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**Bioremediation of diesel contaminated water using  
immobilized bacterial consortium TS1 on fired clay pellet**

**Nilobon Nakornburi<sup>1\*</sup> and Kobchai Pattaragulwanit<sup>1</sup>**

*<sup>1</sup>Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok  
10330 Thailand*

*\*Corresponding author. E-mail: nilobon.nakorn@gmail.com*

**DOI:**

**ABSTRACT**

Diesel is a mixture of alkanes and aromatic compounds frequently used as engine fuel. The spillage of diesel due to accidents during transportation, leakage from storage tanks and human activities leads to contamination of soil, sea water and fresh water. Components of diesel are toxic for the environment and carcinogen for human. Bioremediation is one of the environmentally friendly and high efficient method for remediation of diesel, wherein microbial activity has been used for this purpose. Immobilization of microbial cell is the optimal bioremediation technology because this method protects microbial cells from toxic and harsh environment condition as well as enhances microbial stability and easy to separate from the environment. In the present study, bacterial consortium TS1, isolated from water hyacinth roots, could degrade 1% diesel for 73.41% within 7 days. TS1 consortium was immobilized on fired clay pellets by adsorption method. Scanning electron microscope (SEM) revealed bacterial cells attached on the surface of fired clay pellet. The immobilized cells could efficiently degrade 1% diesel for 89.56% within 7 days. The immobilization of TS1 could also enhanced the biodegradation of diesel at higher concentration, in which 3% and 5% diesel were degraded for 80.83% and 70.90%, respectively within 7 days, which were higher than those of non-immobilized cell.

**Keywords:** Diesel; Bioremediation; Immobilization; Fired clay pellet

## INTRODUCTION

Diesel is a mixture of alkanes and aromatic compounds that used as engine fuel (Robson et al., 2017). Diesel contamination of soil, sea water and fresh water often occur as a result of accidents during transportation, leakage from storage tanks and human activities. Components of diesel, especially aromatic hydrocarbon are toxic for the environment and human health and can cause irritation, headache, nausea and lead to cancer (Zheng et al., 2018).

Bioremediation is one of the methods that proposed as an environmentally friendly, cost-effective and high efficiency strategy, wherein diesel contaminants can be removed by using microorganisms. (Wang et al., 2015; Chen et al., 2017). Using free cell for bioremediation is mostly unsuccessful due to the loss of augmented strains in environment or cell death in higher concentration of contaminants. Immobilization is the optimal bioremediation technology because the method protects microbial cell from toxic and extreme environment condition, enhance microbial stability and are easy to separate back from environments (Chen et al., 2016). Adsorption is an immobilization technique which uses the natural ability of microorganisms to produce biofilms and remain attached on the surface of carriers (Bayat et al., 2015).

In a previous study, *Acinetobacter* sp. F9, was immobilized on calcium alginate (CA)-chitosan. The immobilized F9 cells were able to remove more than 90% of the diesel in the second day, while free F9 cells removed about 60% of the diesel in seawater (Hou et al., 2013). *Rhodococcus pyridinivorans*, *Gordonia alkanivorans*, and *Alcaligenes piechaudii* immobilized in calcium alginate gel beads could removed 78% of diesel, but performed as suspended free cells, achieved only about 46% removal in seawater (Liu et al., 2015). Natural organic carrier could be used for immobilization. *Pseudomonas* YT-11 was immobilized on cinnamon shell. The immobilized YT-11 had the highest diesel degradation rate of 69.57% in 5 days while free cells removed only 48.87% in seawater (Fu et al., 2019). Bacteria consortia, namely: *Pseudomonas* sp., *Acinetobacter* sp. and *Rhodococcus* sp., immobilized on corn straw and corn leaf could enhanced diesel removal rates for more than 40% compared to free cells (Zhixiu et al., 2017). All of the above mention researches demonstrated the potential of immobilized cell for removal of diesel.

