

DIESEL BIOTREATMENT COMPETENCE OF INDIGENOUS METHYLPARATHION DEGRADING BACTERIAL STRAIN OF PSEUDOMONAS AERUGINOSA DOU

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ABSTRACT

Pseudomonas aeruginosa methylparathion degrading bacterial strain previously isolated and identified in our lab was subjected to confirm their ability to diesel oil degradation in synthetic wastewater containing diesel oil (10 % v/v). They are cultured in modified mineral salts agar medium with diesel oil as the sole source of carbon. They were able to grow and distinguish in 7 days of incubation. Biosurfactant was produced in the medium and its effect was detected using emulsification activity, growth estimation and gravimetric analysis as an indicator for this process. Factors affecting hydrocarbon utilization by this isolate were pH, temperature, salt concentration and agitation along with glucose, peptone, phosphate and nitrate concentrations. Gravimetric and FTIR analysis showed that the strain was able to degrade complex hydrocarbon chains to simple ones. The optimum conditions of various factors showed that, neutral pH, with temperature of 32^oC, and agitation rate of 150 rpm gave the optimum conditions to accelerate diesel oil degradations, while additional nutrients of glucose (1 mg/l), peptone (5 mg/l), sodium nitrate (2 mg/l), phosphate (7 mg/l) and sodium chloride (3 mg/l) enhanced optimum effect on the bioprocess. The bacterial strain of indigenous pure monoculture having the degradative potential of xenobiotic compounds of various pollutants includes diesel oil as well as methylparathion pesticide practically. The main conclusion is that *Pseudomonas aeruginosa* dou-1 strain can be able to degrade complex hydrocarbons and make it an ideal applicant in bioremediation.

Keywords: Methylparathion tolerant strain, *Pseudomonas aeruginosa* dou-1, diesel oil, bioremoval, FTIR, wastewater

INTRODUCTION

Environmental pollution with petroleum and petroleum products (complex mixture of hydrocarbons) has been recognized as one of the most serious current problems, hydrocarbons may reach the water table before becoming immobilized in the soil. They spread horizontally on the ground water surface and continue to partition into ground water, soil pore space, air and to the surface of soil particles. Oil contamination of soil and water from industrial sources and other activities are producing ecological disasters and addressing public concerns (Che, 2002). India's oil and gas sectors meet around 42% of the country's primary energy demand and contribute over 15% to the gross domestic product. Oil spill accidents are very common in the areas near the seashore because of shipping of oil tankers and bursting of oil supply pipelines. Recently in India, Mumbai-urban oil spill through the pipeline bursting witnessed about 600 metric tons spillage of crude oil which caused a widespread mortality of sea animals. The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment (Ghosh and Tick, 2013).

Petroleum-based products are the major source of energy for industry and daily life. Petroleum products such as engine oil, petrol, diesel and kerosene are used daily in various forms in mechanic workshops. Petroleum hydrocarbons composed of complicated mixtures of non-aqueous and hydrophobic components such as n-alkane, aromatic, resins and asphaltenes. Oil contamination with petroleum hydrocarbons has caused critical environmental and health defects and increasing attention has been paid for developing and implementing innovative technology for cleaning up this contamination (Yeung, 1997). Diesel, a derivative of Petroleum is one of the major pollutants of water and soil. In areas where oil refineries are present, air, water and soil resources have become contaminated with oil and its by-products namely diesel. Diesel oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Transportation through the railways and roadways causes organic and inorganic contamination. Broken and corroded oil pipelines significantly contribute to oil pollution on a large and rapidly increasing scale. Due to the

adverse impact of these chemicals on human health and environment, they are classified as priority environmental pollutants by the US Environmental Protection Agency (1996). The numbers of spills that have dumped millions of gallons of crude oil into the environment have been steadily increasing over the past decade. This can lead to disastrous consequences. Therefore the pollutants need to be removed or destroyed from the environment.

An array of procedures has been developed including physical, chemical and biological techniques. Among these procedures, bioremediation is currently used alone or associated with physicochemical procedures. Biological methods of rehabilitation of polluted sites represent an interesting alternative. These techniques are based on the microorganism's capacities to degrade petroleum compounds (Harayama, 1999). Bioremediation has become an alternative way to remedy oil polluted sites, where the addition of specific microorganism (bacteria) or enhancement of microorganism already present can improve biodegradation efficiency (Hagwell *et al.*, 1992). These microorganisms can degrade a wide range of target constituents present in oil sludge (Barathi and Vasudevan, 2001; Mishra *et al.*, 2001).

The study of petroleum degrading bacteria therefore becomes very important in deal with problems of oil and petroleum based pollutions. In this study, *Pseudomonas aeruginosa* methylparathion degrading bacterial strain previously identified and stored in our lab was subjected to diesel oil degradation in synthetic wastewater containing diesel oil.

MATERIAL AND METHODS

The methylparathion degrading *Pseudomonas aeruginosa* bacterial strain previously identified and stored in the lab was used for diesel oil degradation. Diesel oil used in this study was refined diesel oil commercially available, which was purchased from local petrol station.

Morphological and Biochemical Characterization

Gram staining revealed that the morphological characters of the isolated bacterial strains. Spore staining shows whether the organism is spore producing or not. Motility test can determine the organism which is motile or non-motile. Catalase test was performed to check the ability of the isolated strains to degrade hydrogen peroxide and Oxidase test for the detection of presence of cytochrome oxidase enzyme. IMViC test is performed for the identification of the organisms. Carbohydrate utilization tests revealed that the ability of the isolated strains in fermentation of sugars like glucose, lactose, sucrose and mannitol. The genus and species of *Pseudomonas aeruginosa* was identified and confirmed by *Pseudomonas* agar medium and King's A medium. The identification was done by on the basis of morphological and biochemical characteristics as per Bergeys Manual of Systemic Bacteriology (Holt et al., 1994).

