Biodegradation of Total Petroleum Hydrocarbon from Al-Daura Refinery Wastewater by Rhizobacteria

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ABSTRACT

Due to the deliberate disposal of industrial waste, a great amount of petroleum hydrocarbons pollute the soil and aquatic environments. Bioremediation that depends on the microorganisms in the removal of pollutants is more efficient and cost-effective technology. In this study, five rhizobacteria were isolated from Phragmites australis roots and exposed to real wastewater from Al-Daura refinery with 70 mg/L total petroleum hydrocarbons (TPH) concentration. The five selected rhizobacteria were examined in a biodegradation test for seven days to remove TPH. The results showed that 80% TPH degradation as the maximum value by Sphingomonas Pauimobilis as identified with Vitek® 2 Compact (France).

Keywords: petroleum wastewater; biodegradation; Phragmites australis; rhizobacteria.

Biodegradación del hidrocarburo total de petróleo de aguas residuales de la refinería Al-Daura por rizobacterias

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RESUMEN

Por el descartado intencional de residuos industriales, una gran cantidad de hidrocarburos de petróleo contamina el suelo y los entornos acuáticos. La bioremediación que depende de los microorganismos en la eliminación de contaminantes es más eficiente y coste-económico. En este estudio, se aislaron cinco rizobacterias de las raíces de Phragmites australis y expuestas a aguas residuales de la refinería Al-Daura con una concentración de 70 mg/L de hidrocarburos de petróleo totales (TPH). Las cinco bacterias seleccionadas fueron examinadas en un ensayo de biodegradación durante siete días para eliminar el TPH. Los resultados mostraron que 80% de la degradación de TPH como valor máximo por Sphingomonas Pauimobilis como identificado con Vitek® 2 Compact (Francia).

Palabras clave: aguas residuales de petróleo, biodegradación; Phragmites australis; rizobacterias.

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1. INTRODUCTION
The crude oil is the most essential energy source in the industrial countries (Xaaldi Kalhor, et al., 2017). In 2015, 97 million barrels of oil were needed per day in order to meet the energy required (dos Santos and Maranho, 2018). During the exploration of oil wells, refining, and transportation of petroleum products, a large amount of crude and refined oil is released into surroundings as a negative approach, (Yavari, et al., 2015). Also, and because of the high cost of treatment, the refinery wastewater is discharged to the rivers and other aquatic bodies without treatment is one of the ways that is occurring in the developing countries (Nayyef M. Azeez 2016; Musa, et al., 2015). The pollution due to crude oil and refinery process has a significant environmental impact on human health and ecosystem quality(Yavari, et al., 2015; Hussain, et al., 2018).

There are numerous techniques, such as physical, chemical and biological for treating the petroleum contamination (Peng et al. 2009). Among them, biological methods are more feasible, cost-effective and easy to apply (dos Santos and Maranho, 2018; Chunli Zheng, et al., 2012). and offer complete degradation of oil to CO₂ and H₂O (Hussain, et al., 2018).

The treatment, which depends on the plant and their associated rhizobacteria is one of the most important biological strategies that can be used for removal of pollutants water, because it is more efficient and cost-effective technology (Liu, et al. 2011; Toyama, et al. 2011; dos Santos and Maranho, 2018; Cai, et al. 2010). The main mechanism in those methods are based on stimulation of rhizobacteria, called rhizoremediation or rhizodegradation (Gerhardt, et al., 2017; Cai, et al. 2010; Al-Baladi, et al. 2013; dos Santos and Maranho, 2018). Rhizodegradation is the combination of phytoremediation and bioaugmentation with rhizobacteria (Hussain, et al. 2018; Pant, et al., 2016). In this process, the plant acts indirectly by providing carbon and energy source to rhizobacteria (the bacteria which concentrate on root surface and in the surrounding area to the root). The plant exudes oxygen and nutrient such as organic acids, amino acids, enzymes and sugars to root zone (Epps 2006; Pant, et al., 2016).

