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ISOLATION AND CHARACTERIZATION OF DIESEL DEGRADING BACTERIA FROM PETROLEUM OIL CONTAMINATED SOIL

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ABSTRACT

Petroleum products are used for energy production and an essential part of our day-to-day lives especially in vehicles, ships, and industries. Accidental leakages occur easily and wastage petroleum is also discarded in the environment without any further processing causing environmental pollution. Diesel contributea big part topetroleum pollution. The current study was aimed to identify diesel degrading bacteria and determine some conditions to evaluate their best degradation capability. We identified *Aeromonas* spp., *Bacillus* spp., and *Enterobacter* spp. from diesel contaminated soil and found that *Aeromona*s spp. and *Bacillus* spp. grow best with 10% to 15% diesel whereas *Enterobacter* spp. can grow quite well with 20% diesel concentration at a higher temperature (40ºC) than the previous two bacteria. *Aeromonas* spp. worked well at low pH (pH 4 to pH 6) whereas *Bacillus* spp. and *Enterobacter* spp. worked best at higher pH (pH 10).

Keywords: Hydrocarbon. Diesel oil. Parameter. Diesel biodegrading bacteria

INTRODUCTION

Diesel is a fuel consisting of aliphatic and aromatic hydrocarbons ranging from C_8-C_{26} and can be obtained from crude oil distillation (**Ramasamy *et al.* 2017; Ciric *et al.* 2009; Gallego *et al.* 2001**). It is also known as Automotive Gas Oil (AGO) (**Ciric *et al*. 2009**).It is often considered as one of the major contaminants in soil and marine environment due to heady spillage of approximately 1.7 to 8.8 million metric tons each year (**Ramasamy *et al.* 2017; Palanisamy *et al.* 2014; Dadrasina *et al.* 2013**). Diesel can be released into the environment during transportation, spillage, accidents, washing of engines, from workshops, disposing of used oils, etc. It can cause devastating damage to the environment, aquatic lives, crops, animals, and even humans (**Usha *et al*. 2015; Ahamed *et al*. 2010; Klingston 2007**). The biodiversity of the affected area is also at risk due to the presence of such contaminants in the environment (**UKHPA 2006**). Inhaling liquid or vaporized diesel can cause poisoning, carcinogenesis with some immediate discomforts like vomiting, dizziness, and headache. Pneumonitis can occur if the diesel compound gets entrance directly into the lungs (**Ramasamy *et al*. 2017; UKHPA 2006**). Diesel in the soil can impart direct toxicity towards the plants, hinders sufficient aeration into the soil, reduces germination (**Kayode-Isola *et al.* 2008; Scullion 2006; Okerentugba and Ezeronye 2003**). Some native soil microorganisms are capable of bioremediating the diesel oil from the contaminated areas. Some such potent bacteria are *Psuedomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Bacillus* sp., *Flavobacterium* sp., *Acromobacter* sp., *Klebsiella* sp., *Actinomycetes* sp., *Acetobacter* sp., *Rhodococcus* sp., *Citrobacter* sp.,*Acinetobacter* sp., *Corynebacterium* sp., *Arthrobacter* sp., *Acromobacter* sp., *Alcaligenes* sp. (**Noor *et al*., 2017; Bhuvaneswar *et al*. 2012; Ahmed *et al.*2010; Al- Saridah *et al.*1999; Singh et al. 2008; Kebria *et al*. 2009; Hong *et al.*2004; Ueno *et al.*2007; Saadoun 2002; Amund 2000; Wackett and Hershberger 2001; Parales *etal*. 2002**) and some fungi like *Fusarium* sp., *Ulocladium* sp., *Candida* sp., etc (**Dhar*et al.*2014; Miranda *et al*. 2007; Binsadiq *et al*. 2014**). These microorganisms can degrade diesel to utilize it as carbon and energy source with the production of enzymes (**Chikere *et al.* 2009; Patil *etal*. 2012**).
The current study was conducted to isolate and identify diesel degrading bacteria from petroleum oil contaminated soil. Different parameters were investigated to determine the efficient diesel degrading condition.