Screening the capability of diesel oil utilization by potential methylparathion degrading bacterial strain

The screening of the diesel oil degrading bacteria was done by inoculating 1 ml of pure culture (*Pseudomonas aeruginosa*) in diesel oil containing nutrient broth (NB) and incubated for 21 days at 35°C on rotary shaker at 150 rpm. After growth in NB, serial dilutions (up to 10⁻⁵) were made and 100µl of the liquid was surface spread on nutrient agar plates containing diesel oil (1% v/v) and plates were incubated at 35°C for 72h. A colony was picked and streaked on nutrient agar plates with increased concentration of diesel oil (up to 10%). Out of these the best grown isolates were selected and were maintained in modified mineral salts (MMS) agar medium (K₂HPO₄ 7.0 mg/l; NaNO₃ 2.0 mg/l; NaCl 3.0 mg/l; Peptone 5.0 mg/l; glucose 1.0 mg/l; agar 22.0 mg/l; Distilled water 1000 ml; pH-7.0 ± 0.2) and stored at 4 °C for further work.

Halo plate diffusion assay

Growth on diesel was determined by halo plate diffusion assay by Bushnell Hass (BH) agar medium (Geetha et al., 2013 and Saadoun, 2002). Colonies of the different bacterial isolates were transferred into 50 ml BH Medium (Leadbetter, Foster, 1958), supplemented with 10% (v/v) diesel oil solutions, filter sterilized through 0.45µm membranes and incubated at 35°C in a water bath shaker at 150 rpm for 9 days. Bacterial growth was determined at intervals by measuring the optical density (OD_{600 nm}). The test to detect the biodegradation of diesel was studied by a monoxygenase biodegradation pathway (Jacob et al., 1983). The test was performed at 28°C in a glass test tubes containing; 20 µl of 0.05 mol/l 2,6-dichlorophenolindophenol (DCPIP) (Himedia, India); 30 µl of 0.05 mol/l 5-methyl-phenazinium methylsulphate (5-MPMS); 25 µl of 0.1% (v/v) diesel; 5 µl of 0.15 M NAD solution and 25 µl of pre-washed bacterial cells. Change in color was visually compared with different controls: no diesel (substrate); no NAD⁺; no cells and heat-killed cells. The reaction was monitored up to 12 hours with intermittent observations (Geetha et al., 2013).

Optimization of growth conditions

The initial enrichment cultures were established in synthetic wastewater containing mineral salts medium amended with the diesel oil as the sole source of carbon and energy. The optimum temperature, agitation, pH and salinity concentration, glucose, peptone, nitrogen, nitrate, phosphate and diesel concentrations for the growth of the bacterial strains were determined. In order to optimize the growth conditions, potential bacterial strains (1% v/v) was grown in a conical flask containing 100 ml MMSM and 1% (v/v) diesel at 35°C for 24 hr and it provides as the inoculum. The MMSM containing flasks were inoculated with 1 ml of inoculum with 0.1 OD culture and incubated the flasks at 35°C for 9 days. After every 24 hr the treated culture medium was analysed for the diesel degradation up to 9 days. The growth in terms of optical density (OD_{600nm}) value was determined by turbidity measurement using UV-Vis spectrophotometer (Shimadzu - UV-3600, Japan).

Effect of different factors on diesel oil utilization

Different factors were tested for optimization of diesel utilization. These were pH, NaCl concentration, temperature, time and agitation (Satpute et al., 2008).

Effect of diesel oil concentration

The concentration of diesel oil as hydrocarbon source (up to 10% v/v) was studied to determine the effect of diesel oil on the growth of bacteria.

Effect of Temperatures on growth.

Temperature is one of the most important physical factors affecting bacterial growth. The potential bacterial strain inoculated MMS medium containing flasks along with control were incubated for 9 days at different temperatures (28°C, 30°C, 35°C and 40°C) in a rotary shaker at 150 rpm. The presence or absence of

growth of the cultures was observed after every 24 hr of incubation. The degree of growth was observed at wavelength of 600 nm using spectrophotometer.

Effect of agitation on growth

The effect of agitation on the growth of bacteria in MMSM was varied at different rpm (100, 120, 150 and 170 rpm) using incubator shaker and the optimum growth was monitored.

Effect of pH on growth

To study the effect of pH on the growth of bacteria, the pH of the MMSM was varied (pH 5, 6, 7, 8, 9) using 1N HCl and 1N NaOH and the growth was continuously monitored. The pH was adjusted and monitored using pH meter (Fisher Accumet pH meter).

Effect of salt concentrations on growth.

The effect of salt concentration on the growth of the bacteria was studied by growing the culture in MMSM with different salt concentration (4%, 8%, 12%, 16% and 20% of NaCl). One ml of the potential culture strain was added into the MMSM with different salt concentration and incubated for 24-48 hours at 35°C in a rotary shaker at 150 rpm.

Effect of carbon source and glucose concentration on growth

The effect of carbon source was investigated by growing the culture in MMSM supplemented with different carbon sources includes glucose, sucrose and fructose. Glucose was selected as the source which can best enhance the growth of bacteria. The concentration of the glucose was varied (0.5%, 1%, 2%, 3% w/v) to probe the influence of concentration of carbon on the growth of bacteria.

