

## Plasmid Curing of a Novel Hydrocarbon Degrading *Bacillus cereus* Strain DRDU1 Revealed its Involvement in Petroleum Oil Degradation

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### Abstract

Plasmid curing of a novel hydrocarbon degrading *Bacillus cereus* strain DRDU1 was carried out to confirm the possible involvement of plasmid genes in hydrocarbon degradation. Plasmid curing was done by using 100 µg/mL of ethidium bromide, which is approximately 12 kb in size. Hydrocarbon degradation potential of the plasmid cured strain was compared with that of the control and the percentage hydrocarbon degradation of diesel, kerosene, crude oil and used engine oil after 28 days of incubation was confirmed by gas liquid chromatographic (GLC) analysis. The percentage hydrocarbon degradation for diesel, kerosene, crude oil and used engine oil (2% v/v) in Bushnell and Haas (BH) broth was found to be decreasing up to 30%, 21%, 13%, and 6% respectively for the plasmid cured strain, from its initial values of 99%, 96%, 84%, and 29% by the uncured strain. This study clearly confirms that the respective petroleum hydrocarbon degradation in this case is both plasmid and chromosomal encoded as considerable amount of degradation can be seen by the plasmid cured strain also. Due to this the strain is beneficial for in-situ bioremediation purpose, as the loss of plasmid due to any environmental or biological factor will not stop its potential to degrade hydrocarbon oil.

**Keywords:** *Bacillus cereus* strain DRDU1; Plasmid curing; Petroleum oil degradation

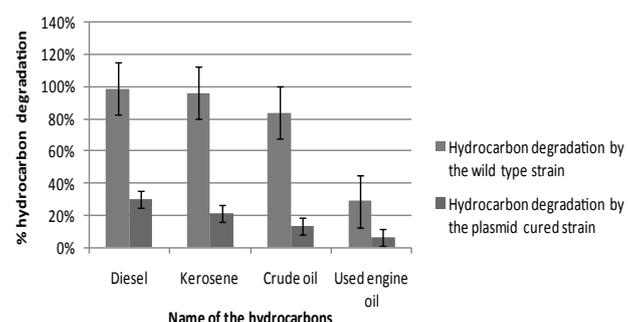
### Introduction

Nowadays, contamination of soil and water by hydrocarbon is a major environmental issue due to deliberate use of crude oil, diesel and used engine oil. Polycyclic aromatic hydrocarbons (PAHs) constitute about 3-30% of crude oil [1] and are a major suspect of cancerogenicity and mutagenicity [2]. Restoration of petroleum based oil-contaminated soil by microorganisms by removing the environment toxins viz., volatile organic hydrocarbons (VOCs), total petroleum hydrocarbons (TPHs), PAHs, heavy metals etc. is a well-documented process [3-7]. Molecular characterization and plasmid DNA curing studies of hydrocarbon degrading bacteria puts some light in determining role of genomic and plasmid DNA on hydrocarbon degradation, although only a limited number of such studies were found to be carried out with the evidence of publication [7-10]. Previous study showed a novel *Bacillus cereus* strain DRDU1, in terms of its ability to degrade hydrocarbon even under nutrient stressed conditions isolated from an automobile engine [11]. In the current study, plasmid curing of the strain was carried out to evaluate its possible role in the degradation of diesel, kerosene, crude oil, and used engine oil.

### Materials and Methods

The possible involvement of plasmid DNA in hydrocarbon degradation was determined by plasmid curing by inoculating 0.1 mL of overnight *B. cereus* strain DRDU1 in LB (Luria Bertani) broth (O.D.  $\geq 1$ ) in 100 mL of nutrient broth containing ethidium bromide (100 µg/mL). The exact mechanism of plasmid curing by intercalating agents is still unknown, however hypothesized by researchers that the inhibition of plasmid replication results from the relaxation of superhelical plasmid DNA by single nick caused outside the replication origin by the intercalating agents such as ethidium bromide [12]. Plasmid curing was confirmed by comparing antibiotic susceptibility test results of the control and test strain (basically antibiotic resistance in bacteria is plasmid encoded) using standard antibiotic discs of Methicillin (30 mcg) and Penicillin (30 mcg) on Muller Hinton agar plates. Cured strain was subcultured and 1 mL overnight broth was

inoculated in 100 mL BH broth supplemented separately with 2% (v/v) diesel, kerosene, crude oil, and used engine oil in 250 mL air tight Erlenmeyer flask. These were than incubated for 28 days at 37°C and 135 rpm. The petroleum oil degradation was confirmed by gas liquid chromatographic (GLC) analysis. Plasmid DNA isolation was carried out from overnight LB broth of the control and test bacterial cells by osmotic lysis method [13] Plasmid curing was finally confirmed by isolating and resolving the plasmid DNA from test and control strain in 1.2% agarose gel. The size of the plasmids was estimated relative to a 1 kb step up DNA ladder.



