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PETROLEUM HYDROCARBON DEGRADATION OF HUSK BIOCHAR PRODUCT CARRYING BIOFILM-FORMING BACTERIA

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SUMMARY

Environmental pollution problems caused by petroleum and its derivatives such as polycyclic aromatic hydrocarbons (PAHs) has remarkably increased and become a major global threat to human health and ecological equilibrium, resulting in a crucial need for remediation. Recently, in order to solve this problem, biofilm is one of the biodegradation approaches which could degrade and transform oil components effectively. Moreover, to enhance the petroleum hydrocarbons removal efficiency and easy to apply in any place bio-carriers, biochar was used to attach biofilm-forming microorganisms. Biochar is not only used as a carrier for microorganisms but also a source of substrates to help absorb a part of aromatic compounds. Therefore, in this investigation, we used the mixture of multiple petroleum-degrading and biofilm-forming bacterial strains immobilized on husk biochar to create an oil-degrading product. The ability of oil-degrading bacteria immobilized on biochar, planktonic bacteria and biochar without bacteria on the elimination of total petroleum hydrocarbons and several aromatic hydrocarbons was investigated. The results indicated that using husk biochar as a carrier for biofilm-forming bacteria to attach on could considerably enhance the removal efficiency of oil components. At 50 kg/batch scale, the formed product could remove 99% of total petroleum hydrocarbons with the initial amount of 4.786 mg/l and over 96% of aromatic hydrocarbons including anthracene, naphthalene, phenanthrene and pyrene with the initial amount of 250 mg/l. The obtained product is porous, black, with particle size of 1-3 mm, cell density of ≥ 10^9 CFU/g(ml), stable for use ≥ 6 months and safe for the environment. In general, the obtained results highpoint the great possible of applying this product in the treatment of oil contaminated soil and water.

Keywords: Biodegradation, fermentation, oil polluted waste water, oil polluted soil

INTRODUCTION

Currently, with sharp increasing demand of fossil fuel energy, the extensive exploration of petroleum oil extraction and processing industry has caused remarkable environmental problems all over the world and in Vietnam. Soil and water contamination with oil is a enormous environmental problem and a human health concern, resulting in an urgent need for effective and sustainable solutions (Qingren *et al.*, 2011). In Vietnam, a number of oil pollution treatment technologies have been applied including physical and chemical approaches and indicated certain effects. However, these approaches are energy-consuming, expensive and create side products. Meanwhile, in recent years, biodegradation using microbial strains to degrade hydrocarbons has been used widely due to its efficiency, inexpensive, and eco-friendly nature (Xingjian Xu *et al.*, 2018). Bacteria degrade and metabolize most petroleum hydrocarbons for their energetic and carbon need for growth and reproduction. Especially, bacteria-forming biofilm have been proved being able to decompose hydrocarbons more effective than bacteria in single cell form (Wang *et al.,* 2012; Mollaei *et al.,* 2010).

Biofilm is a collection of microorganisms that adhere to a solid surface, forming a film covers the surface (Watnick, Kotter, 2000). Forming biofilm helps microorganisms to withstand harsh environmental conditions (pH, temperature, cell permeation or dehydration, etc.), support better metabolism and limit the competition of other microorganisms, increasing the efficiency of pollutants decomposition (Shemesh *et al.,* 2010). Thus, using biofilm-forming bacteria has been successfully applied in oil-contaminated wastewater and soil in some countries around the world and promises to become a new technology with high economic efficiency and environmental friendliness.

In order to enhance the growth, development and biofilm formation of microorganisms, an effective method is using proper carrier material to immobilize the hydrocarbon-degrading bacteria to increase the pollutants biodegradation (Ahmad *et al.,* 2014). Biochar has recently been seen as a potential material for environmental bioremediation because it has rich carbon content, good adsorption ability, high stability and optimal adherence capacity for bacteria (Zhang *et al*., 2019).