Effect of nitrogen source and its concentration

The effect of nitrogen source was investigated by growing the culture in MMSM supplemented with different nitrogen sources; urea, ammonium nitrate, sodium nitrate. Sodium nitrate was selected as the nitrogen source which can best enhance the growth of the bacteria. The concentration of the sodium nitrate was varied (0.5%, 1%, 2%, 3% w/v) to probe the influence of concentration of nitrogen on the growth of bacteria. The effect of peptone concentration was also studied with various concentration of peptone (0.5, 1-6 mg/l) to investigate the growth of bacteria.

Screening of oil degrading Biosurfactant producing bacterial strains

The isolates of the bacteria was screened for production of oil degrading biosurfactant. The screening was carried out by the following methods. Biosurfactant production and diesel degradation capabilities of the isolates were determined by using drop collapse method, oil spread method and measurement of emulsification index (E₂₄). The determination of diesel oil degradation ability of the selected potential bacterial strain was done by gravimetric analysis and FTIR analysis.

Drop collapse method

Biosurfactant production was screened using the qualitative drop collapse test. 2µl of diesel was added to 96 well micro-titre plates. The plates were equilibrated for 1 hour at 30°C and 5µl of the respective culture supernatant obtained was added to the surface of the oil in the well. The shape of the drop on the oil surface was observed for 1 minute. If the culture supernatant makes the drop collapse, it indicated positive result for biosurfactant presence and if the drop remained as such it indicated negative result (Bodour et al., 2003).

Oil spread method

The petridish base was filled with 50 ml of distilled water. On the water surface, 20µl of diesel and 10µl of culture were added respectively. The culture was introduced at different spots on the diesel which is coated on the water surface. The occurrence of a clear zone was an indicator of positive result (Morikawa et al., 2000).

Emulsification Index (E₂₄)

The emulsifying capacity was evaluated by an emulsification index. The E₂₄ of the culture samples was determined by adding 2 ml of diesel and 2 ml of the inoculum in a test tube and it was shaken for 2 minutes. Then, water and diesel were added and shaken for 2 minutes to obtain maximum emulsification and allowed to stand for 24 hours. This was taken as control (Desai and Banat, 1997).

The percentage of the E₂₄ index is calculated by the following formula:

eqn.[1]

$$E_{24} = \frac{\text{Height of the emulsified layer (cm.)}}{\text{Total height of the column (cm.)}} \times 100$$

Analytical Characterization

Batch degradation of diesel oil by potential bacterial strains in SWW

Biodegradation of diesel oil using *Pseudomonas aeruginosa* dou-1 was evaluated in synthetic wastewater. The potential bacterial strains at the log phase overnight cultures with 1 O.D (at 600 nm) were transferred to 250 ml conical flasks containing 100 ml of sterile defined modified mineral salts medium with 10% diesel oil in triplicates. All flasks were incubated at 32°C in a rotary shaker at 150 rpm for 10 days. The uninoculated medium containing 10% diesel oil and MMSM broth serves as control. At 24 hr intervals, sets of flasks were used for the extraction of residual oil. Whole sample contained in the individual flasks were extracted at initial 0 time, and every 24 hrs up to 10 days for the purpose of measuring residual concentration (Bishnoi et al., 2009). The total hydrocarbons in the treatments were determined gravimetrically.

Gravimetric analysis

The oil degradation was quantified by measuring the oil recovery after 16th days of incubation using the gravimetric analysis (Marquez-Rocha et al., 2001). The amount of oil in wastewater was estimated using the Gravimetric method. About 4ml of treated culture sample was taken from flask. Petroleum ether and acetone were taken in the ratio 1:1 and was mixed with the sample in a separating funnel. The mixture was shaken for about 45 minutes and then was left undisturbed for about 10 minutes. The upper solvent along with oil was separated from the lower extract. The solvent with the oil layer was then kept in the hot air oven at about 50° C until the solvent gets evaporated. After the complete evaporation, the oil residue obtained was weighed and taken as the gravimetric value for further calculation. Analysis of sample before and after treatment was done using this Gravimetric method.

The percentage of diesel oil degraded was determined from the following formula:

eqn.[2]

$$PDOD = \frac{\text{Weight of diesel oil degraded}}{\text{Weight of diesel oil present originally}} \times 100$$

Where,

PDOD = Percentage of diesel oil degraded,

the weight of diesel oil degraded = (original weight of diesel oil) – (weight of residual diesel oil obtained after evaporating the extractant).

Weight of residual oil = (Weight of beaker containing extracted oil) – (Weight of empty beaker).

FT-IR Analysis

After 7 days of growth, cell biomass was removed by centrifugation of broth culture at 10, 000 rpm for 15 minutes. Then the supernatant was precipitated overnight with ice cooled ethanol. The precipitated medium was centrifuged and the pellet was collected, dialyzed against distilled water for 24 hours and then lyophilized. A lyophilized sample was ground with potassium bromide (KBr) powder and pressed with 7,500 kg for 30s to obtain a translucent pellet. The infrared spectra were recorded on an FT-IR system (Perkin Elmer 783, Germany) within the range of 400-4,000 cm⁻¹ wave number. A KBr pellet was used as background.

