Systematic degradation mechanism and pathways analysis of the immobilized bacteria: Permeability and biodegradation, kinetic and molecular simulation

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Article history:
Received 25 February 2020
Received in revised form 5 April 2020
Accepted 6 April 2020

Keywords:
Immobilized bacteria
Biodegradation
Bioremediation
Kinetics
Molecular simulation

1. Introduction

Marine oil spill remediation is a complex process, and has long been an important subject of academic study [1–3]. Microbial remediation has been the primary focus in recent years due to its economic advantages, high efficiency, and benefits as a clean technology with minimal production of pollutants [4,5]. However, the degradation rate of crude oil by free bacteria is extremely low due to harsh marine environments and low nutrient availability [6,7]. Enhancing the biodegradability of crude oil requires suitable conditions for growth and reproduction of microorganisms to attain a sufficient biomass [8–10]. Compared with free bacteria, organic components are adsorbed on the surface or pores of immobilized bacteria, increasing the contact probability between the degrading bacteria and the petroleum, further improving degradation rate. Consequently, immobilized microorganism technology can effectively solve the issues of low microbial density and low treatment efficiency caused by environmental factors [11–13].

Studies have found that prepared Eichhornia crassipes dried straw-immobilized bacterial community were used to remove crude oil [14]. Furthermore, total petroleum hydrocarbon in soil degraded by 51.7% in 30 days. Zhang et al. investigated two kinds of immobilization carriers—Enteromorpha residue and kelp residue—to study immobilized marine oil-degrading bacteria, and found that immobilized of Bacillus sp. E3 had an oil degradation rate of greater than 65% over 21 days [15]. Nevertheless, Acinetobacter venetianus immobilized on porous materials was reported to provide enhanced degradation of diesel oil, with a degradation rate could of as much as 94% over 3 days [16]. Therefore, the degradation efficiency of immobilized bacteria can differ greatly based on their preparation on different carriers [17,18]. This means that carrier selection plays a vital role in the degradation of oil pollution [19]. However, many reports also indicate that the some degradation characteristics difference of immobilized bacteria on different immobilized carriers may lower. The differences in degradation by

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immobilized bacteria observed between different carriers may instead be due to the permeability of immobilized bacteria. More recently, the permeability of immobilized bacteria has been studied [20,21]; however, the lower permeability of immobilized bacteria has largely been ignored. Current methodologies involve immersing immobilized bacteria in red ink instead of oil to determine the permeability of the immobilized bacteria. The mass transfer performance of immobilized bacteria is not objective or accurate due to the difference in physical and chemical properties between ink and petroleum. Consequently, it is a favor to improve the biodegradation and bring innovative changes to microbial remediation technology that the oil permeability of immobilized bacteria is explained. However, the degradation mechanism used by immobilized bacteria has not been systematically analyzed, especially with respect to permeability and the association of oil and carrier.

In this paper, a new method to systematically analyze the degradation mechanism used by immobilized bacteria is proposed. Combined with cinnamon shells or peanut shells, sodium alginate, and calcium chloride, a high-efficiency oil-degrading Pseudomonas sp. YT-11 strain used in our previous research was immobilized through an adsorption-embedding method [19]. In our previous study, using cinnamon shells or peanut shells as carriers yielded optimal immobilization effects. The permeability and interactions of immobilized bacteria were analyzed for surface adsorption, internal uptake, gas chromatography-mass spectrometry (GC-MS) analysis, molecular simulation, and adsorption kinetics. Based on these results, the degradation mechanism was systematically analyzed. The results may help improve the bioremediation of oil-pollution in seawater.

2. Materials and methods

2.1. Chemicals

In this study, diesel was used as a target substance for microbial degradation of oil hydrocarbons. We obtained # diesel from PetroChina (Qingdao 151th Station), Huangdao District, Qingdao, Shandong Province, P.R. China. The diesel was obtained from PetroChina (Qingdao 151th Station), Huangdao District, Qingdao, Shandong Province, P.R. China. The diesel was filtered with a 0.22 μm filter prior to usage for the experiments.

2.2. Sampling sites

Oil-contaminated samples from oil-spilled seawater were collected from north latitude 37°34′28″ and east longitude 121°15′3″. The samples were collected in sterile glass bottles and stored at 4 °C until use.

