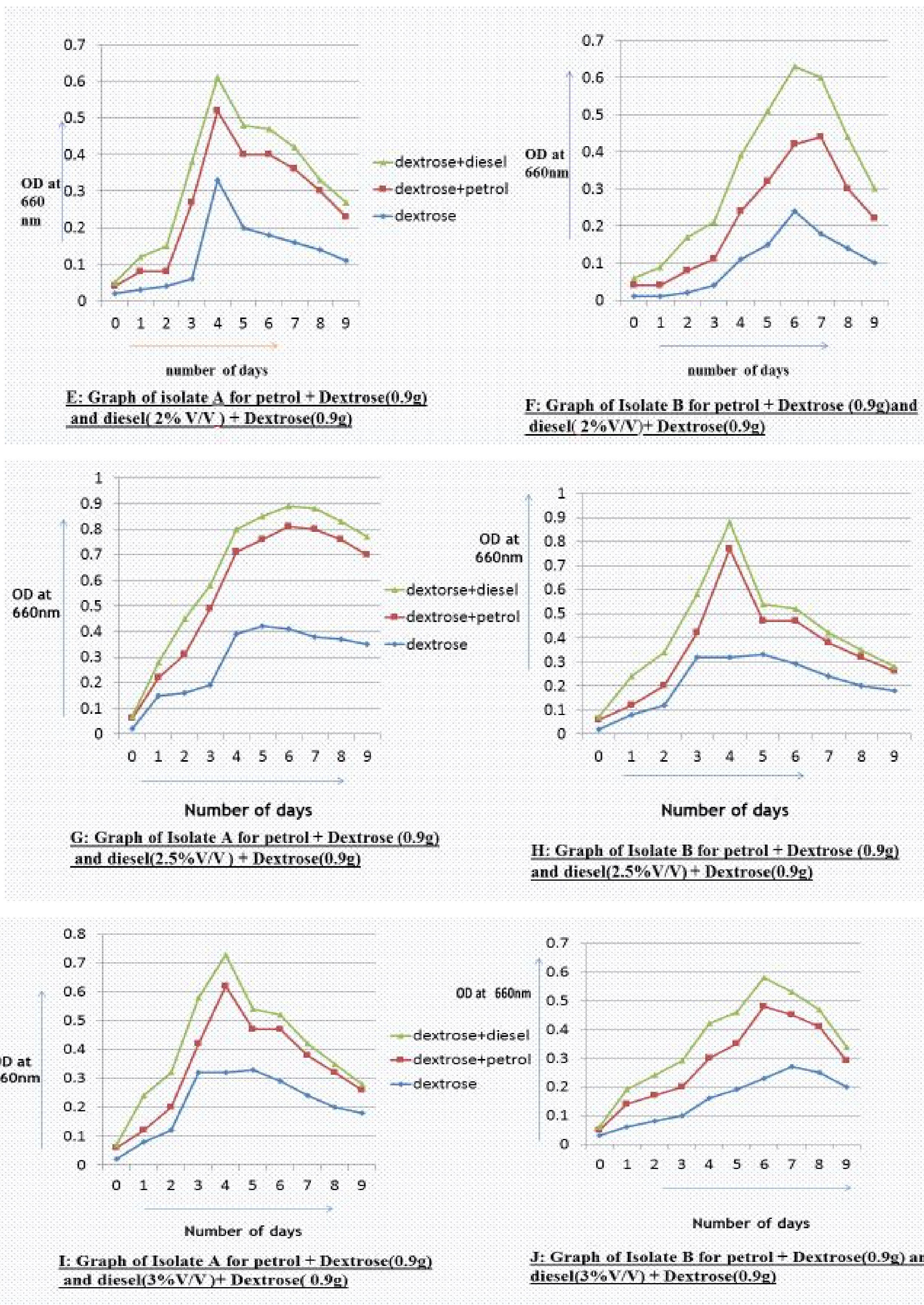


Fig 9 (A to D)
Comparative turbidometric assay of Par.a and Par.b in presence of dextrose and variable quantity of crude oil.



E: Graph of isolate A for petrol + Dextrose(0.9g) and diesel(2% V/V) + Dextrose(0.9g)

F: Graph of Isolate B for petrol + Dextrose (0.9g)and diesel(2%V/V)+ Dextrose(0.9g)

G: Graph of Isolate A for petrol + Dextrose (0.9g) and diesel(2.5%V/V) + Dextrose(0.9g)

H: Graph of Isolate B for petrol + Dextrose (0.9g) and diesel(2.5%V/V) + Dextrose(0.9g)

I: Graph of Isolate A for petrol + Dextrose(0.9g) and diesel(3%V/V)+ Dextrose(0.9g)

J: Graph of Isolate B for petrol + Dextrose(0.9g) and diesel(3%V/V) + Dextrose(0.9g)

Fig 9(Eto J)

Comparative turbidometric assay of Par.a and Par.b in presence of dextrose and variable quantity of crude oil.

Cleaning up of petroleum hydrocarbons in the subsurface environment is a real world problem. A better understanding of the mechanism of biodegradation has a high ecological significance that depends on the indigenous microorganisms to transform or mineralize the organic contaminants. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods. This is possible because microorganisms have enzyme systems to degrade and utilize different hydrocarbons as a source of carbon and energy. By using biological processes, as in the case of bioremediation, usually lowers the costs as compared to chemical treatment processes for various contaminated sites. It is also less disturbing to the environment. The toxicity and fertility of the soil before and after treatment was also assessed, thereby proving that biostimulation is an effective method of reducing environmental pollution.

CONCLUSION

Bioremediation is one of the most potential area of environmental biotechnology for the cleaning up of pollutants from crude oil contaminant side. Our isolated both the organisms Par.a and Par.b can be used in near future as one of the potent living agent for bioremediation.

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