Husk biochar is formed by rice husk substrate produced through pyrolysis under low or zero oxygen conditions at 480-530^{o}C (Zhang *et al.,* 2010). Rice husk is an abundant agricultural waste in Vietnam and often discarded or used for cooking with limit effect or even caused a significant impact on the environment. Recently, with new biochar production technology, it is possible to utilize good source of this material. Hence, husk biochar has been used as a carrier for selected bacterial strains in the present study.

A number of studies indicated biochar's ability to absorb petroleum hydrocarbon compounds in soil and water contaminated with oil. For instance, biochar has been shown to be applicable in the treatment of several compounds such as naphthalene (B. Chen *et al.*, 2009), phenanthrene (H. Kong *et al.*, 2011), PAH (L. Beesley *et al.*, 2010), pyrene (S. E. Hale *et al.*, 2011). Furthermore, the huge particular surface, well-developed absorbent structure and abundance superficial functional group of biochar could

increase the adsorption of bacteria ability to degrade petroleum hydrocarbons (Sun K. *et al.*, 2011; Cao *et al.*, 2010; Ahmad *et al.*, 2014). Thus, the combination biofilm-forming bacteria with biochar may be a potential way to remove effectively petroleum hydrocarbons.

However, to our knowledge, only few studies reported about using biochar as a bacterial carrier or an oil-contaminated waste water and soil treatment approach in Vietnam so far. Therefore, in this study, we built a standard process for the production of petroleum hydrocarbon treatment product by utilizing biofilm-forming bacteria immobilized on husk biochar. A 50 kg/batch scale has been produced in order to investigate its capacity to remove total petroleum hydrocarbons and several aromatic hydrocarbons. The total petroleum hydrocarbons and several aromatic hydrocarbon including anthracene, naphthalene, phenanthrene and pyrene were used as substrates to evaluate the degradation capacity of the product.

MATERIALS AND METHODS

Microbial strains

The biofilm-forming and hydrocarbon-degrading bacterial strains used in this study were obtained from the microbial collection of Environmental Biotechnology Department, Institute of Biotechnology, VAST. The selected strains expressed an excellent ability to decompose petroleum hydrocarbons and were safe for environment (Table 1).

Table 1. Characteristics of bacterial strains used in the study.

No.	Strains	Hydrocarbon utilization					Isolated location
		DO	Naphthalene	Phenol	Pyrene	Toluene	
1	*Rhodococcus* sp. BN5	+++	++	+++	+++	+++	Hanoi
2	*Stenotophomonas* sp. QNG02	+++	+	+++	++	+++	Quang Ngai
3	*Acinetobacter baumannii* QN01	+++	+++	+++	+++	+++	Quang Ngai
4	*Rhizobium* sp. DG2	++	+	++	++	+++	Hanoi

(+++: great growth; ++: good growth; +: growth)

Husk biochar

Biochar from rice husk used in the study is black with a size of 1x3x0.5 (mm); the humidity ≤ 10%; formed through the pyrolysis at 480-530^{o}C, the specific surface area of 1.5 m^2/g, the ash content of 42% and contains a large number of C-O groups, e.g. phenolic, hydroxyl and ether radicals.

Activation of biofilm-forming bacterial strains

The strains were reactivated by growing

on total aerobic medium in a shaker at 150 rpm for 24-48 h at 28°C ± 2.

Creating the mixture of bacterial cells with biochar

Husk biochar were sterilized at 121°C for 30 minutes before using as a carrier for bacterial strains. Bacterial strains after growing to achieve the cell density of 10^8 CFU/ml were attached to biochar by adsorption method and incubated at 28°C ± 2 for 5 days. The product is dried at different temperatures to determine the appropriate humidity for the product.

Assessment of microbial cell density of the product

The MPN method and CFU method were used to evaluate the microbial cells present in 1 g of product or 1 cm^3 of carrier (Oblinger, Koburger, 1975).