Fired clay pellets is a special type of clay that has been pelletised at 1,100 °C. The resulting pellets are porous, light weight and chemically inert. The pellets are used in gardening to retain moisture in the soil. The use of fired

clay pellets for removal of pollutants such as phenanthrene, pyrene and phosphate in water has also been reported (Nkansah et al., 2012; Ifelebuegu et al., 2016). In this study, we therefore aimed to immobilize bacterial consortium TS1, isolated from water hyacinth roots (Surengrit, 2015), on fired clay pellet as natural organic carrier. The ability to degrade diesel in liquid media was investigated and compared with those of free cell.

## MATERIAL AND METHODS

### Preparation of diesel degrading bacteria

Bacterial consortium TS1, consists of *Pseudomonas auraginosa*, *Stenotrophomonas* sp., *Ochrobactrum* sp., *Microbacterium* sp., *Shinella* sp., *Pusillimonas* sp. and *Pseudomonas* sp., were used in this study (Surengrit, 2015). Luria-Bertani (LB) was used as enrichment medium. The culture was incubated at room temperature with agitation of 200 rpm for 24 hrs. 10% of bacteria culture was inoculated to Carbon Free Mineral Medium (CFMM) (Mahanty et al., 2006) supplemented with 10% of LB and diesel 0.5%. The cultures were incubated at the same conditions. 10% of bacteria culture were inoculated in CFMM with 1% of diesel, incubated at the same condition for 7 days.

### Diesel degrading efficiency of bacterial consortium TS1

Bacterial cell cultures were centrifuged at 8000 rpm 4°C for 10 minutes and washed twice with 0.85% NaCl. The pellet was adjusted with CFMM to OD<sub>600</sub>= 1 (10<sup>8</sup> CFU/ml). 4 ml of bacterial solution were inoculated to CFMM 16 ml containing 1% diesel in 50 ml Erlenmeyer flasks. As control experiments, CFMM containing diesel without bacterial cells were used. The culture was incubated at room temperature with agitation at 200 rpm for 7 days. Samples were analyzed for the remaining of diesel by Gas Chromatography (GC).

### Preparation of immobilized bacterial consortium TS1

**Supporting materials.** Fired clay pellet (9 mm in diameter) were purchased from Chatuchak market in Bangkok, Thailand. Fired clay pellet were smashed to half pieces. 1 g of fired clay pellet were soaked in water overnight and autoclaved at 121°C for 15 min.

**Immobilization of TS1 cells.** 1 g of carriers were added into the flasks containing 5 ml of the bacterial cultures (OD<sub>600</sub>= 1). Cells were immobilized at room temperature with agitation at 200 rpm for 0.5, 1, 2 and 24 hours. Then

Immobilized pellets were put on sterile tissue paper until dried. Bacterial cells were counted by viable plate count method.

### **Diesel degrading efficiency of immobilized cells**

Immobilized cells were added into the flasks with 20 ml of CFMM containing 1% of diesel in 50 ml Erlenmeyer flasks. Diesel degradation occurred at room temperature with agitation at 200 rpm for 7 days. Then, samples were collected and analyzed for diesel remaining by Gas Chromatography (GC). As controls, CFMM with 1% diesel, CFMM with fired clay pellet containing 1% diesel and CFMM with TS1 cells containing 1% diesel were used. All experiments were performed in triplicate.

### **Electron Microscopy (SEM).**

Fired clay pellet as well as immobilized pellet at 0 day were investigated using Scanning Electron Microscope and energy dispersive X-ray Spectrometer – SEM- EDS (IT300) performed by Scientific and Technological Research Equipment Centre (STREC).

### **Gas Chromatography (GC)**

GC analysis was performed with 6890 Agilent technologies Equipped with 5% Phenyl Methyl Siloxane HP-N5 capillary, flame ionization detector (FID) and detector temperature was 325°C. The oven program started at 80°C was raised to 160°C for 3 minutes then raised to 240°C for 2 minutes and raised to 300°C for 10 minutes and keep at 80°C for 1 minutes. Helium flow rate was 2.1 ml/minutes. Area under the graph with 18 peak in different times: 2.5, 3.0, 3.6, 4.2, 5.0, 6.1, 7.4, 9.4, 11.7, 14.3, 17.2, 20.1, 23.0, 26.0, 28.8, 31.7, 34.5, and 36.0 minutes was used for calculating diesel remaining.

$$\% \text{diesel remaining} = \frac{\text{The total area under the graph of sample}}{\text{The total area under the graph of day 0}} \times 100$$

### **Analysis of total bacteria**

Total bacterial cells on carriers were sonicated for 2 minutes in 5 ml 0.85% NaCl. This step was repeated three times. Total of 0.85% NaCl solution containing bacteria were centrifuged at 8000 rpm 4°C for 10 minutes. Samples were ten-fold diluted with 0.85% NaCl and spread on LB agar, incubated at 30°C for 24-48 hours.

