

Biodegradation of Petroleum Compound Using the Bacterium *Bacillus subtilis*

¹K.V. Darsa, ²A. Joseph Thatheyus and ²D. Ramya

¹Postgraduate Department of Immunology and Microbiology,

²Postgraduate and Research Department of Zoology, American College, Madurai, 625 002, Tamil Nadu, India

ABSTRACT

Background: With increasing demands of fossil fuel energy, extensive exploration of natural sources has caused a number of large scale accidental spills of crude oil and resulted in environmental disasters. The consequence of oil pollution to environment and human health has brought a serious challenge to environmental scientists. The aim of the present study is to evaluate the biodegradation of petroleum compound by *Bacillus subtilis* isolated from automobile workshops. **Methods:** Soil samples were collected from petrol bunks and workshops and subjected to serial dilution and plating. From the developed bacterial colonies, one was selected and identified as *Bacillus subtilis* based on the biochemical tests. The isolated strain was able to grow in minimal broth along with 2.5, 5, 7.5 and 10% concentrations of petrol which indicated the capability of the organism in degrading petrol and utilizing it as a source for growth. **Results:** The isolated strain's efficiency was determined by analysing the parameters pH, optical density and CO₂ released during petrol degradation. HPLC analysis also confirmed the degradation of petrol by *Bacillus subtilis*. **Conclusion:** The isolate *Bacillus subtilis* has the ability to tolerate the petrol concentrations and grow on them. Hence, this strain can be used in cleaning oil polluted sites.

Key words: Biodegradation, petroleum compound, *Bacillus subtilis*, petrol

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INTRODUCTION

Oil contaminated wastewater has been identified as one of the most concerned pollution sources. This kind of wastewater is widely produced from a variety of sources such as crude oil production, automotive garage, oil refinery, petrochemical industry, metal processing, lubricant and car washing. These sources serve as the major contributor to the environmental problems especially in soil and water¹. Both the waste and unused compounds from the prescribed sources are grouped as oily waste which is difficult to treat or recycle². Petroleum contaminated soil contains various hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons and they are potentially toxic, mutagenic and carcinogenic³.

Multiple initiatives have been developed to resolve the problem of petroleum pollution. An array of procedures has been developed including physical, chemical and biological techniques. Among these procedures bioremediation is currently gaining importance. The demand of petroleum as a source of

energy and as a primary raw material for chemical industries in recent years has resulted in an increase in world production. This dramatic increase in production, refining and distribution of crude oil has brought with it an ever increasing problem of environmental pollution⁴. The persistence of petroleum pollution depends on the quantity and characteristics of hydrocarbon mixture and on the properties of the affected ecosystem. The ability to isolate high numbers of certain oil degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are active degraders in that environment⁵.

Contamination of water with hydrocarbon wastes stimulates indigenous microbial populations, which are capable of utilizing the hydrocarbon substrates as their sole carbon and energy sources thereby degrading the contaminants. Several bacterial species have been identified as having the ability for oil degradation. In general, *Bacillus* sp. has been identified as petroleum hydrocarbon degrader⁶ and is known as naphthalene and pyrene degrader^{7, 8}. According to Sorkhoh⁹, 368 isolates belonging to the genus *Bacillus* were isolated from desert samples and two strains of *Bacillus* were able to degrade 80-89% of crude oil (5 g LG¹) within five days at 60°C.

Corresponding Author: K.V. Darsa, Postgraduate Department of Immunology and Microbiology, American College, Madurai, 625 002, Tamil Nadu, India Tel: 91- 9487424820

Recently, Obuekwe *et al.*¹⁰ identified *Bacillus* sp. and *Paenibacillus* sp. as two of the most prominent crude oil degraders in the Kuwait desert environment. The ability of *Bacillus* species in hydrocarbon degradation has consistently been observed by Antai¹¹ and associated competent hydrocarbon enzyme system of the organism and its ability to form spores and emulsify hydrocarbons¹². The aim of the present study is to evaluate the biodegradation of petroleum compound by *Bacillus subtilis* isolated from automobile workshops.