SEM (Scanning electron microscopy) observation

The samples were mildly engaged on a glass plate coated with poly-L-lysine. The sample was stable with glutaraldehyde and OsO4, dehydrated in ethanol, isoamyl acetate and CO2, then coated with platinum for observation under a scanning electron microscope (Glauert, 1975).

Analysis of total petroleum hydrocarbon degradation efficiency by the product

In order to evaluate hydrocarbon degradation productivity of the product, all treatments were carried out with 100 g contaminated soil or water (contaminated sample) and incubated for 7 days. The treatments were as follows: (1) – (4) each of 4 screened strains was amended to contaminated sample to examine the biostimulatory effect of the only bacteria without carriers; (5) the mixture of 4 screened strains was amended to contaminated sample; (6) the obtained product which was the immobilized bacteria were mixed with contaminated sample to evaluate the effectiveness of the combining of biochar and oil utilizing bacteria; (7) the biochar was added to contaminated sample to study the bioaugmentaion effect of the biochar without bacteria; (8) the contaminated sample without carrier and microorganism addition was applied as the control (details as shown in Table 2).

Table 2. Samples were set up for evaluation of petroleum hydrocarbons degradation.

No.	Description
Sample 1	*Rhodococcus* sp. BN5 + 100 g contaminated sample
Sample 2	*Acinetobacter baumannii* QN01 + 100 g contaminated sample
Sample 3	*Stenotophomonas* sp. QNG02 + 100 g contaminated sample
Sample 4	*Rhizobium* sp. DG2 + 100 g contaminated sample
Sample 5	The mixture of 4 selected bacterial strains + 100 g contaminated sample
Sample 6	1 g of the product + 100 g contaminated sample
Sample 7	1g biochar + 100 g contaminated sample
Sample 8	100 g contaminated sample

Eight treatments were conducte in three replicates and cultured at 28-30^{o}C for 7 days.

The product was evaluated for its ability to decompose diesel oil at different initial concentrations by gravimetric analysis according to TCVN 4582-88 standard.

Analysis of petroleum hydrocarbon degradation by high-performance liquid chromatography (HPLC)

The analysis was performed as described in our previous publication (Nhi-Cong *et al*., 2016). These experiments were conducted independently at least three times and calculated standard deviation (SD) values.

RESULTS AND DISCUSSION

Production of biochar carrying biofilm-forming and hydrocarbon-degrading bacteria at scale of 50 kg/batch

Husk biochar carrying biofilm-forming and hydrocarbon-degrading bacteria (the product) was obtained by mixing 1,930 ml of stock bacterial broth, 19,300 ml of LB, HKTS or MPA medium and 28,950 grams of husk biochar at a ratio of 1:10:15 (volume/volume/weight respectively). After well mixing, the mixture was dehydrated to achieve 50 kg (50,000 grams) of mixed product with the humidity of 40%. The product created from this process at scale of 50 kg is porous, black, with particle size of 1-3 mm, cell density of $\geq 10^9$ CFU/g(ml), stable for use $\geq$ 6 months and safe for the environment (Table 3). The scan electron microscopy image confirms the bacteria were successfully immobilized on biochar.

Hydrocarbon degradation of the product

Total petroleum hydrocarbons analysis

After 7 days of incubation, gravimetric method was performed to analyse total petroleum hydrocarbons extracted from each treatment. As shown in Figure 2, with the initial supplement diesel oil amount of 4.786 mg/L, the total petroleum hydrocarbons removal efficiency was 74.5, 60.0, 51.5, 43.0, 82.0, 99.0, 18.8 and 1.5 % in the treatment 1 to 8, separately. There was no significant difference of each result ($p<0.05$). The obtained product at 50 kg scale indicated the highest efficiency of total petroleum hydrocarbons removal (99%), which could be a reason of the combination of hydrocarbon utilizing bacteria and carrier could highly enhance the biodegradation.