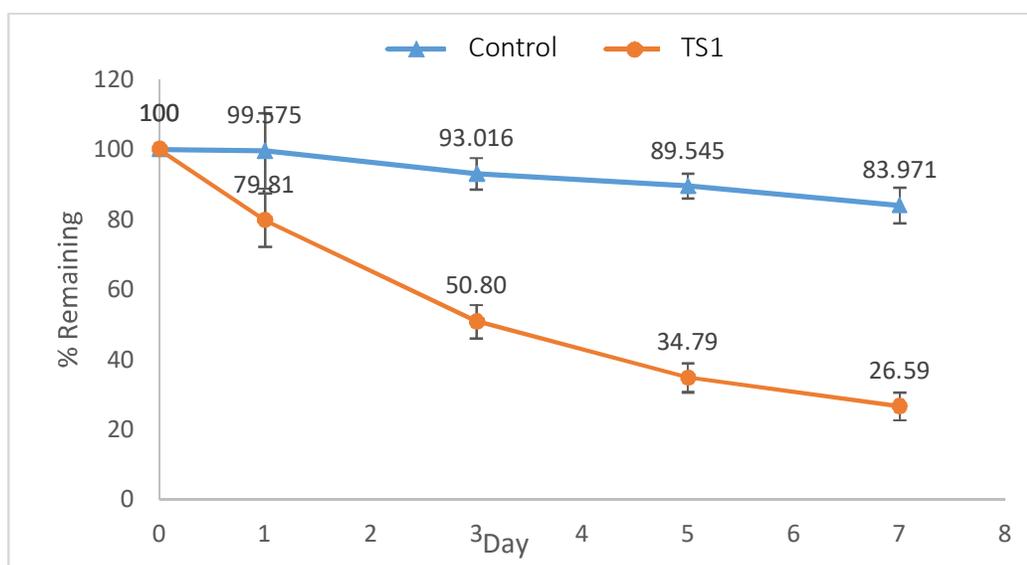
## RESULTS

### Biodegradation of diesel by bacterial consortium TS1

Bacterial consortium TS1 could remove diesel to the remaining of 26.59% within 7 days (Figure 1).

### Immobilization of TS1 on fired clay pellet

To optimize the incubation period for immobilization, fired clay pellets were incubated with TS1 culture for various time. The viable cells immobilized on carriers were counted. The result revealed that the number of immobilized cell reached the highest of 8.61 log CFU/g of fired clay pellet within 24 hours. Immobilization of TS1 for two hours resulted in the viable cell count of 8.42 log CFU/g which were about 97.8% of the highest immobilized cell. Therefore, the optimal time for immobilization used in this experiments was two hours.