RESULTS AND DISCUSSION

Morphology and Biochemical Characterization

The selective potential bacterial strain was analyzed taxonomically. The colony morphology of the strain was abundant, thin, white growth with medium turning green, earthy odour and rod shape. The strain was found to be Gram negative and rod shaped bacterium (Figure. 1). Based on the biochemical characteristics, the selected potential bacterial strain was identified as *Pseudomonas aeruginosa* dou-1 (Table. 1).

Screening the competence of diesel oil utilization by potential methylparathion degrading bacterial strain (*Pseudomonas aeruginosa* dou-1).

The bacterial strain used for the biotreatment process was *Pseudomonas aeruginosa* dou-1 (Table.1 and Figure 1), which is a methylparathion degrading bacterial strain previously identified according to Bergy’s manual of determinative bacteriology (Holt et al., 1994; Cappuccino and Sherman, 2010) stored in the lab was subjected to diesel oil degradation in synthetic wastewater containing diesel oil at 10% (MMSM). The bacterial strain which was found to

persist and grow under high diesel oil concentration was screened from this study and named as *Pseudomonas aeruginosa* dou-1. In Figure.1, there was not much difference between the colony forming units found in nutrient agar (NA-36 x10² CFU/ml) was) medium when compared to modified mineral salts agar medium (MMSM- 34 x10⁻² CFU/ml).

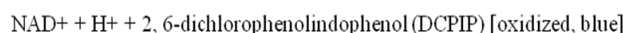
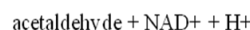
Table 1 Identification of potential bacterial strain of biosurfactant activity

Biochemical Tests	Characters
Colony morphology on Nutrient Agar medium	Abundant, thin, white growth with medium turning green, earthy odour
Gram stain	Negative, Rod shaped
Spore	Negative
Motility	Positive
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl Red	Positive
Voges Proskaur	Positive
Citrate	Positive
Nitrate	Positive
Glucose	Positive
Lactose	Negative
Sucrose	Negative
Mannitol	Negative
Rhamnose	Negative
H ₂ S production	Negative
Gelatine liquefaction	Positive
Casein hydrolysis	Negative
Lipid hydrolysis	Positive
Starch hydrolysis	Negative
Growth: at (28 - 40°C)	Positive
at (pH 5– 8)	Positive
at (100-170 rpm)	Positive
Growth in Selective medium Cetrinide agar medium	Positive
Pigment production: <i>Pseudomonas</i> agar medium and King’s A medium.	Positive
Identified Bacterial strain	<i>Pseudomonas aeruginosa</i> dou-1

Hole-plate diffusion test using diesel as a carbon source showed bacterial growth around the holes containing the diesel. Growth of hydrocarbon degrading bacteria using different concentration of hydrocarbons as carbon source was analysed. The bacterial growth was observed in different concentration of hydrocarbons upto 10%. The potent hydrocarbon degrading isolate (based on hole-plate diffusion method) were selected for the diesel degradation assay, by a monooxygenase biodegradation pathway. The test is based on the following reactions (Geetha et al., 2013).



alcohol dehydrogenase



5-methyl-phenazinium methylsulphate



But while considering the economic feasibility and applicability in wastewater treatment, the MMS medium was selected for further biotreatment study. The isolates grew maximally on the diesel substrate when supplied as the sole source of carbon and energy. In a similar research by Rahman et al. (2002), the total viable count method was used to confirm the potential of different kind of bacteria utilizing hydrocarbon. This technique was used in several studies to show the ability of bacteria utilizing hydrocarbons (Emtiazi and Shakarami, 2004).

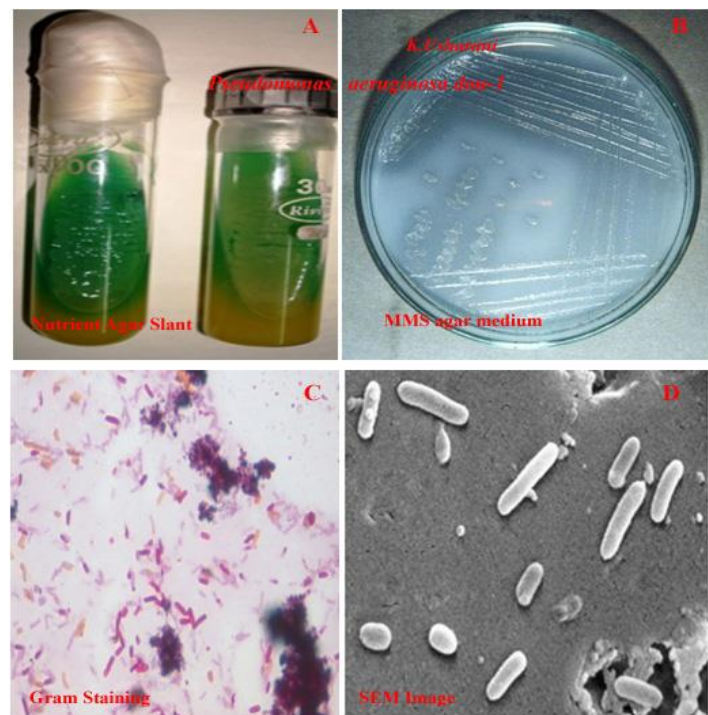


Figure 1 Identified potential bacterial strain of *Pseudomonas aeruginosa dou-1* (A) on NA slant (B) on MMS agar medium (C) Gram Staining (D) SEM Image

Optimization of growth conditions

Effect of diesel oil with respect to time on the growth of selected bacterial strain (*Pseudomonas aeruginosa dou-1*)