However, great attention to bacteria that able to degrade petroleum hydrocarbons in water and soil has been received. Yasseen (2014) has listed 25 bacterial genera that act as petroleum hydrocarbons degraders, such as Acidovorax, Mycobacterium, Pseudomonas, Rhodococcus, Sphingomonas, Xanthomonas, Achromobacter, Micrococcus and Bacillus. This study aimed to explore plant–rhizobacteria interaction in the rhizosphere zone to improve the biotreatment of petroleum hydrocarbons of refinery wastewater. Rhizobacteria used was isolated from the root of Phragmites australis exposed to real wastewater from Al-Daura refinery contains hydrocarbons, and identified by Vitek® 2 Compact systems (bioMérieux, France).

2. MATERIALS AND METHOD
2.1 Isolation and identification of rhizobacteria
The bacteria strains used in this study were isolated from roots of Phragmites australis which was exposed to wastewater containing petroleum hydrocarbons that obtained from Al-Daura refinery, Iraq. Ten grams of Phragmites australis root was inserted in 250 mL Erlenmeyer flask containing 100 mL sterilized distilled water and then incubated in a rotary shaker at 30°C with 150 rpm for one hour. Five dilutions were obtained for planting by applying serial dilution method. 1 mL of water from Erlenmeyer flask was mixed with 9 mL sterile saline water (0.85% NaCl) until reaching
serial dilution up to $10^{-5}$. 0.1 mL of each dilution ($10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$) was pipetted in a petri dish with Tryptic Soya Agar (TSA) and spread with glass cell spreader (hockey stick). The planted Petri dishes were incubated at 30°C overnight (Al-Baldawi, et al. 2015).

A pure culture of more appearance colonies was obtained on TSA Petri dish. The isolated rhizobacteria were coded according to color or shape of the colony (W = white, Y = yellow, T = tree). Then, the selected rhizobacteria were identified using Vitek® 2 Compact systems (bioMérieux, France) after biochemical test of gram stain was examined. Cultures to be examined were regrown from storage on TSA and incubated in 30°C before one day of testing. By a sterile swab, numbers of pure colonies were suspended in 0.3 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a test tube until reach 0.6 NTU which was measured by turbidity meter (Pincus, 2010).

2.2 Biodegradation of petroleum hydrocarbon by isolated rhizobacteria

Bushnell Haas medium prepared using refinery wastewater with characteristics as shown in Table 1. was inoculated with rhizobacteria to determine the ability of rhizobacteria to degrade petroleum hydrocarbon. The medium was described by (Anon, 2011), as follows (g/L): 0.2 MgSO$_4$.7H$_2$O, 0.02 CaCl$_2$.2H$_2$O, 1.0 KH$_2$PO$_4$, 1.0 K$_2$HPO$_4$, 0.1 NH$_4$NO$_3$, and 0.05 FeCl$_3$. 20 mL of sterilized Tryptic Soya Broth (TSB) was prepared and inoculum with pure cultures of each rhizobacterium and incubated in a rotary shaker (JSR, Korea) at 30°C with 150 rpm for 24 h (Plaza, et al. 2008). 2 mL of inoculum with 4 McFarland (1200×10$^6$ CFU/mL) was added to a 100 mL Erlenmeyer flask contained 48 mL of Bushnell Haas medium and kept in a rotary shaker at 30°C and 150 rpm for seven days, and then the biodegradation was tested.