MATERIAL AND METHODS

Study area and sample collection

Soil samples in 5 cm depth of surface soil were collected in pre-sterilized bottle from different petroleum oil contaminated areas (soil from some garages in Dhaka city were collected where many vehicles are kept and wasted/used and burnt diesel are discarded) of Dhaka city. Collected samples were taken back to the microbiology laboratory as soon as possible and stored at -4ºC until further processing. Diesel was collected from a local petroleum oil station.

Enrichment of soil samples

Bushnell Haas (BH) broth was used for the enrichment of the soil. 100 ml BH (Bushnell Haas) broth was autoclaved.. After that, 10 gm soil samples were mixed with 100 ml of BH broth with 10% (v/v) diesel as the carbon source. The conical flasks were then put in shaker incubator for 24-48 h for incubation at 32 ºC at 100 rpm (**Singh *et al.* 2008**). After incubation, the soil suspension was used for the isolation of bacteria.

Isolation and identification of diesel resistant bacteria from enriched soil samples

To isolate diesel resistant bacteria, at first 1 ml of enriched sample was mixed with 9 ml sterile water and mixed thoroughly. BH agar plates were prepared where 0.1 ml of the suspension from the mixed enriched oil and water was added to the agar plate followed by 100 µl of diesel as the sole source of carbon. Then the plates were incubated at 32 ºC for 24 hours (**Singh *et al.* 2008**). After incubation distinct colonies were selected as bacteria capable of using diesel as sole carbon source.
The identification of the selected bacteria was determined by some biochemical tests including triple sugar iron agar test (TSI), indole production test, catalase, oxidase, Methyl red test (MR), Voges proskauer test (VP) and citrate utilization test (**Cappuccino and Sherman, 1996**).

Effects of different parameters on the growth of diesel degrading bacteria

Effect of different concentrations of diesel

The bacterial suspension was prepared in normal saline and incubated for 24 hours at 37ºC. After incubation when bacterial suspension reached 1.5 OD (optical density), 1 ml suspension of each bacterium was inoculated into BH broth mixed with 5%, 10%, 15%, 20%, and 25% v/v diesel in separate conical flasks. All the flasks were then put on shaker incubator (10 rpm) at 32ºC (**Palanisamy *et al.* 2014**). OD was measured at 600 nm after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours respectively.

Effect of different temperature

Bacterial suspension was prepared in normal saline with the optical density of 1.5. 2 ml bacterial suspension of each bacterium was inoculated into 100 ml BH broth with 1% v/v diesel in separate conical flasks. The flasks were then incubated at 25ºC, 30ºC, 35ºC, 40ºC and 45ºC. OD was measured at 600 nm after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours respectively (**Palanisamy *et al.* 2014**)..

Effect of pH

The bacterial suspension was prepared in Luria Bertani (LB) broth and centrifuged at 5000×g for 10 minutes. Pellets were washed twice with normal

saline to remove all the traces of LB broth. The final bacterial pellet was mixed with normal saline to make a suspension of 1.5 OD. 2 ml suspension of each bacteria was inoculated into 100 ml BH broth with 1% v/v diesel in separate conical flasks adjusted to pH 4, 5, 6, 7, 8, 9, 10. The flasks were then incubated at 37ºC. OD was measured at 600 nm after 24 hours, 48 hours, and 72 hours respectively (**Palanisamy *et al.* 2014**).

Effect of inoculum size

BH broth supplemented with 1% v/v diesel was inoculated with bacteria suspension with the optical density of 0.15, 0.20 and 0.25. The flasks were then incubated at 37ºC. OD was measured at 600 nm after 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours respectively (**Palanisamy *et al.* 2014**)..