2.3. Preparation of immobilized bacteria

Aliquots of bacterial culture (10 mL) were added to 100 mL of solution containing sodium alginate (SA) and cinnamon shells or peanut shells (particle diameter of <0.15 mm) in a 100 mL beaker. This mixture was added drop-wise by a dropper into a CaCl₂ solution, forming the immobilized bacterial preparations. Then, the immobilized bacteria were immersed in 3 g/L CaCl₂ solution of 4 °C for 36 h and washed three times with saline solution (8.5‰). Finally, the immobilized bacteria were immersed in distilled water and stored at 4 °C [10].

2.4. Experimental set-up

The high-efficiency oil-degrading Pseudomonas sp. YT-11 was enriched and separated from collected samples and used to prepare bacteria immobilized on cinnamon or peanut shells for marine oil pollution degradation [19]. The degradation characteristics of immobilized bacteria on different carriers were studied by measuring surface adsorption of diesel, internal uptake, and degradation rate. Then, adsorption kinetics of diesel degradation by the immobilized bacteria were studied. Organic components were compared and analyzed by GC-MS before and after degradation. In addition, molecular simulation software (Molecular Operating Environment) was used to analyze the adsorption and binding of diesel to the immobilized bacteria.

2.5. Analyzing degradation characteristics of immobilized bacteria

The composition of minimal salt medium (MSM) is as follows: Na₂HPO₄ (0.6 g/L), KH₂PO₄ (0.5 g/L), NH₄NO₃ (3.0 g/L), NaCl (30.0 g/L), yeast extract (2.0 g/L), 1 mL CaCl₂ (1.11 g/L), 1 mL FeSO₄ (1.52 g/L), and 2 mL MgSO₄ (3.60 g/L). The pH level was adjusted to 7.2 – 7.5.

2.5.1. Degradation rate measurement

Bacteria immobilized on cinnamon shells or peanut shells (6.5 g) were added to MSM, and diesel (7.5 g/L) was used as the carbon source. The degradation efficiency of bacteria immobilized on different carriers in culture medium at days 1, 3, 5, 7, and 10 at 29 °C and 120 rpm were analyzed. Three parallel samples were prepared for all experiments [22,23].

Diesel degradation rate was measured as described previously [10,24] using the following equation:

\[ D = \left( \frac{C_0 - C_1}{C_0} \right) \times 100\% \]  

where D (%) is the degradation rate of diesel, C₀ (mL/L) is the initial concentration of diesel, and C₁ (mL/L) is the final concentration of diesel.

2.5.2. Surface adsorption of diesel by immobilized bacteria

Surface adsorption experiments were carried out at different time points to determine the adsorptive capacity of immobilized bacteria. Quantitative immobilized bacteria and diesel were added to MSM. The adsorption of diesel on the surface of immobilized bacteria at different time points was determined using the shaking flask culture method, in which the culture medium was removed leaving behind the immobilized bacteria. The bacteria were then washed once with sterilized inorganic salt medium, twice with n-hexane, and an additional two times with sterilized inorganic salt medium. Finally, all the washing liquids were combined. The supernatant was diluted and the diesel content in the solution was determined by ultraviolet spectrophotometry [25,26]. Three parallel samples were prepared in all experiments.

2.5.3. Internal uptake of diesel by immobilized bacteria

Washed immobilized bacteria were crushed, resuspended in Tris-HCl (pH 7.4, 10 mM), and sonicated in an ice bath for 10 s at intervals of 10 s for 3 min. Finally, n-hexane was added to extract diesel, and the diesel content was measured as the content of diesel enriched by immobilized bacteria. The samples were cultured in a shaker for 1, 3, 5, 7, and 10 days, and the diesel content in the supernatant was determined by ultraviolet spectrophotometry. Three parallel samples were prepared in all experiments.