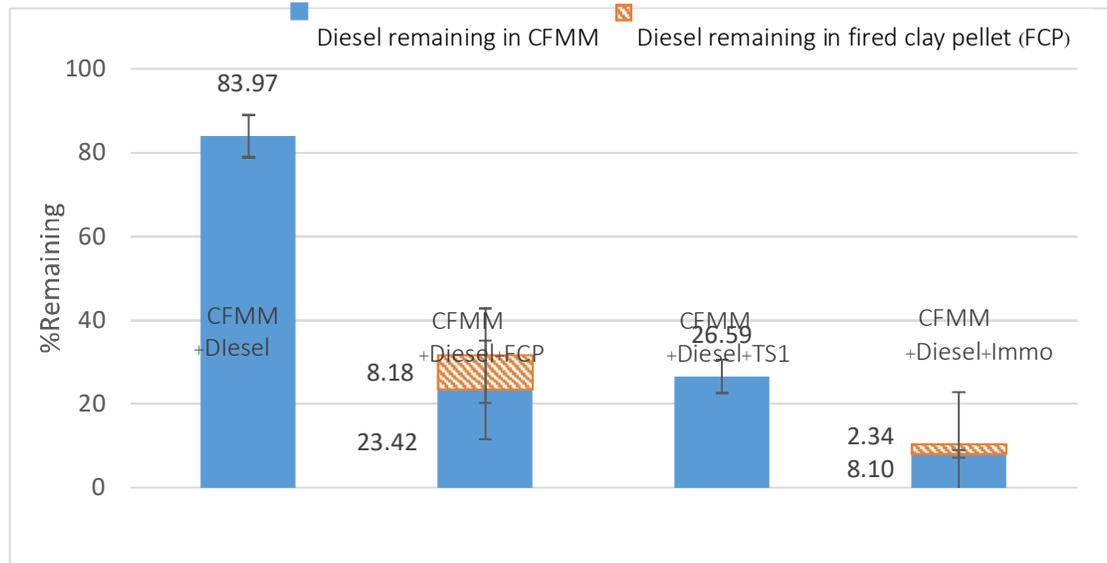


**Figure 1.** Biodegradation of 1% diesel by TS1

### Diesel removal by the immobilized TS1 cells

The diesel removal efficiency of immobilized TS1 cells and free TS1 cells have been tested in CFMM for 7 days. The results shown in Figure 2 revealed that immobilized TS1 on fired clay pellet could best degrade 1% diesel to the remaining of 10.44% within 7 days which was better than the use

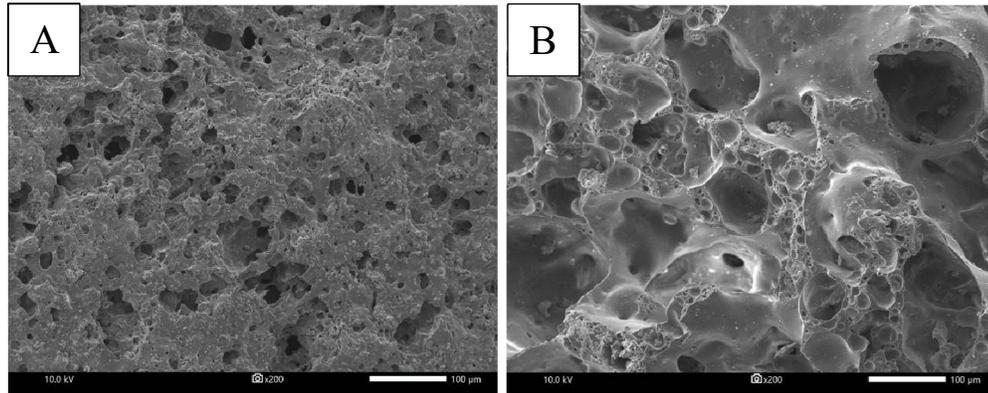
of free cells. The loss of diesel due to physical factors was as minimum as 16%. The adsorption of diesel by the pellet was only 8.18%.



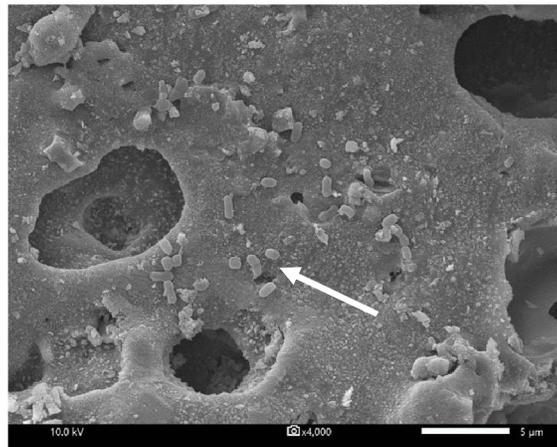
**Figure 2.** 1% diesel removal by immobilized TS1 compared to controls for 7 days.