RESULTS AND DISCUSSION

From petroleum contaminated soil, bacterial isolates were collected using BH media with diesel as the sole source of carbon. After the biochemical identification of the bacteria grown on the media, we found three bacterial species designated as *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp. (Table 01). As these bacteria were present with diesel as the only carbon source and survived, we can assume that these bacteria are capable of utilizing diesel and are resistant to the damaging effect of it as well. As BH does not contain any carbon source, diesel will act as the sole carbon source or the bacteria which will grow on the medium eventually. Those who can not utilize diesel as sole carbon source, will not appear on the media (**Palanisamy *et al.* 2014; Ganesh and Lin, 2009**).

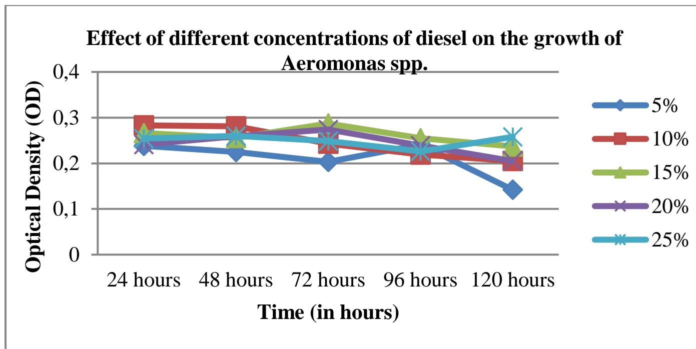
Table 1 Biochemical identification of diesel resistant isolates

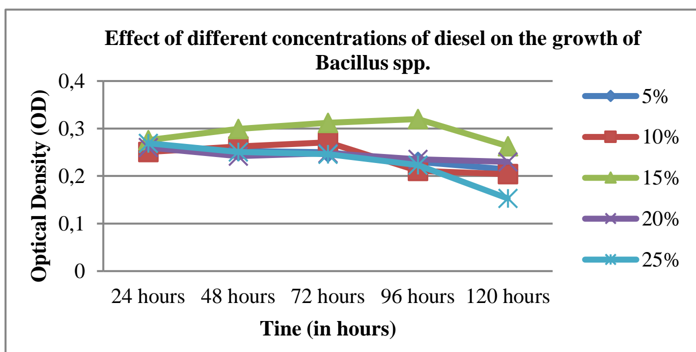
Isolates	TSI				Citrate	Indole	MR	VP	Oxidase	Catalase	Bacterial identification
	Slant	Butt	Gas	H_2S							
01	Y	Y	+	+	+	+	+	-	+	+	*Aeromonas* spp.
02	Y	Y	+	+	+	-	+	+	+	+	*Bcillus* spp.
03	R	Y	+	-	+	-	-	+	-	+	*Enterobacter* spp.

After identification of the bacteria, we intended to determine the most favorable conditions for their growth in which situation the degradation would be better. We have selected four parameters like different concentrations of diesel (as carbon source), temperature, pH, and inoculum size for all of the three bacterial isolates.

While studying with the different diesel concentrations (Fig. 1), *Aeromonas* spp. grew better with 15% diesel concentration comparing other concentrations after 72 hours. 25% showed the least effectiveness. 10% diesel concentration was suitable for growth only up to 48 hours.

Bacillus spp. showed to grow markedly well with 15% diesel especially until 96 hours of time. Where *Aeromonas* spp. and *Bacillus* spp. grew well with 15% diesel, *Enterobacter* spp. grew profoundly with 20% diesel which was the highest amongst all the isolates. Growth was maximum after 96 hours. In a study, it has been found that *Enterobacter* spp. Can produce lipopeptide C3 which can efficiently degrade diesel with maximum value of 48% (**Jemil *et al.*, 2018**). Aeromonas hydrophila was also found to be able to degrade diesel by 78% in 21 days (**Kaczorek *et al.*, 2010**). *Bacillus amyloliquifaciens* AN6 was also tested psoitive for their potential to degrade diesel (**Ayed *et al., 2015***).