The effect of different concentration of diesel oil (1, 2, 4, 6, 8 and 10 %) on the growth of potential bacterial strain was studied. The bacterial strain *Pseudomonas aeruginosa dou-1* showed an increase in cellular density with respect to growth tolerance occurring at maximum 10 % (v/v) of diesel concentration (Figure.2A). Cell density considerably reduced at diesel concentrations higher than this. The selected bacterial strain *Pseudomonas aeruginosa dou-1* was able to tolerate high diesel concentration; this suggests that the strain is a better applicant for diesel biodegradation. Biodegradation studies on diesel are carried out using diesel concentrations ranging from 0.5 to 1.5% (Mukherji et al., 2004; Hong et al., 2005; Lee et al., 2006; Ueno et al., 2007). Degradation at a much higher concentration (6% v/v diesel) has been reported but degradation requires glucose (0.2% w/v) and yeast extract (0.1% w/v) (Kwapisz et al., 2008). Diesel is essential as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel that could obliterate cell membrane (Shukor et al., 2009).

Effect of agitation with respect to time on the growth of bacteria

The effect of different rate of agitation (rpm) on diesel degradation from the oil contaminated wastewater was studied. The bacterial strain *Pseudomonas aeruginosa dou-1* showed optimal growth at 150 rpm (Figure. 2B).

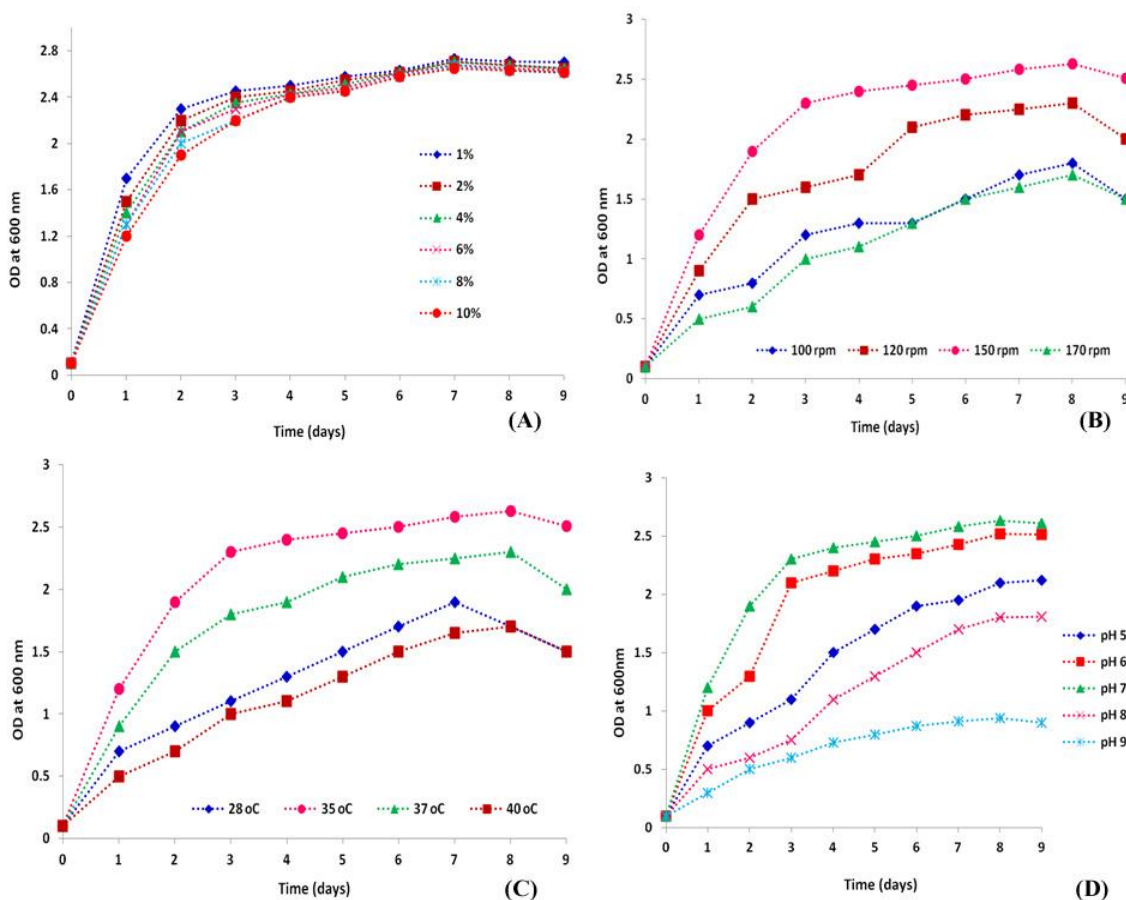


Figure 2 Effect of initial diesel oil concentration, agitation, temperature and pH on the growth of *Pseudomonas aeruginosa dou-1*