MATERIALS AND METHODS

Collection of samples: The soil samples were collected from automobile workshops and petrol bunks from Madurai in sterile containers and transported to laboratory for analysis.

Isolation of petrol degrading bacteria: The collected soil samples were serially diluted up to 10G⁷ and 0.1 mL from the dilutions 10G⁵ and 10G⁶ were spread plated on bushnell hass mineral salt medium (magnesium sulphate 0.2 g, calcium chloride 0.02 g, mono potassium phosphate 1 g, di potassium phosphate 1 g, ammonium nitrate 1 g and ferric chloride 0.05 g) containing 2.5% of petrol¹³. The plates were incubated at 37°C for 24 h and from the developed colonies, one was selected for further experiments.

Identification of petrol degrading strain: Gram staining and biochemical tests like catalase, oxidase, Voges proskauer, indole production and sporulation tests were carried out for strain identification¹⁴.

Biodegradation studies: One hundred mL of minimal medium (dextrose 1 g, ammonium sulphate 1 g, dipotassium phosphate 7 g, monopotassium phosphate 2 g, sodium citrate 0.5 g and magnesium sulphate 0.1 g) with petrol and one mL inoculum from the overnight culture maintained in nutrient broth during the logarithmic phase were added to 250 mL erlenmeyer flasks which were subjected to a period of sixteen days. Flasks were incubated in a shaker at 30°C at 100 rpm. After specified time, flasks were taken out. The ability of the isolated strain to degrade petrol was studied by determining the parameters pH, OD and CO₂ in the culture medium every four days.

pH estimation: Sample from the culture medium was checked for pH after 0, 4, 8, 12 and 16 days of treatment with the pH meter.

Optical density (growth rate) determination: The optical density of the sample from culture medium

was determined at 610 nm after 0, 4, 8, 12 and 16 days of treatment using a spectrophotometer.

Carbon dioxide estimation: From each petrol concentration one ml of sample was taken after 4, 8, 12 and 16 days of treatment and titrated against 0.05 N NaOH solution. Phenolphthalein was used as the indicator and appearance of stable pink colour was considered as the end point. The amount of CO₂ was calculated using the following equation¹⁵:

$$\text{Free CO}_2 \text{ (mg L}^{-1}\text{)} = \frac{\text{Titre value} \times \text{Normality of NaOH} \times 1000 \times 44}{\text{Volume of the sample}}$$

Statistical analysis: Two way ANOVA was performed for the parameters pH, optical density and carbon dioxide released using MS Excel. Variability was considered only when the calculated F value was greater than the tabulated F-value at P is less than or equal to 0.05¹⁶.

RESULTS

The bacterial strain isolated from the soil was identified as Gram positive rod and the isolate was identified as *Bacillus subtilis* on the basis of results obtained in biochemical tests (Table 1). The strain exhibited positive results for Gram's staining, Voges Proskauer and sporulation tests. Negative results were noted for carbohydrate fermentation, catalase, oxidase, mannitol, nitrate reduction, methyl, indole and litmus tests.

The efficiency of the bacterial strain was studied using different concentrations of petrol (2.5, 5, 7.5 and 10%). Figure 1 illustrates the changes in pH recorded after 4, 8, 12 and 16 days of treatment with *Bacillus subtilis*. Initially the pH showed a decline indicating the formation of organic acids except 7.5% petrol concentration. Later, pH showed an increase in all the concentrations except 5% and the highest pH was observed for 10% petrol concentration.