2.6. Adsorption kinetics

A rate constant was obtained using the adsorption kinetic equation by applying the experimental kinetic data in order to study the adsorption rate and to construct a kinetic model. Adsorption kinetics were investigated using pseudo-first-order and the pseudo-second-order kinetic models. The basic theory is
determined. The adsorption of diesel by the immobilized bacteria may have been the determining factor of the adsorption rate, indicating that the force between the covalent bonds generated by the chemical interactions of two phases is the controlling factor. The pseudo-first-order kinetic equation is often applied to the adsorption kinetics of liquid-phase reactions. The pseudo-second-order kinetic equation is based on the assumption that the adsorption rate is controlled by chemisorption mechanisms designed to share electrons or electron transfer between the adsorbent and the adsorbate. In this study, immobilized bacteria (6.5 g) were added to 100 mL diesel solution with a concentration of 7.5 g/L for adsorption. The diesel adsorption capacity at varying timepoints was determined. The adsorption of diesel by the immobilized bacteria was calculated using the following formula:

\[
q_t = \frac{(C_0 - C_t)}{m} V
\]

where \(q_t\) (mg/g) is the adsorption capacity at time \(t\); \(C_0\) and \(C_t\) are the concentrations (g/L) of diesel at initial time and time \(t\), respectively; \(V\) (L) is the solution volume; and \(m\) (g) is the mass of the immobilized bacteria.

2.7. GC-MS analysis

Saturated hydrocarbons content of the oil was determined by GC-MS analysis, which provided a basis for analyzing the components of oil degraded by immobilized bacteria [29]. Using GC-MS analysis to compare organic components before and after degradation, the degradation efficiency of immobilized bacteria was investigated [30]. For GC, we used an initial temperature of 60 °C for 2 min, gradually increased to 120 °C at a rate of 15 °C/min, gradually increased again to 180 °C at 8 °C/min, incubated for 2 min, increased the temperature to 280 °C at 5 °C/min, and incubated for 4.5 min. MS was performed at an ionization energy of 70 eV and ion source temperature of 250 °C with a solvent delay of 4 min. Scanning mode was performed using a mass scan range between 35 amu and 650 amu.

2.8. Molecular operating environment

The MOE (Molecular Operating Environment) is a comprehensive software system for pharmaceutical and life sciences developed by Chemical Computing Group Inc. (Canada). It is a comprehensive application environment and technology development platform that integrates visualization, simulation, and application development [31]. MOE supports drug design in a unified operating environment through molecular modeling, protein structure analysis, small molecule data processing, and protein and small molecule docking research. In this study, the sodium alginate macromolecule and some small molecule components in diesel were simulated to study the adsorption of diesel oil by immobilized bacteria.

3. Results and discussion

3.1. Biodegradability of immobilized bacteria on different carriers

It can be seen from Fig. 1(a) that the immobilized bacteria on both cinnamon and peanut shells have an increasing degradation rate. The diesel degradation rates reached 69.94% for bacteria immobilized on cinnamon shells and 64.41% for bacteria immobilized on peanut shells over 10 days. The surface adsorption of diesel by immobilized bacteria is shown in Fig. 1(b). The amount of diesel adsorbed reached 30.01% for bacteria immobilized on cinnamon shells and 28.97% for bacteria immobilized on peanut shells. The amount of diesel adsorbed by the immobilized bacteria stabilized after 5 days and was close to zero. Nevertheless, the internal uptake of diesel by the immobilized bacteria increased with time. As shown in Fig. 1(c), compared with the surface adsorption of diesel, the internal uptake of diesel was substantially lower. The maximum internal uptake was only 2%.

In summary, the pathways for diesel removal by immobilized bacteria included surface adsorption, internal uptake, and biodegradation. Surface adsorption played an important role in the initial stages of degradation, and then gradually, biodegradation became the dominant method. However, internal uptake by immobilized bacteria was very low. This may be due to the poor permeability of immobilized bacteria following adsorption and embedding methods. A dense spherical shell structure may have been formed near the outer surface due to the penetration of the crosslinking agent, which may have made it difficult for oil to penetrate. Similarly [24], studied the mechanism that free bacteria use to degrade diesel and found that the first step of diesel degradation involved rapid surface adsorption followed by cell uptake.

3.2. Degradation mechanisms used by immobilized bacteria

To better explain the above removal pathways, we studied the mechanism of degradation by analyzing kinetics and molecular simulation.