**Table 1.** The main characteristics of Al-Daura refinery wastewater.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Turbidity</td>
<td>NTU</td>
<td>31.3</td>
</tr>
<tr>
<td>TSS</td>
<td>mg/L</td>
<td>142</td>
</tr>
<tr>
<td>Phenol</td>
<td>mg/L</td>
<td>2.5</td>
</tr>
<tr>
<td>TPH</td>
<td>mg/L</td>
<td>70</td>
</tr>
<tr>
<td>COD</td>
<td>mg/L</td>
<td>370</td>
</tr>
<tr>
<td>BOD$_5$</td>
<td>mg/L</td>
<td>120</td>
</tr>
<tr>
<td>ORP</td>
<td>mV</td>
<td>179</td>
</tr>
<tr>
<td>TDS</td>
<td>mg/L</td>
<td>904</td>
</tr>
</tbody>
</table>
2.3 Evaluation of TPH Degradation by Rhizobacteria

After seven days of incubation, the remaining TPH was measured using oil content analyzer (Horiba, OCMA-350, USA). 10 mL of liquid medium was extracted by equal volume of Tetrachloromethane (CCL₄) and then the solvent layer which contains residual TPH was passed through anhydrous sodium sulfate (Na₂SO₄) to remove the remaining water. During extraction the liquid sample was adjusted at pH=2 (USEPA, 1996).

The removal percentage of TPH was determined according to equation (1):

\[
\%\text{Biodegradation} = \frac{TPH₀ - TPH₇}{TPH₀} \times 100 
\]

where TPH₀= total petroleum hydrocarbon at 0 days, and TPH₇= total petroleum hydrocarbon at seven days.

The ability of rhizobacteria to degrade n-alkanes (C₁₀-C₂₆) was evaluated using gas chromatography with a flame ionization detector (GC-FID) (GC-Shimadzu, Japanese company, model 2010) and dichloromethane (DCM) as solvent. The column was programmed to stay for 30 seconds at 100°C, and then ramp 10°C every minute until reach 330°C for 10 minutes.

3. RESULTS AND DISCUSSION
3.1 Isolation and identification of rhizobacteria

After 24h of growth on TSA, pure cultures of 5 species of rhizobacteria were obtained on TSA medium, as shown in Fig. 1.

![Figure 1. Code and pictures of isolated bacteria.](image)

Then, the identification of the five selected rhizobacteria was made using Vitek® 2 Compact systems (bioMérieux, France), the results as shown in Table 2.
Table 2. Rhizobacteria identification by vitek® 2 compact.

<table>
<thead>
<tr>
<th>Code</th>
<th>Bacteria name</th>
<th>Probability</th>
<th>Gram stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Pseudomonas stutzeri</td>
<td>97%</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>Sphingomonas paucimobilis</td>
<td>94%</td>
<td>-</td>
</tr>
<tr>
<td>Y</td>
<td>Micrococcus luteus</td>
<td>99%</td>
<td>+</td>
</tr>
<tr>
<td>W</td>
<td>Staphylococcus aureus</td>
<td>86%</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>Staphylococcus lentus</td>
<td>93%</td>
<td>+</td>
</tr>
</tbody>
</table>

The results of identification show that all rhizobacteria were hydrocarbon-degrading bacteria and that explained by (Toyama, et al. 2006) as due to ability of plant (Phragmites australis) to select and enhance accumulation of contaminants degrading bacteria (TPH) to enhance removal efficiency. Where, Pseudomonas Stutzeri have high ability to degrade crude oil contaminant soil and water (Kaczorek, et al., 2012; Hassan Shahian, et al., 2012; Biology, et al., 2008; Celik, et al., 2008). Sphingomonas paucimobilis has been reported as poly-aromatic hydrocarbons (PAHs) degrading bacteria (Story, et al. 2004; Haritash and Kaushik, 2009) and also have the ability to degrade diesel (Ipung Fitri Purwanti, et al. 2012; Moliterni, et al. 2012. Zhou, et al. 2016) show that Sphingomonas acts a key role in the degradation of PAH also, (San Miguel, et al. 2009), study the ability of Sphingomonas paucimobilis to degrade naphthalene in water and showed that 80% of naphthalene was degraded during three weeks. (Ojo, 2006), studied the capability of Micrococcus Luteus with other bacteria as a consortium in the utilization of Petroleum hydrocarbon and the result shown the major effect of the consortium in the biodegradation. Moreover,( Mustapha, et al. 2015), isolate Micrococcus Luteus from sub-surface wetland treating refinery wastewater. Also, Micrococcus Luteus able to degrade different organic contaminant such as nitrobenzene (Zheng, et al. 2009). aliphatic and aromatic hydrocarbons Benedek, et al. 2010; Toledo, et al. 2006, and Pesticide( Kanjilal, et al., 2015. Musa, et al. 2015 isolate Staphylococcus aureus from petroleum refinery wastewater and Length, 2010). revealed that Staphylococcus aureus is the best in degradation of kerosene. (Moliterni, et al. 2012) isolated Staphylococcus Lentus and other bacteria from oil refinery soil to study biodegradation of diesel by them, and then the result shows 80% of diesel was degraded after 40h.

