[Research Note]

Degradation of Alkane by Bacteria Immobilized on Polyurethane Foam

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Immobilization of alkane-degrading bacteria by physical adsorption on polyurethane foam support was examined for application to the repeated degradation of alkane. Presence of hydrophobic substrate during cultivation promoted the attachment of alkane-degrading bacteria, such as *Brevibacterium*, *Rhodococcus*, and *Pseudomonas* sp., possibly due to the absorption of hydrophobic substrate on the polyurethane foam, followed by bacterial attachment to the substrate. Efficiency of immobilization depended on the hydrophobicity index, log $P$ of the substrate. Various substrates, such as saccharides, triglycerides and fatty acids, were tested with log $P$ in the range of ~3.24 to 23.7. Triolein was the optimum substrate with the efficient immobilization and assimilation of microorganisms. Using cultivation with commercial salad oil as an inexpensive substitute for triolein, repeated batch degradation of $n$-tetradecane with the selected immobilized bacterium, *Brevibacterium ketoglutaricum* ATCC15587, could be continued for over 300 h with only slight loss of activity.

Keywords
Hydrocarbon degradation, Cell immobilization, Coryneform bacterium, Alkane, *Brevibacterium* sp.

1. Introduction

Bioremediation is a biological process to degrade, break down, transform, and/or essentially remove contaminants or quality impairments from soil and water. Such an eco-friendly application can clean up contaminants caused by oil spills since complete *in-situ* mineralization of pollutants can be achieved. Petroleum hydrocarbons including industrial petroleum refinery wastewater are complex mixtures of non-aqueous and hydrophobic components such as $n$-alkanes, aromatics, resins and asphaltenes. Bioavailability might be the limiting factor controlling the biodegradation of such compounds. Microorganisms capable of degrading hydrocarbon-related compounds are important for petroleum contaminated site remediation, such as soil and (sea) water, either by biostimulation or bioaugmentation. Immobilized microorganisms have several advantages over free microorganisms, such as high cell density, avoidance of cell washout even at high dilution rates, easy separation of cells from the reaction system, repeated use of cells, and better protection of cells from harsh environments. Many methods of immobilization have been tested for the degradation of petroleum hydrocarbons. Bacterial cells entrapped in a hydrophobic gel was examined for repeated diesel oil desulfurization. Bacterial spores entrapped in chitosan beads cross-linked with glutaraldehyde was also reported for $n$-hexadecane degradation in a batch experiment. Pentane-degrading *Arthrobacter* sp. was immobilized on a macroporous polystyrene particle matrix by promoting flocculation with the addition of Ca$^{2+}$ at the end of the growth phase. Earlier studies in our lab for fatty-oil containing wastewater treatment have shown that a bacterial consortium in an activated sludge formed into a biofilm showed good physical adsorption on polyurethane foam even with large pore size.

In this study, hydrocarbon-degrading bacterial cells were efficiently immobilized on macroporous polyurethane foam by treatment with hydrophobic substrate and evaluated for repeated batch degradation of $n$-tetradecane ($n$C14).

2. Experimental

2.1. Strains

Microorganism strains used in this study were purchased from the culture collections (ATCC: American Type Culture Collections, USA, and NBRC: National Bioresource Center, Japan) as listed in Table 1. The cultures were stored at ~80 °C with 15% (w/v) glycerol and maintained on Nutrient Agar (Oxoid) slants with 1% glucose (NAG) at 4 °C until use, as described previously.

2.2. Immobilization of Microorganisms

Polyurethane foam (PUF, No. QPB13, 1 cm × 1 cm ×
1 cm, containing about 5 pores per cm, Bridgestone Diversified Chemical Products Co., Ltd., Tokyo, Japan) was used as the immobilization support. Ten particles of the treated PUF were added to 100 mL of minimum mineral (MM) medium6) containing 1 % (w/v) of the defined substrate in a 500 mL Erlenmeyer flask followed by bacterial inoculation and aerobic shaking at 30°C for 7 days.

2.3. Repeated Batch Degradation

Seed culture (0.4 mL) grown on MM medium containing 1 % glucose at 30°C for 24 h was inoculated into 24 mm i.d. test tubes with PUF and 5 mL MM medium containing 1 % nC14 with aerobic shaking at 30°C. Duplicate cultured test tubes were harvested for nC14 and PUF-bound cell amount analysis at the defined time intervals. After confirming residual nC14 concentration below 1 g/L, PUF immobilized cells in the culture tubes were transferred to a fresh medium containing 1 % nC14 to repeat the culture cycle.

2.4. Analysis

Weight of the cell mass on the support was measured after washing twice with deionized water and drying at 80°C for 48 h. Weight of the support without cell culture was measured after the same treatment for the calculation of adsorbed cell mass on the support. Substrate concentration was analyzed by gas chromatography (GC) after extracting the culture broth with diethyl ether at a pH below 1 using n-undecane as the internal standard. The GC machine was equipped with a flame ionization detector and a 3 mm i.d. x 2 m length glass column packed with Silicone SE-30 3 % on Chromosorb WAW DMCS (GL Sciences Inc., Japan).

2.5. Chemicals

All materials were of the highest grade commercially available and were used without further purification.

3. Results and Discussion

Gel entrapped immobilization enhances the adsorption capacity, but physical adsorption immobilization could be feasible for efficient contact and aeration of microorganisms although the adsorption capacity on the support is poor. Therefore, alkane-degrading microorganisms were immobilized by physical adsorption to evaluate the degradation activity. Table 1 shows the degradation of n-tetradecane (nC14) concentration after 7 days incubation. Adsorption means dry cell weight on a polyurethane form after 7 days cultivation. Specific activity is calculated by the following formula, "Specific activity = Degradation/Adsorption."