Table 1: Biochemical tests conducted for the identification of the natural isolate *Bacillus subtilis*

Tests	Results
Gram's staining	+
Carbohydrate fermentation	-
Catalase	-
Mannitol	-
Nitrate reduction	-
Urease	-
Methyl	-
Indole	-
Litmus	-
Voges proskauer	+
Oxidase	-
Sporulation	+
Result	<i>Bacillus subtilis</i>

+ : Positivem, -: Negative

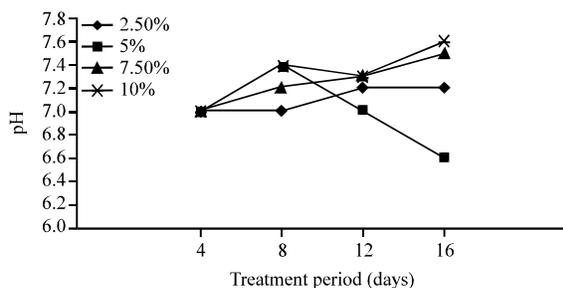


Fig. 1: Changes in pH of the culture medium during the degradation of petrol by the bacterium *Bacillus subtilis*

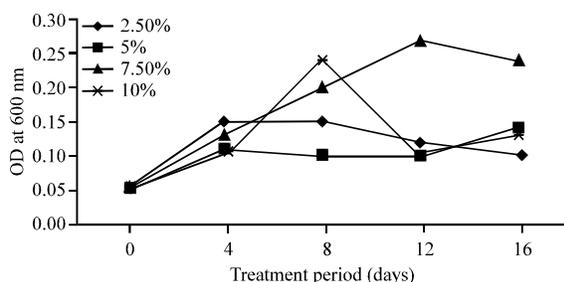


Fig. 2: Changes in the optical density of the culture medium during the degradation of petrol by the bacterium *Bacillus subtilis*

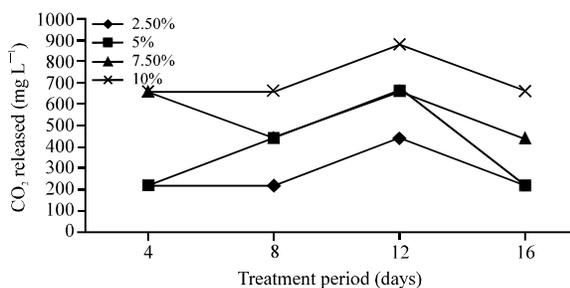


Fig. 3: Changes in the levels of carbon dioxide released (mg L^{-1}) during the degradation of petrol by the bacterium *Bacillus subtilis*

Changes in the optical density of medium during the sixteen days of treatment of petrol by *Bacillus subtilis* are illustrated in Fig. 2. Optical density values exhibited an increase during the initial period of treatment upto eight days and afterwards showed a decline. Maximum optical density was observed for 7.5% petrol concentration after eight days of treatment.

Changes in the carbon dioxide levels of medium during the sixteen days of treatment of petrol by

Bacillus subtilis are shown in Fig. 3. The biodegradation of petrol resulted in the production of carbon dioxide which was found to increase linearly with increasing concentration of petrol. During degradation of 10% petrol by *Bacillus subtilis*, more amount of carbon dioxide was released on 12th day of incubation. CO_2 released during petrol degradation showed an increase during initial period and later remained in the asymptote level except for 5% petrol concentration. Highest level was found for 10% petrol concentration after twelve days of treatment.

The variations due to petrol concentration and treatment period were statistically significant for the factors optical density and carbon dioxide released, while they were not significant for pH (Table 2). Figure 4 shows the HPLC analysis report for control having 10% of petrol without inoculum. It shows only the two peaks with the retention time of 1.933 and 2.247 min. In Fig. 5, HPLC analysis report for 10% petrol treated with *Bacillus subtilis* after sixteen days is shown. Both the peaks observed in the control were missing and the appearance of several new peaks with different retention time, indicate the degradation of petrol.

DISCUSSION

Biodegradation with the metabolic utilization of organic compounds by microorganisms requires oxygen, nutrients, water and specified physico-chemical conditions such as moderate temperature and pH¹⁷. Microbial populations increase rapidly in the water after an oil spill. Oil spilled on the ground can rapidly disappear completely under optimal conditions often within a year or two as a result of microbial oxidation of the hydrocarbons. Consequently it may be useful to employ the natural microflora in cleaning up of oil spills¹⁸.