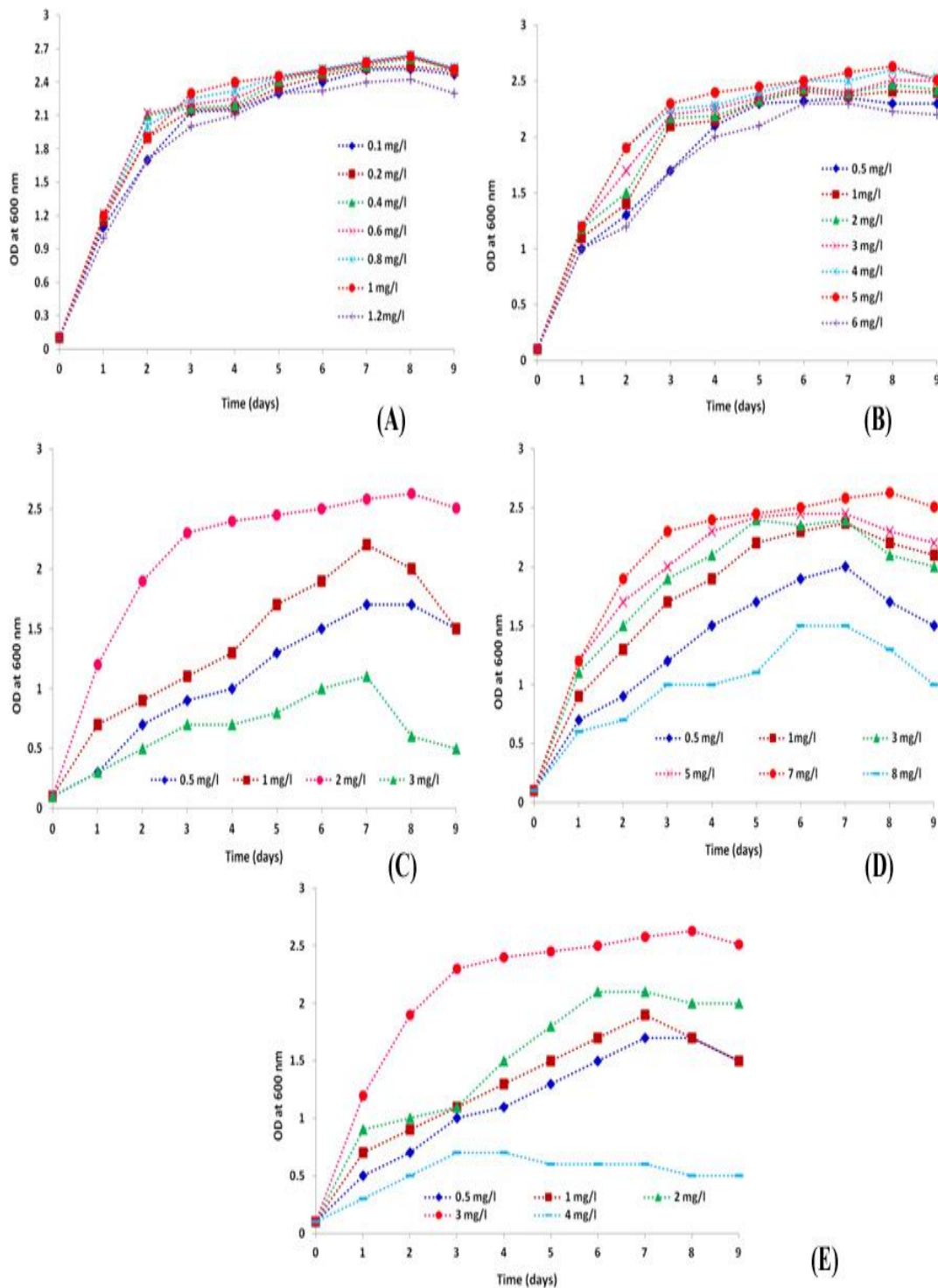


Figure 3 Effect of glucose, peptone, nitrate, phosphate, sodium chloride concentration, on the growth of *Pseudomonas aeruginosa* dou-1

Effect of temperature with respect to time on the growth of bacteria

Among factors limiting microbial activity, temperature is one of the most significant (Atlas and Bartha, 1993). The effect of different temperature on diesel degradation in wastewater was studied. The bacterial strain *Pseudomonas aeruginosa* dou-1 showed optimal growth at 35°C (Figure. 2C). The influence of temperature on the biodegradation of diesel oil was favourable at optimum temperature of 35°C.

Effect of pH with respect time on the growth of bacteria

The optimization of growth conditions showed that pH 7 was the best for the growth of *Pseudomonas aeruginosa* dou-1 (Figure. 2D) and followed by pH6, pH5, pH8. Optimal growth of the strain *Pseudomonas aeruginosa* dou-1 occur at

pH 7.0 in MMS medium with significantly higher growth was attained. Minimal growth was observed at pH 9.0 and at pH 5.0 (Figure. 2D).

The isolate showed an optimum growth requirement indicating that pH control is very important to get optimum results during bioremediation. Therefore, the optimization of environmental conditions is important for the enhancement of bacterial growth and for designing effective bioremediation strategy (Davey, 1994). The requirement of neutral or near neutrality for optimal growth of bacteria on diesel is also exhibited by many other diesel-degrading bacteria (Marquez-Rocha et al., 2005; Rajasekar et al., 2007; Kwapisz et al., 2008).

Effect of glucose concentration on the growth of bacteria

To study the effect of carbon and nitrogen sources on degradation, MMSM supplemented with 10 % of diesel oil along with carbon source such as glucose and nitrogen source such peptone. The effect of different glucose concentration

on the growth of bacterial strain for diesel degradation in wastewater showed glucose concentration of 1mg/l gave up the optimum growth of *Pseudomonas aeruginosa* dou-1 (Figure.3A). Glucose served as the most favourable carbon source in comparison with sucrose and fructose. The carbon source is mainly used as a cellular constituent, and for synthesis of new cells and production of polysaccharide as an energy source.