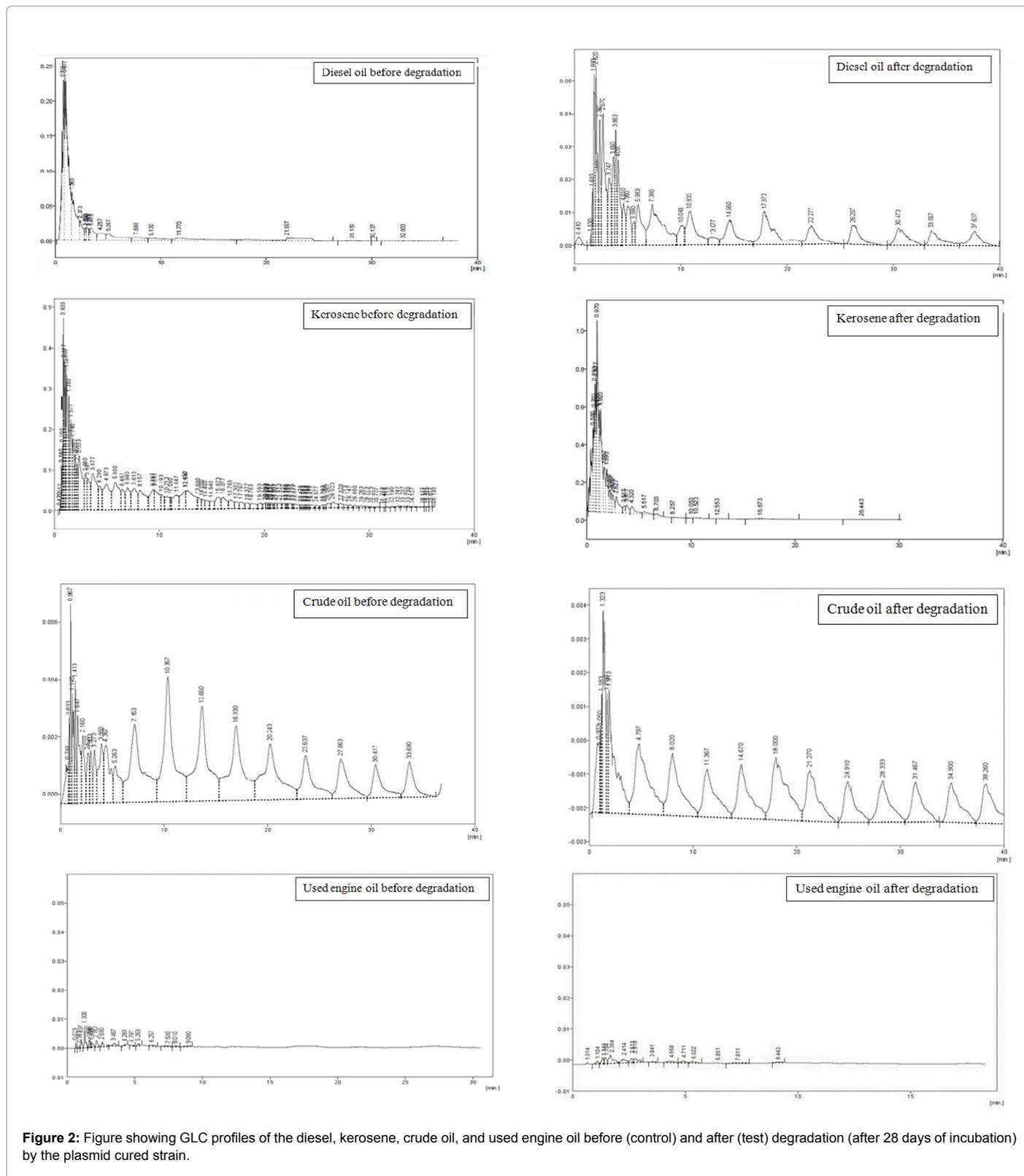
**Figure 1:** Percentage hydrocarbon degradation by the wild type strain and plasmid cured strain after 28 days of incubation at 135 rpm and 37°C at 2% (v/v) substrate concentration (petroleum oil). Results are in mean  $\pm$  S.D., where n=3.

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## Statistical Analysis

All the experiments were performed in triplicate and the results were expressed in mean  $\pm$  S.D.