According to Alesandrello *et al*. (2017), a mixture biofilm of *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 attached on polyurethane foam could remove 75% of petroleum oil with the initial concentration of 0.1 g per 100 mL medium after 7 day-incubation at 30 ^{o}C. Nunal *et al*. (2014) demonstrated that a biofilm of bacterial consortium formed on cocopeat and rice husk powder significantly degraded both aliphatic and aromatic fractions after 60-day *in vitro* experiments. These results showed the effectiveness of biofilm adherence on several kinds of biocarriers and in agreement with our obtained results.

Table 3. Microbial cells density of the produced biochar.

Sample	Number of microoganisms (CFU/g)
Sample at the beginning	$10^8 – 10^9$
Sample after 7 days	$10^9 \pm 10$

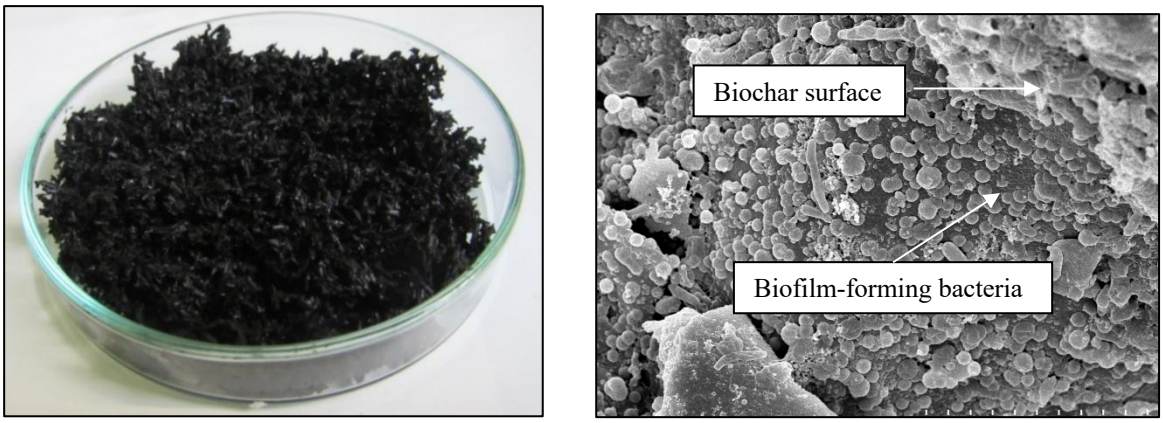

Figure 1. The obtained product (A) and SEM image immobilized bacteria on the biochar surface of the product (B).

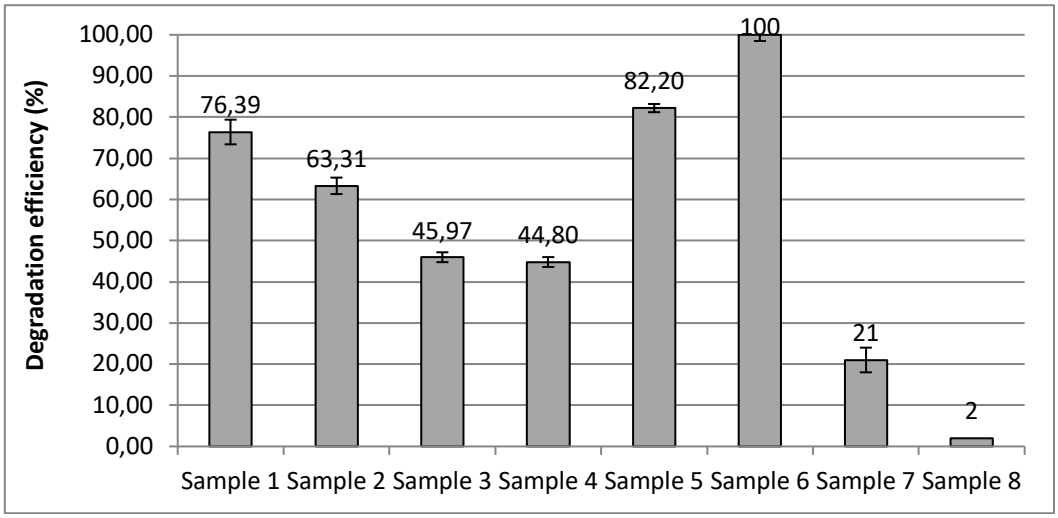

Figure 2. Total saturated hydrocarbons degradation efficiency of the product at the scale of 50 kg/batch after 7 day-incubation.