Effect of peptone concentration on the growth of bacteria

The effect of different peptone concentration on the growth of bacterial strain for diesel degradation in wastewater confirmed that the optimal concentration that enhances the maximal growth was found to be 5 mg/l (Figure. 3B). The peptone rich of nitrogen source influence the growth and metabolism of the selected strain of *Pseudomonas aeruginosa* dou-1.

Effect of nitrate concentration on the growth of bacteria

The nitrogen source affects the growth and metabolic activities of the organism. Various nitrogen sources such as urea, ammonium nitrate, sodium nitrate were used at an initial concentration of 1% (w/v) in MMS media supplemented with up to 10 % diesel to study their effects on bacterial growth. Sodium nitrate was observed to be the most suitable nitrogen source for the growth of the tested bacterial strain. The effect of different concentration of sodium nitrate on the growth of bacterial strain for diesel degradation in oil contaminated wastewater showed that the optimal concentration that supports maximal growth was at 2 mg/l (Figure. 3C). Sodium nitrate was selected as the nitrogen source which can best enhance the growth of the bacteria, was chosen as the principal nitrogen source due to its widespread usage as a cheap source of nitrogen for bioremediation (Sugumar et al., 2014).

Effect of phosphate concentration on the growth of bacteria

The effect of different concentration of phosphate on diesel degradation from the oil contaminated wastewater confirmed that the optimal concentration that supports maximal growth was at 7 mg/l (Figure. 3D).

Effect of salt concentration on the growth of bacteria

The effect of different NaCl concentration on the growth of bacterial strain for diesel degradation in wastewater showed that increasing NaCl concentration in wastewater had decreasing effect on diesel degradation. The amount of oil degraded by *Pseudomonas aeruginosa* strain increased initially to a maximum level at 3 m/l NaCl, but thereafter decreased with increasing salt concentration for the substrates (Figure. 3E).

Growth and screening of oil degrading biosurfactant producing bacterial strain *Pseudomonas aeruginosa* dou-1 in MMSA medium at optimum condition

From the Figure.4A, the growth in terms of optical density (2.68 OD) was found to be optimum on 7th day. Thus bacterium growth reached the stationary phase and moved into the death phase and this is probably due to the chemistry of the diesel degradation. The utilization of the diesel oil as sole carbon and energy source by the isolate resulted in the growth with a resultant production of acid. This is probably as a result of chemical change of the diesel oil, hydrocarbons and production of by products and ability of isolated *Pseudomonas aeruginosa* dou-1 to use diesel oil and generates organic acids. The biodegradation effectiveness of diesel oil in terms of growth by bacterial strain *Pseudomonas aeruginosa* dou-1 increased in efficiency from the 1st day to 7th day of incubation, increasing from 1 to 10 % of DO. Moreover, the growth studies indicated that diesel degradation ability was correlated with cellular growth of the organism. Almost complete removal of diesel oil (83%) as seen after 7days of incubation. The initial pH of the medium used in this experiment was neutral; a decreasing trend of pH was detected in the experimental flasks within the incubation period, as growth increases in the presence of the diesel oil was observed (Figure.4B). A change in pH is largely due to the production and accumulation of bacterial waste products. Maintenance of pH is important since pH strongly affects bacterial growth (Sugumar et al., 2014).

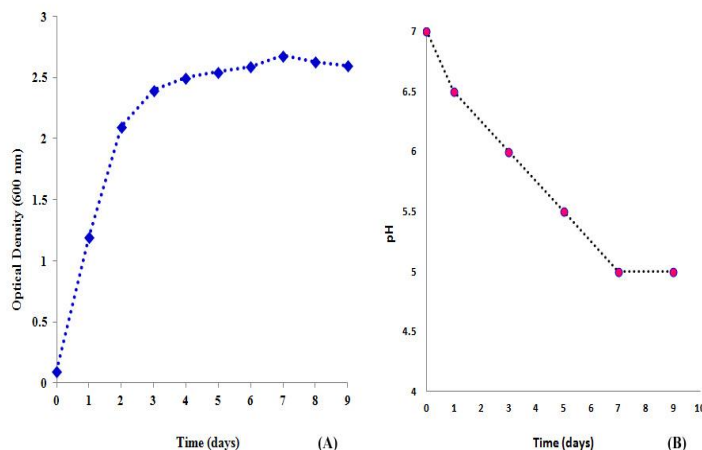


Figure 4 (A) Growth of diesel oil utilizing bacterial strain *Pseudomonas aeruginosa* dou-1 and (B) pH change in culture medium (MMS) at optimized conditions after biotreatment

Drop collapse method

The bacterial strain selected out of enrichment technique in MMS agar medium was used for screening of biosurfactant production purpose. A significant biosurfactant activity was reported when *Pseudomonas aeruginosa* dou-1 strain was used for analysis (Table.2). Yousef et al. (2004) stated that drop-collapse test expresses the surface activity and indicates production of biosurfactants.

Oil spread method

The study revealed a high biosurfactant activity of the bacterial strain (*Pseudomonas aeruginosa* dou-1) by oil spread method (Table.2). The increase in biosurfactant activity was also reported by other researchers (Ranjana Julias and Jeya Rathi, 2013).