### Scanning Electron Microscopy (SEM)

The inner and outer of fired clay pellets were imaged using SEM. Figure 3a and 3b respectively revealed that the inner surface was more porous than the outer surface of fired clay pellet. The high porosity of this carrier was therefore suitable for immobilization. After immobilization, SEM (Figure 4) showed the cells of TS1 consortium on the surface of fired clay pellet. The results in Figure 4 revealed that the TS1 cells were rod-shaped and easily immobilized on the fired clay pellet.



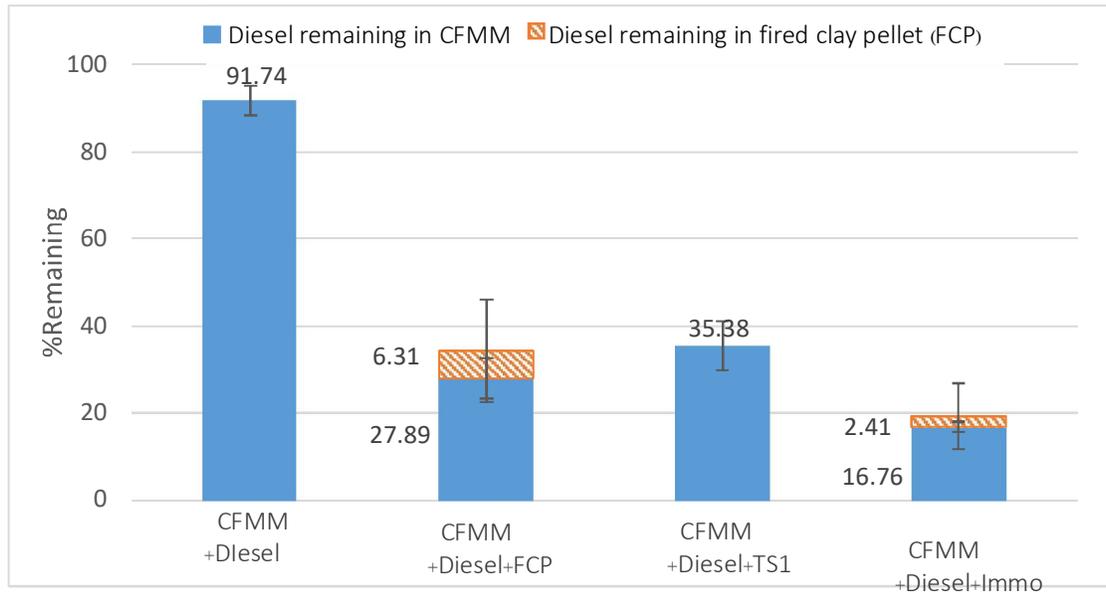
**Figure 3.** SEM images of fired clay pellet surface. (A) outer surface. (B) inner surface.



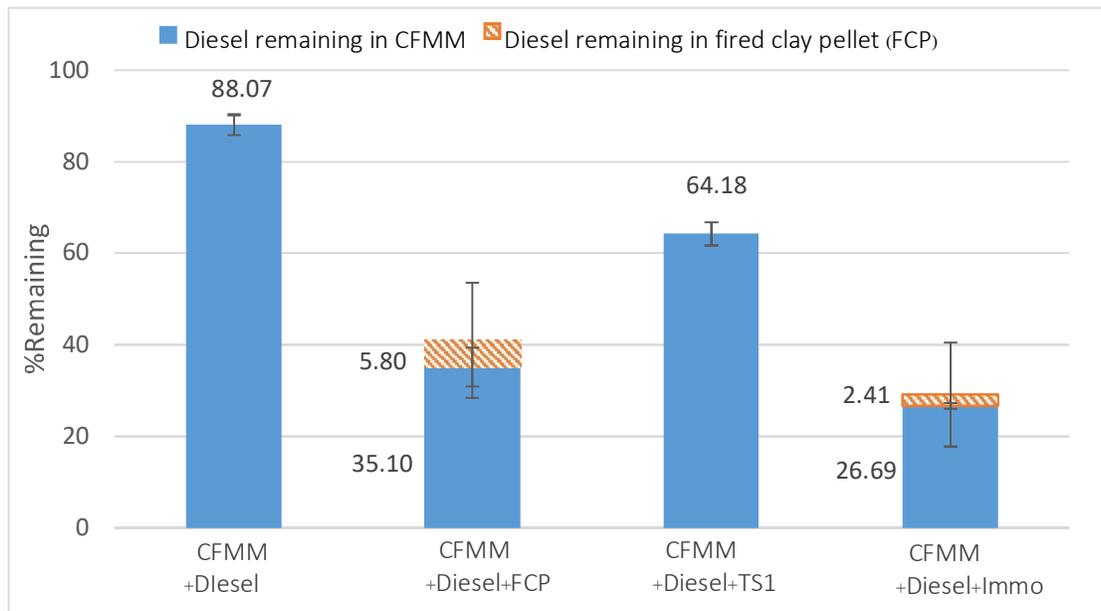
**Figure 4.** SEM images of the immobilized cells on inner surface after immobilization process. The arrow showed immobilized cells on fired clay pellet.