Bacteria have the ability to exploit carbon compounds in oil as a necessary source of energy¹⁹ and have large capability to adapt to the environment that live in it by using its own special enzymatic system²⁰. There are many different varieties of hydrocarbons and over millions of years bacteria have evolved catalytic enzymes that are specific for particular degradation reactions. Some of the simpler compounds can be degraded by a very wide variety of bacteria but the ability to degrade hydrocarbons is found in fewer species. No single bacterium can make all the different enzymes, instead; each kind of bacterium specializes in a few hydrocarbons as preferred food sources²¹. Most microbial oil degradation occurs by aerobic degradation. In the absence of oxygen, microbes have other mechanisms to degrade hydrocarbons for energy. Biodegradation of oil constituents without oxygen is much slower but anoxic processes may be relevant to the long-term restoration efforts.

Table 2: Two way analysis of variance (ANOVA) for the factors, pH, optical density and carbondioxide with the variables, treatment period and petrol concentration for treatment with the bacterium, *Bacillus subtilis*

Factor	Source of variation	df	MS	Calculated F-value	Table F-value	Level of significance
pH	Treatment period	3	0.5229	0.90396	3.862548	Not significant
	Petrol concentration	3	0.8562	0.85625	3.862548	Not significant
Optical density	Treatment period	3	0.0070	4.80428	3.490295	Significant
	Petrol concentration	4	0.0070	4.79695	3.259167	Significant
Carbon dioxide	Treatment period	3	59491	7.08	3.862548	Significant
	Petrol concentration	3	14822	17.64	3.862548	Significant

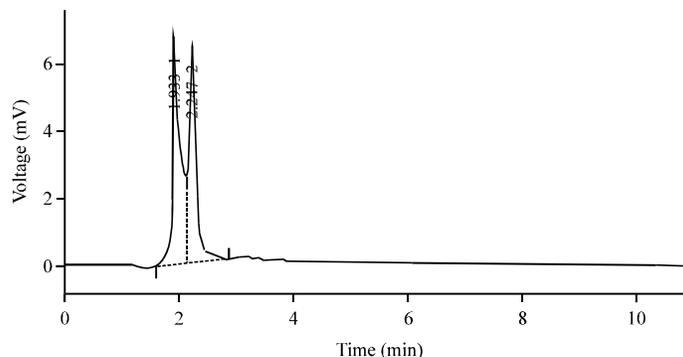


Fig. 4: High pressure liquid chromatographic analysis report for 10% petrol concentration in minimal medium before treatment with the bacterium, *Bacillus subtilis*

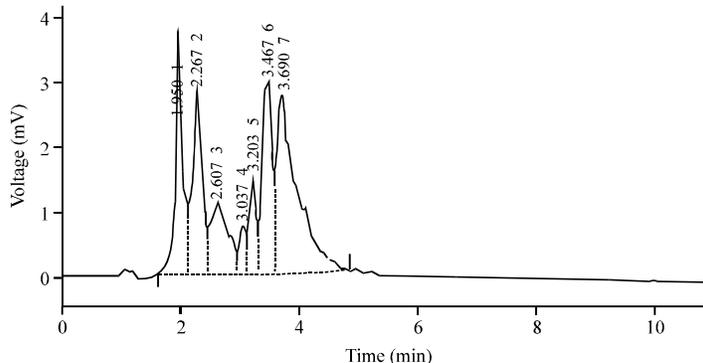


Fig. 5: High pressure liquid chromatographic analysis report for 10% of petrol concentration in minimal medium treated with the bacterium, *Bacillus subtilis* for sixteen days