Emulsification index(E₂₄)

The release of biosurfactants is a strategy used by microorganisms to affect the uptake of hydrocarbon compounds (Obayori et al., 2009). Consequently, measurement of emulsification activity (E₂₄) experiment was conducted for all of the bacteria which were successful at drop-collapse and oil spread (displacement) tests. The emulsifying capacity of the selected bacterial strain was evaluated by an emulsification index (E₂₄). The emulsification activity test revealed that the emulsification index (E₂₄) was observed to be maximum 72.72% for the diesel oil . The bacteria with emulsification indices higher than 50 % have been defined as potential biosurfactant producers (Rodriguez-Rodriguez et al., 2012). This method was reported previously by many researchers in their investigations (Sugumar et al., 2004 and Grazyna et al., 2006).

Table 2 Evaluation of biosurfactant activity by drop collapse and oil spread method

Bacterial strain	Drop collapse method	Oil spread method
<i>Pseudomonas aeruginosa</i> dou-1	positive	positive

Biodegradation efficiency

Diesel oil degradation ability of the isolates was investigated by using both gravimetric and FTIR analysis.

Gravimetric analysis of oil degradation

Gravimetric analysis was used to determine the diesel degradation ability of the potential bacterium and it was found to be 83% degradation by *Pseudomonas aeruginosa* strain (dou-1). Even though the gravimetric analysis is not as sensitive as GC analysis, it is comparatively helpful method for the preliminary determination. Therefore, the isolated bacteria found out to be successful in preceding experiments were subjected to gravimetric analysis of diesel oil degradation. The residual diesel oil amounts in samples were calculated by using the equation (2). Olsen et al. (1999) reported 75% for the n-alkane fraction of total extractable petroleum hydrocarbons in diesel oil after 35 days in batch experiments.

FTIR Analysis

The functional group or the chemical species of the biosurfactant was examined using FTIR analysis (Figure 5). The wave number at 2955, 2924 and 2852 cm^{-1} indicate the C-H stretching vibrations of hydrocarbon chain position.

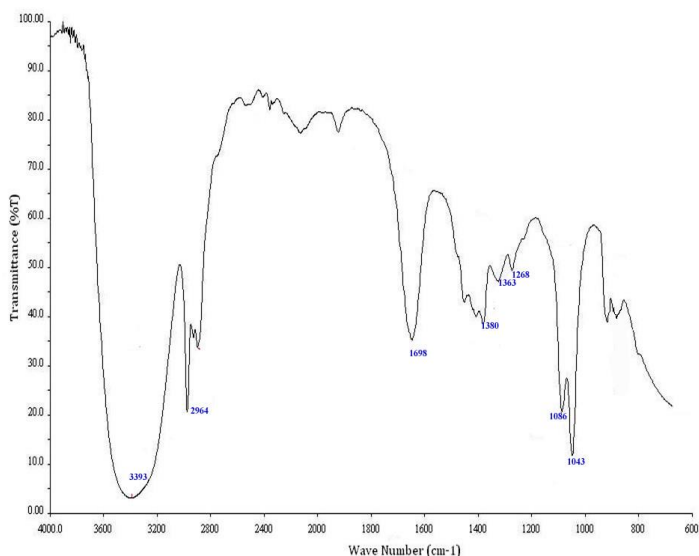


Figure 5 FTIR analysis of biosurfactant produced by the potential bacterial *Pseudomonas aeruginosa* dou-1

The bands at 2964 cm^{-1} resulting from the C – H stretching mode indicate the fatty acid part of lipopeptide. 1268 cm^{-1} exhibits the C-N stretching in peptide bond (amide III band frequency). Hydrogen bending on an aromatic ring could be observed at 800 cm^{-1} . The wave number at 1363 cm^{-1} indicates the chemical structure identical to those of rhamnolipids which are composed of rhamnose rings and long hydrocarbon chains. 1268 cm^{-1} exhibits the C-N stretching in peptide bond (amide III band frequency). The peaks in the range of 1080-1043 cm^{-1} corresponded to C-O-C stretching in the rhamnose. Similarly Pornsunthorntawe et al. (2008) and Bondarenko et al. (2010) reported similar results. The use of pure cultures in this study, in addition provides practical advantages by removing the ambiguity associated with constantly variable microbial populations (Ghazali et al., 2004). There is a possibility to increase the level of degradation by increasing the number of degradation days. The diesel oil was degraded by *P. aeruginosa* to utilize oil as the sole source of carbon and energy (Mandri and Lin, 2007). Hydrocarbon does not have any lethal effect on the growth of the bacteria. This may be due to the ability of the organisms to produce exopolysaccharides, which may protect them from the lethal effects of the hydrocarbons.

CONCLUSION

Soils contaminated with hydrocarbon are good sources for the isolation of diesel oil (DO) degrading bacteria which can then be used for the elimination of such compounds from the environment. The selected bacterial strain in this study from pesticide contaminated soil was recommended that the isolates may be well adapted for use because of prior exposed and acclimatized to the xenobiotic contaminants, which can be used efficiently in bioremediation of the diesel oil contaminated sites. Screening of the isolates (*Pseudomonas aeruginosa* dou-1) at different pH, temperature and agitation indicates the optimum pH of 7, temperature of 35°C and agitation of (150 rpm) in modified mineral salts agar medium at 10% diesel oil (v/v) with optimum concentration of additional nutrients of glucose (1 mg/l), peptone (5 mg/l), nitrate (2 mg/l), phosphate (7 mg/l) and sodium chloride (3 mg/l). The bioremoval and its biodegradation efficiency of *Pseudomonas aeruginosa* was 83% after seven days of incubation. From the study, it was concluded that the bacterial strain having the degradative potential of various pollutants include diesel oil as well as methylparathion. The bacterial strain has the ability to degrade the xenobiotic compounds practically in indigenous pure monoculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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