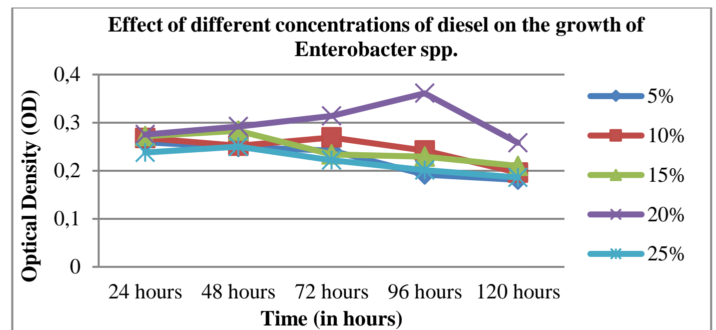
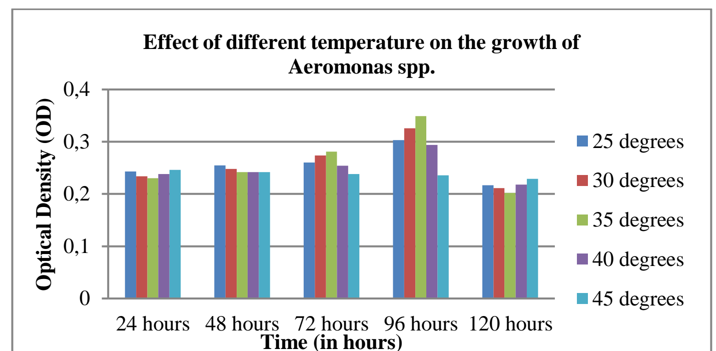


Figure 1 Effects of different concentrations of diesel on the growth of *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp.

In case of different temperatures (Fig. 2), we can see from the bar chart that, the growth of *Aeromonas* spp. was highest compared to *Bacillus* spp. and *Enterobacter* spp. for different temperatures (25ºC, 30ºC, 35ºC, 40ºC) after 72 and 96 hours. Growth was low after 120 hours for all temperature conditions. From 24 to 48 hours the growth rate was moderate.

For *Bacillus* spp. 40ºC temperature was best suited only after 96 hours.30ºC and 35ºC provided suitable growth conditions during 72 hours. From the beginning to 48 hours no change in the growth pattern was observable for any of the temperatures.

Enterobacter spp. was found to grow inadequately for 25ºC, 30ºC, 35ºC, 40ºC, and 45ºC (between 0.25 and 0.25 optical density) up to 96 hours which further decreased after 120 hours. This result reflects the similarity of other bacteria with the degradation capabilities at different teperatures (**Ramasamy *et al.* 2017; Palanisamy *et al.* 2014).**