Aromatic hydrocarbons degradation

The HPLC was used to evaluate the aromatic hydrocarbon degradation and the results are shown in Table 4. Compared to the other treatments, the obtained product also had ability to degrade aromatic hydrocarbons most effective, which were 99, 96.2, 99.5 và 98.5% of anthracene, naphthalene, phenanthrene and pyrene after 7 day-incubation, respectively with the initial amount of 250 mg/l each aromatic hydrocarbon. The results revealed that the addition of biochar to immobilized degrading bacteria in the obtained product could enhance the removal efficiency of aromatic hydrocarbons.

Table 4. Aromatic hydrocarbon degradation productivity of the product at a scale of 50 kg after 7 days.

Sample	Hydrocarbon degradation productivity (%)			
	anthracene	**naphthalene**	**phenanthrene**	**pyrene**
Sample 1	77	63.1	75.1	71.2
Sample 2	74	67.1	76.5	70.5
Sample 3	74	64	78.5	72.1
Sample 4	75	60	72.6	75.1
Sample 5	84	72	84.8	78.9
Sample 6	**99**	**96.2**	**99.5**	**98.5**
Sample 7	25	23	21.1	20
Sample 8	0	0	0	0

In this investigation, 3 of 4 selected strains including *Acinetobacter baumannii* QN01, *Rhodococcus* sp. BN5, and *Stenotophomonas maltophilia* QNG02 may be considered as human disease bacteria. But there is no publication reported that all species of *Acinetobacter baumannii, Rhodococcus* sp. nor *Stenotophomonas maltophilia* are disease bacteria. They only suggested that these bacteria may cause disease on human. Moreover, according to Chang *et al*. (2011), Arulazhagan *et al* (2019), Larik *et al*. (2019), and Zhang *et al*. (2021) these bacteria have high capacity in crude oil removal and were widely applied in oil pollution treatment.

The results provided in Table 4 indicated that the sample 6 including 1 g of the product with 100 g contaminated sediment has the better degradation capacity in comparison with biofilm from single species (sample 1 to sample 4) or mixture biofilm of 4 strains without carrier (sample 5) or the carrier without bacteria (sample 7) or the control without neither biocarrier nor bacteria. Bacteria formed biofilm on carriers improved the survival and retention of biodegradation factors in oil contaminated areas (Nunal et al., 2014). The attachment enhanced the aromatic and aliphatic hydrocarbon degradation (Alesandrello *et al*., 2017). A number of carriers such as sawdust, styrofoam, wheat bran chitin, chitosan, peanut hull, zeolite and activated carbon (Liang et al., 2009) presented increased oil bioremediation by improving and sustaining a high number of oil-degraders. However, there is a few publications on using husk biochar as biocarrier to reduce oil pollution concentration. Therefore, our results may give a strategy in application of petroleum hydrocarbon removal.

CONCLUSION

The obtained product is porous, black, with particle size of 1-3 mm and high cell density of $\geq 10^9$ CFU/g(ml), stable for use ≥ 6 months and safe for the environment. At a 50 kg/batch scale, the product could well degrade petroleum hydrocarbons with its capacity to remove of 99.0, 99.0, 96.2, 99.5 and 98.5% of total saturated hydrocarbons, anthracene, naphthalene, phenanthrene and pyrene, respectively. In brief, our study reveals the

great potential of applying this product in the treatment of oil polluted soil and water.

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