The optimum pH for biodegradation of hydrocarbons is around 6-8²². Biodegradation of crude petroleum in an acid soil (pH 4.5) could be doubled by limiting to pH 7.4. pH is an important factor which influences the microbiological metabolic activity and growth of microorganisms. Different microorganisms can grow over a wide pH range and every organism has its own tolerance level. Generally fat, oil and grease degrading organisms have optimum growth between pH 5.5 and 8.0 with maximization at 7.5²³. From the observed results the isolate, *B. subtilis* actively uptake the hydrocarbons at pH 6-7.6 and it coincides with the above

finding. Microorganisms promoting fouling of oil can live in a wide range of pH from 4 upto 9, however, they tend to prefer a neutral pH²⁴. The utilization of the crude oil as sole carbon and energy source by these microorganisms resulted in their growth with a concomitant production of acid. These acidic metabolic products might account for the decrease in pH of the culture medium⁵.

Thamer *et al.*²⁵ proved when conducting studies on *B. thuringiensis* biomass level reaching 5 g LG¹ indicated that such bacteria are highly efficient in oil degradation. All the bacterial isolates produced turbidity indicating an

ability of each to utilize the crude oil. The *Bacillus* species had the highest optical density followed by the *Micrococcus* sp. and thirdly by the *Proteus* species²⁵. In this study, the maximum optical density was observed for 7.5% petrol concentration after eight days of treatment with *B. subtilis* So. *B. subtilis* can be highly efficient in the process of degradation of oil. Microbes break oil down into simple carbon compounds that are used to make the sugars, fats and proteins needed for growth and energy production, ultimately the byproducts become carbon dioxide and water. The simple carbon compounds are incorporated into new cellular constituents, in other words, they are used to make more microbes. So, the carbon has been used to produce additional biomass²⁶.

The biodegradation of petroleum products by microbes produce ultimately microbial cells and carbon dioxide. The maximum release of CO₂ was found during degradation of petrol at 10% concentration. Balba *et al.*²⁷ stated that mineralization studies involving measurements of total CO₂ production can provide excellent information on the biodegradability potential of hydrocarbons in contaminated soils. The approach provided rapid, relatively unequivocal time-course data suitable for testing different biological treatment options, like the effect of nutrient supplementation and microbial inoculation. The test can be useful for confirming active hydrocarbon degradation during full scale bioremediation.

Few researchers have reported on *Bacillus* sp. for oil degradation potential. *B. subtilis* is more tolerant to high levels of oils due to their resistant endospores. There is growing evidence that *B. subtilis* could be effective in clearing oil spills²⁸. The initial decline in pH may indicate the formation of organic acids as a result of petrol degradation by *B. subtilis*. The higher concentrations of petrol, during initial period of treatment exhibited an increase in biomass confirming the isolate being capable of exhibiting growth by breaking down petroleum hydrocarbons. This is also confirmed by the increased release of carbon dioxide which may be a product of petrol degradation. HPLC analysis also offers further confirmation exhibiting new peaks which represent the metabolites of petrol degradation.

B. subtilis showed an increase in degradation up to eight days. When relating this trend to the increase in microbial cell count, it was observed that there was a rapid increase in the cell biomass of *B. subtilis* in the first eight days of incubation. The initial high rate of biodegradation observed is attributed to the increase in microbial biomass and nutrient availability²⁹. From this it can be understood that the efficiency of the organism and its stage of growth should be taken into consideration in clean up or removal of oil from the environment. This study has also revealed that the removal of oil from an

environment is effective within the first two weeks of microbial growth. Survival of microorganisms in oil medium after their inoculation is a key deciding factor in the rate of biodegradation of oil either in soil or in liquid phase³⁰. The necessity for oil degrading bacteria might have arisen from the fact that introduction of efficient oil degraders would be essential in order to effectively degrade the oil mixture³¹.

CONCLUSION

Bacillus subtilis has the potential to degrade petroleum compound because of its growth in high concentrations of petrol. It is confirmed by the changes in pH, optical density and CO₂ levels of the medium and HPLC analysis.

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