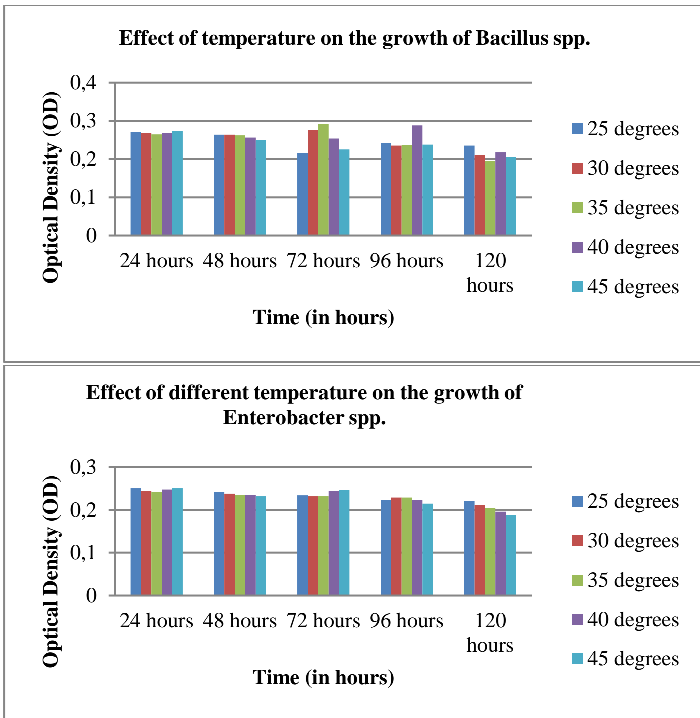
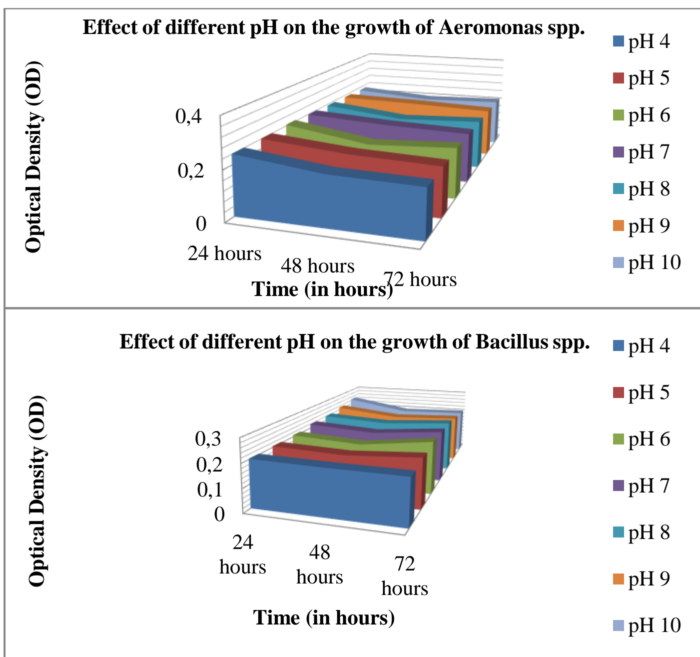


Figure 2 Effects of different temperatures on the growth of *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp.

While studying the effect of different pH (Fig. 3), we found maximum growth of *Aeromonas* spp. from pH 4 to pH 6.After that as pH increased, the growth decreased accordingly. For all pH condition, best results were showed only up to 24 hours. On the other hand, *Bacillus*spp. was found to increase their growth as the pH rises and we have found such results up to pH 10- the highest pH we used in this study. All these conditions resulted in better growth (up to pH 8) after 72 hours with a slight decrease after 48 hours. Finally, we studied *Enterobacter* spp. at different pH and found it grew well with increasing pH (from 7 to pH 10). Growth was maximum after 24 hours only. In a study, Bacillus spp.and Enterobacter hormaechei were found to degrade diesel at pH 7 (**Sivagamasundari *et al.,* 2017**).



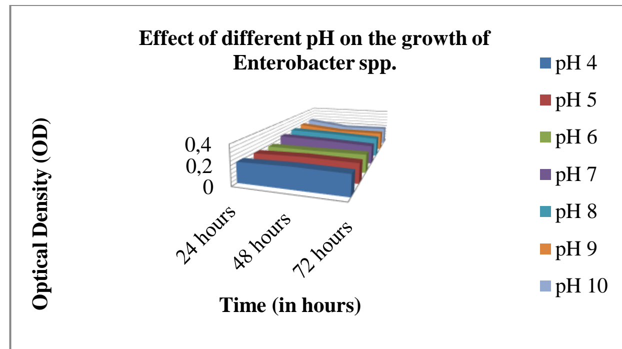


Figure 3 Effects of different pH on the growth of *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp.