### **Degradation of higher concentration by immobilized TS1**

Degradation of diesel at high concentrations using immobilization of TS1 demonstrated that immobilized TS1 on fired clay pellet could degrade 3% and 5% diesel to the remaining of 19.17% and 29.10% within 7 days which was much better than the use of free cells. The loss of 3% and 5% diesel due to physical factors were only 9% and 12% of 3% and 5% diesel, respectively. The adsorption of diesel by the pellet were 6.31% and 5.80%, respectively (Figure 5 and 6).



**Figure 5.** 3% diesel removal by immobilized TS1 compared to control for 7 days.



**Figure 6.** 5% diesel removal by immobilized TS1 compared to control for 7 days.

## DISCUSSION

Bioremediation is a high efficiency strategy and environmentally friendly technology. The limitation of this method is the loss of bacterial activity in real environment (Chen et al., 2016). Cell immobilization could solve the problem due to the loss of free cells during pollutant degradation (Nie et al., 2016). In this study, bacterial consortium TS1 could degrade 1% diesel for 73.41% within 7 days. Diesel degradation of bacterial consortium TS1 is higher than that of *Bacillus niacini* and *Ochrobactrum anthropic*, which degraded 1% diesel for 71.59 % and 60.25 %, respectively (Ramasamy and Chandran, 2018). Immobilized TS1 on fired clay pellet efficiently degraded diesel to the remaining of 10.44% within 7 days which was better than those of free cell. The amount of diesel removed by immobilized bacteria was always more than that removed by free bacteria (Huang et al., 2016; Zhixiu et al., 2017). One reason for better degradation by immobilized cells might be that the carrier materials absorb and facilitate its degradation (Hou et al., 2013). The adsorption of diesel by fired clay pellet was about 8.18% which are less than those of crab shell biochar which absorbed more than 40% of diesel. (Cai et al., 2019). Moreover the immobilized consortium TS1 could degrade 3% and 5% diesel more efficiently than free cell, as reported that immobilized *Pseudoxanthomonas* sp. could degraded higher concentrations of diesel more than 1000 mg/l<sup>-1</sup> while free cell remove about 400 mg/l<sup>-1</sup> and 700 mg/l<sup>-1</sup> of 1200 mg/l<sup>-1</sup> and 1500 mg/l<sup>-1</sup> of diesel (Nopcharoenkul et al., 2013).

Therefore, fired clay pellets, especially inner surface are suitable for bacterial cell immobilization because of its high porosity, chemically inert, large adsorption surface, neutral pH, easy handling, floatable and cost effectiveness (Nkansah et al., 2012). This technology not only allows bacteria to be adsorbed on the carrier surface but also protect bacteria from harsh environment, which promotes the efficient removal of diesel (Xue et al., 2017).

## CONCLUSION

In this study, fired clay pellet were used as natural organic carrier to immobilize bacterial consortium TS1. Immobilized TS1 cells showed the highest degradation efficiency of diesel for 89% compared with free cell only 73% within 7 days. The special characteristics of fired clay pellet and immobilized TS1 cells showed great potential for bioremediation of diesel

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