## Results and Discussion

GLC analysis confirmed the decline in percentage hydrocarbon degradation upto 30%, 21%, 13%, and 6% respectively for diesel, kerosene, crude oil and used engine oil by the plasmid cured strain, from its initial values of 99%, 96%, 84%, and 29% Figure 1. GLC profile of the petroleum hydrocarbon before and after degradation by the cured strain is shown in Figure 2. Curing of plasmid was confirmed by comparing the antibiotic susceptibility results of the control with that of the plasmid cured strains. Plasmid cured strain showed zone of inhibition of  $31 \pm 3$ , and  $28 \pm 2$  mm respectively against methicillin and penicillin, whereas the control was resistant to both the antibiotics (Table 1). The absence of any plasmid after the incubation period of 28 days was also confirmed to make sure that there was no contamination. The absence of plasmid DNA bands in Lane no. 3 (from left) clearly indicates the successful curing of plasmids and Lane no. 2 shows the plasmid DNA band for the control strain in Figure 3. Antibiotic resistance is showed by the strain is exclusively plasmid encoded and hence the plasmid cured strain showed its sensitivity against all the three antibiotics used in the study. The size of the plasmid of the strain *Bacillus cereus* DRDU1 was found to be approximately 12 kb. Most of the previous studies have shown plasmid DNA as responsible for hydrocarbon degradation [14-17]. But, there are some other shows involvements of genomic DNA in hydrocarbon degradation [7,18,19]. Catabolic pathways, involved in aromatic hydrocarbon degradation routes are located on large plasmids in most of the cases although degradative genes can be located on either chromosome or plasmid or on both Coral and Karagoz [20]. Recent study confirms the inability of the plasmid cured Nitrifying and Nitrosomonas hydrocarbon degrading strains to grow in hydrocarbon supplemented media, as the hydrocarbon degrading genes are found only plasmid encoded [10] Besides only a limited literature describes the hydrocarbon degrading genes and their location in case of *Bacillus cereus*. Previous literature on *Bacillus cereus* 2479 shows the presence of such genes on both chromosomal and plasmid DNA [21] which correlates the findings of the current study. In the current study, the plasmid cured strain confirmed their ability to degrade petroleum oil even in the absence of

plasmids. Moreover the degradation pattern by the cured strain follows the same order of percentage degradation as in case of the wild type. These results, clearly indicates that both the genomic and the plasmid DNA of the strain has their more or less contributions in hydrocarbon degradation. Some degradation, including the breakdown of C5 to C12 n-alkanes, naphthalene and toluene pathways have been extensively characterized and are generally located on large catabolic plasmids. Similarly, several environmental isolates of *Acinetobacter sp.* and *Alcaligenes sp.* [22] *Arthrobacter sp.* [23] and *Rhodococcus strains* Malachowsky [24] have been found to degrade both alkanes and naphthalene, although the genes and catabolic pathways responsible were not described. On the other hand, some of the study showed plasmid encoded genes as responsible for hydrocarbon degradation [25] and some other showed chromosomal encoded genes responsible for hydrocarbon degradation by *B. cereus* [26].

Some of the previous studies have shown plasmid DNA as responsible for hydrocarbon degradation [14-17]. Research also shows involvement of genomic DNA in hydrocarbon degradation [7,18-19]. But the involvement of both genomic (chromosomal) and plasmid DNA in hydrocarbon degradation by the bacterial isolate *Acinetobacter venetianus* VE-C3 isolated from Venice Lagoon. The replica devoid of plasmid showed delayed growth as compared to the wild type on BH agar supplemented with hydrocarbon. These results, clearly indicates that both the genomic and the plasmid DNA of the strain has their more or less contributions in hydrocarbon degradation. Therefore, the current study confirms the presence of hydrocarbon degrading genes on both the genomic and plasmid DNA of the novel isolates *Bacillus cereus* strain DRDU1. These findings are highly promising to deploy the strain for *in-situ* hydrocarbon degradation, as the strain is degrading some amount of hydrocarbon even in absence of plasmid. This property will help the strain to continue the degradation process *in-situ* even in loss of plasmid due to any environmental factor, or if loses it by transferring to other competent strains.

## Conflict of Interest

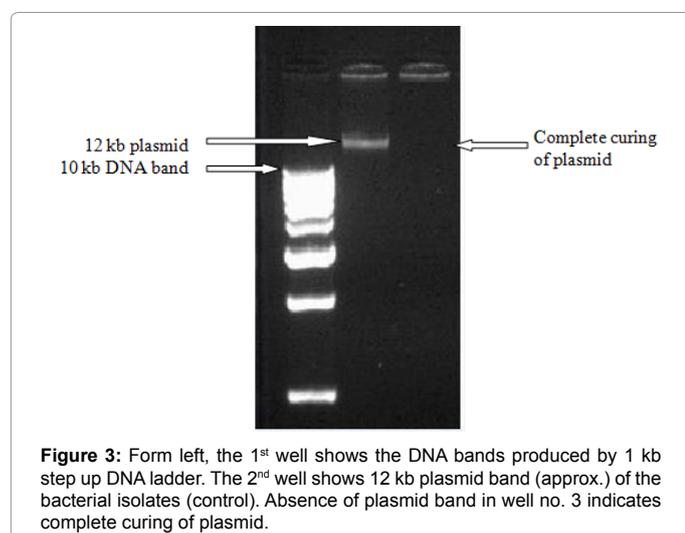
The authors declare that they have no conflict of interest and do not have any financial relationship with the organization that sponsored the research in the manuscript.

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