During the study with different inoculum size (Fig. 4), we observed a simultaneous increase in growth of *Aeromonas* spp. starting from 24 hours to 120 hours of the time period with initial 0.28 OD inoculum. Fluctuated growth has been seen for initial inoculum 0.20 OD and 0.15 OD. *Bacillus* spp. showed maximum growth with all three initial inoculums after 48 hours which gradually decreased up to 120 hours. In the case of *Enterobacter* spp., all initial inoculum showed increased growth up to 96 hours and then decreased. Growth was highest at 96 hours of incubation. Similar study was also done with similar studies (**Palanisamy *et al.* 2014**).

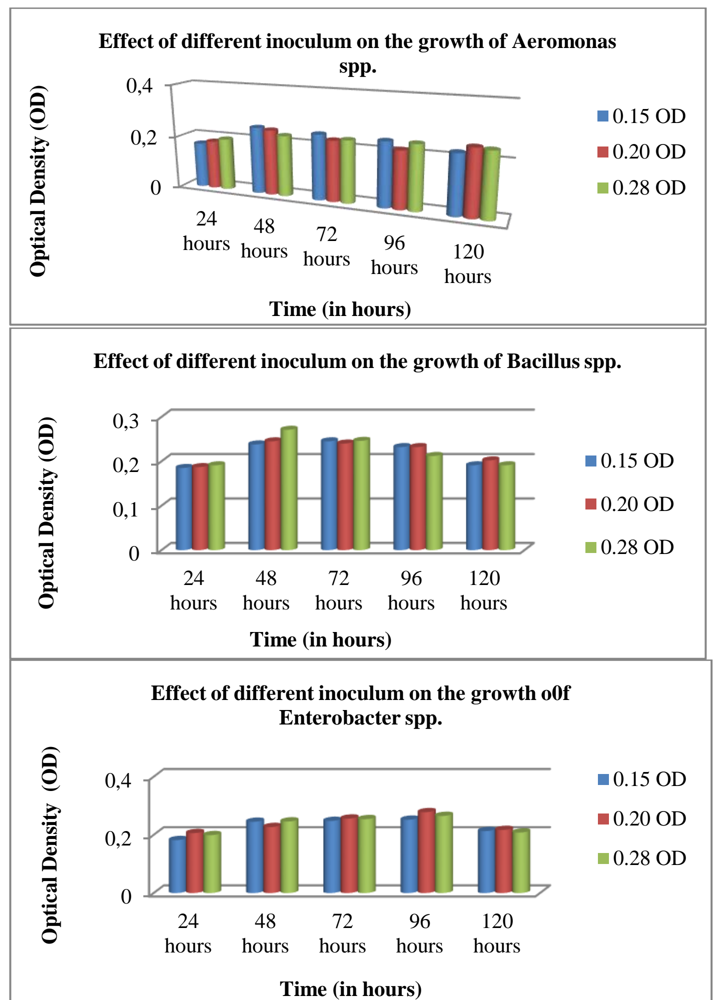


Figure 4 Effects of different inoculum on the growth of *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp.

CONCLUSION

The present study was conducted to determine the capability of degrading diesel oil by *Aeromonas* spp., *Bacillus* spp. and *Enterobacter* spp. isolated from diesel contaminated soil. The successful bioremediation depends on various factors among which carbon source, temperature, pH, and different inoculum was studied. *Aeromonas* spp. worked maximum with 10% to 15% diesel concentration, with environmental temperature 35ºC, pH range 4 to 6, and initial inoculum 0.15 OD. *Bacillus* spp. worked maximum with 15% diesel concentration, with environmental temperature between 35ºC to 40ºC, higher pH condition (pH 10), and initial inoculum 0.28 OD. Finally, *Enterobacter* spp. worked maximum with 20% diesel concentration, with environmental temperature between 40ºC to 45ºC, higher pH condition (pH 10) and initial inoculum 0.20 OD. During practical application, these three bacteria can show their best results of diesel oil degradation under the suggested conditions.

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