Table 1 Alkane Degradation by Various Bacteria Immobilized on Polyurethane Foam

<table>
<thead>
<tr>
<th></th>
<th>Degradation [g/L]</th>
<th>Adsorption [mg-cell/support]</th>
<th>Specific activity [g-alkane/mg-cell]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brevibacterium ketoglutaricum ATCC15587</td>
<td>5.6</td>
<td>93.33</td>
<td>19</td>
</tr>
<tr>
<td>B. ketoglutaricum ATCC15588</td>
<td>5.8</td>
<td>96.67</td>
<td>21</td>
</tr>
<tr>
<td>Mycobacterium phlei ATCC15610</td>
<td>5.6</td>
<td>93.33</td>
<td>20</td>
</tr>
<tr>
<td>Rhodococcus rhodochrous ATCC21197</td>
<td>5.4</td>
<td>90.00</td>
<td>21</td>
</tr>
<tr>
<td>R. rhodochrous ATCC21198</td>
<td>5.5</td>
<td>91.67</td>
<td>21</td>
</tr>
<tr>
<td>R. rhodochrous ATCC29675</td>
<td>5.2</td>
<td>86.67</td>
<td>20</td>
</tr>
<tr>
<td>R. rhodochrous ATCC4273</td>
<td>1.1</td>
<td>18.33</td>
<td>5.5</td>
</tr>
<tr>
<td>Pseudomonas putida NBRC13696</td>
<td>4.9</td>
<td>81.67</td>
<td>17</td>
</tr>
<tr>
<td>P. putida NBRC14164</td>
<td>4.9</td>
<td>81.67</td>
<td>21</td>
</tr>
<tr>
<td>No cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Degradation means decrease of n-tetradecane (nC14) concentration after 7 days incubation.
Adsorption means dry cell weight on a polyurethane form after 7 days cultivation.
Specific activity is calculated by the following formula, "Specific activity = Degradation/Adsorption."

Fig. 1 Photographs of Immobilized Cells of B. ketoglutaricum (ATCC15588) on Polyurethane Support as Grown on Salad Oil (left) and on Glucose (right)
on the support.

As shown in Fig. 2, treatment with hydrophobic substances, such as triolein, hexadecane, or oleic acid, resulted in high amounts of cell adsorption. However, hydrophilic substances, such as glucose, tributyrin or triacetin, had little effect on cell adsorption. These results could be summarized as the effect of log $P$, an index for hydrophobicity, of the treatment substances on the amount of cells adsorbed on the polyurethane support as shown in Fig. 2. Treatment with salad oil, an inexpensive hydrophobic substance that contains more than 50% of triolein\(^5\), was further examined for immobilization. Cells were successfully immobilized using salad oil, and the suspension liquid remained clear (Fig. 1). The immobilized cell weight was 36 mg-cells/support, which was comparable to the outcome with triolein (Fig. 2). Based on the above results, salad oil was used for support treatment for further study.

Enhancement of adsorption in immobilization by soaking with hydrophobic substances has been also reported for lipase immobilization on pig bone support\(^7\). In this case, immobilization of cells on polyurethane foam was also enhanced by treatment with hydrophobic substances. This study showed that pretreatment of the support for the enhancement of immobilization could also be applied for microbial cells. The effect of salad oil treatment was further studied using various alkane-degrading bacteria. Table 1 shows the degradation of \textit{n}-tetradecane in MM medium in the presence of various alkane-degrading bacteria immobilized on polyurethane foam. Specific degradation activity was similar for all strains tested, but the amount of cells immobilized on the support varied, resulting in different extents of degradation.

Repeated batch degradation of \textit{n}C14 was further examined using the immobilized microorganisms as shown in Fig. 3. During the first and second batches, cells adsorbed on the PUF increased with degradation, followed by stable degradation with constant adsorbed cells, despite slight amounts of cell detachment from the support. After the 3rd repeated batch, the degradation rate decreased in spite of slight decrease in cell detachment, possibly due to reduced viability of the cells. Pore size of the PUF support was not affected by cell degradation or adsorbed cell amount (data not shown).

The present study demonstrated immobilization of alkane-degrading microorganisms by physicochemical adsorption with the aid of hydrophobic substances, and application for repeated batch alkane degradation. This technique could also be applied for biodegradation of wastewater containing hydrophobic substances, such as grease oil or fatty oil\(^5\).

References


![Fig. 2](image1.png)

Fig. 2 Effect of Solvent Presoaking on Immobilization of \textit{B. keto-glu\textit{t}amicum} (ATCC15588)

![Fig. 3](image2.png)

Fig. 3 Repeated Batch Degradation of \textit{n}-Tetradecane with \textit{B. keto-glu\textit{t}amicum} Immobilized on PUF
要 旨

ポリウレタン担体に固定化された細菌によるアルカン分解

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吸着法によりポリウレタン発泡体へアルカン分解細菌を固定化し、繰り返し分解処理について検討した。疎水性化合物を共存培養させることにより、*Brevibacterium, Rhodococcus, Pseudomonas* 属のアルカン分解細菌の担体への吸着が促進された。共存化合物の吸着効果は、log $P$ で示される疎水性パラメーターに従って促進されることが明らかとなった。アルカン分解菌によるクリセリド、トリグリセリド、脂肪酸を用いて log $P$ 値が -3.24（グリコース）から 23.7（トリオレイン）の範囲で吸着効果を維持しながら増殖を示す基質について検討した結果、トリオレインが最も高い効果を示した。トリオレイン含油の高い市販油脂としてサラダオイルを使用し、最も高いアルカン分解活性を示した*Brevibacterium ketoglutamicum ATCC15587* の固定化担体を用いて 300 時間の $n$-テトラデカンの繰り返し分解が可能であった。