3.2 Biodegradation of Petroleum Hydrocarbon by Selected Rhizobacteria

The five selected rhizobacteria were tested for biodegradation of TPH in refinery wastewater during seven days. The degradation of TPH was ranged between 68.6 to 80% as shown in Fig. 2. The higher TPH removal was for Sphingomonas Paucimobilis while the lower TPH removal was for Pseudomonas stutzeri.
**Figure 2.** Removal Percentage of TPH by the five selected rhizobacteria monoculture.

The changes in concentration of TPH with carbon number range C10-C26 showed using GC-FID. **Fig. 3** shows gas chromatography of petroleum hydrocarbon extracted at 0 days and seven days of the biodegradation study of Pseudomonas stutzeri, Sphingomonas Paucimobilis, Micrococcus luteus, Staphylococcus aureus, and Staphylococcus Lentus.
Figure 3 Biodegradation of TPH: (a) Control (0 day), (b) Pseudomonas stutzeri- 7 days, (c) Sphingomonas paucimobilis- 7 days, (d) Micrococcus luteus- 7s day, (e) Staphylococcus aureus- 7s day, and (f) Staphylococcus lentus- 7s day.

The results of GC analysis after biodegradation show that each isolated rhizobacteria have the ability to degrade specific n-alkanes more than others. Where, *Pseudomonas stutzeri* shows high ability to degrade C<sub>10</sub>-C<sub>12</sub>, C<sub>17</sub>, C<sub>19</sub>-C<sub>20</sub>, and C<sub>24</sub>-C<sub>26</sub>. *Sphingomonas paucimobilis* show high efficiency to degrade all carbon range stander C<sub>10</sub>-C<sub>26</sub>. *Micrococcus Luteus* show high efficiency to degrade C<sub>10</sub>-C<sub>13</sub>, C<sub>19</sub>-C<sub>21</sub>, and C<sub>24</sub>-C<sub>26</sub>. While *Staphylococcus aureus* could degrade all carbon stander range spatially C<sub>10</sub>-C<sub>12</sub>, and C<sub>17</sub>-C<sub>18</sub>. Also, *Staphylococcus lentus* can efficiently degrade C<sub>10</sub>-C<sub>17</sub>, C<sub>19</sub>-C<sub>4</sub>, and C<sub>26</sub>.

4. CONCLUSIONS

Five colonies were isolated from the root of Phragmites australis after being exposed to 70 mg/L TPH. The selected rhizobacteria were classified by morphology and biochemical tests, then identification using Vitek® 2 compacts as *Pseudomonas stutzeri*, *Sphingomonas paucimobilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus lentus*. The identification process confirmed the ability of these rhizobacteria to degrade n-alkanes, which is important for bioremediation processes.
Staphylococcus lentus. The biodegradability of petroleum hydrocarbons using isolated rhizobacteria was tested by monoculture. The maximum removal of TPH was 80% and achieved by Sphingomonas paucimobilis. The results indicate that the rhizodegradation of an organic contaminant in water by rhizobacteria is excellent technology.

5. REFERENCES:


• Toledo, F L, C Á Calvo, B Rodelas, and J Gonza. 2006. Selection and Identification of Bacteria Isolated from Waste Crude Oil with Polycyclic Aromatic Hydrocarbons Removal Capacities 29:244–52.