3.2.1. Adsorption kinetics

Fig. 2 shows the adsorption kinetic curves of the immobilized bacteria. We found that the adsorption of diesel fit the pseudo second-order kinetic model, with a correlation coefficient of greater than 0.99. The force between the covalent bonds generated by the exchange between the immobilized bacteria and diesel was found to be the determining factor of the adsorption rate, indicating that adsorption kinetics were mainly controlled by chemical interactions. Similarly, the adsorption of diesel by immobilized Acinetobacter venetianus matched the pseudo second-order model \(R^2 > 0.99\) [16].
3.2.2. Molecular simulation of surface adsorption

To better simulate the adsorption and binding of diesel on the surface of immobilized bacteria, organic components present during degradation were analyzed. The chromatographic peaks shown in Fig. 3 indicate that all organic components were degraded. Compared with organic component composition before degradation, the majority of C11–C25 were degraded, with highest degradation efficiency found for C15.

Other than hydrocarbons, petroleum also contains small amounts of non-hydrocarbon compounds, including sulfur compounds, nitrogen compounds, and oxygenates, such as mercaptan and thiophene. Therefore, in line with the GC-MS analysis, organic components and diesel additives were classified as small molecules, the structure of which was acquired from www.zinc.docking.org. Additionally, sodium alginate from the immobilized bacteria simulated large molecules, the structure of which was acquired from the RCSB protein database. The binding sites of sodium alginate macromolecule and diesel components (benzoic acid, dimethyl carbonate, pentadecane, and phenylmercaptan) were simulated using MOE software.

It was found that the main binding force of sodium alginate with benzoic acid and dimethyl carbonate was hydrogen bond, and the preferential binding mode of sodium alginate with them was studied. The combination model with the lowest binding energy was selected from the various possible structures generated. According to the energy calculation results, hydrogen bonds played a major role in mediating the interaction. Fig. 4(a and b) shows the minimum energy docking structures of sodium alginate with benzoic acid and dimethyl carbonate, respectively. Hydrogen bonds were formed during the binding process. A large pocket in sodium alginate provides a binding groove for benzoic acid and dimethyl carbonate to approach the active site. The most probable composite conformations are shown in Fig. 4(c and d). Sodium alginate formed relatively weaker bonds with pentadecane and phenylthiol. Docking structures and composite conformations for these components are shown in Fig. 4(e and f) and (g and h), respectively. These results suggest that immobilized bacteria adsorb diesel mainly through hydrogen bonds formed with sodium alginate. Sodium alginate is an organic molecule, and the production of weak bonds might be due to the principle of similar compatibility. It was previously found that hydrogen bonds and van der Waals forces also play a major role in the interaction of BPA with acid phosphatase [23].

Fig. 1. Study of the degradation characteristics of bacteria immobilized on different carriers. (a) Degradation rate of diesel by immobilized bacteria over time. (b) Surface adsorption of diesel over time. (c) Internal uptake of diesel by different immobilized bacteria.

Fig. 2. Adsorption kinetics of bacteria immobilized on cinnamon shells (a) and peanut shells (b).
Fig. 3. GC-MS analysis of undegraded diesel (a) and diesel extracted from a culture of bacteria immobilized on cinnamon shells (b).

Fig. 4. The molecular docking results of planar graph (a, b, c, d) and the space diagram (e, f, g, h).
Adsorption kinetics show that the adsorption of diesel on the surface of the immobilized bacteria is controlled by chemical interactions. Molecular simulation models show that hydrogen bonds are formed during the adsorption process of diesel by sodium alginate on the surface of the immobilized carrier, and hydrogen bond is a type of chemical bond. Together, these results suggest that the adsorption of diesel on the surface of the immobilized bacteria is a chemical process mediated by hydrogen bonds, which enable efficient and stable binding to facilitate further biodegradation.

3.3. Discussion

A summary of our systematic study on the degradation process of diesel by immobilized bacteria is illustrated in Fig. 5. Our results revealed that diesel was quickly adsorbed on the surface of immobilized bacteria. Sodium alginate on the surface of the immobilized bacteria interacted with the small molecules of diesel components through hydrogen bonds to promote the adsorption of diesel through chemical processes. Residual diesel was removed largely by biodegradation. Diesel was decomposed by immobilized bacteria into small molecular substances, and then slowly degraded into CO₂ and H₂O. Additionally, the immobilized bacteria mainly degraded C₁₁–C₂₅, and the greatest degradation efficiency was observed for C₁₅.

4. Conclusion

In this study, bacteria were immobilized on cinnamon shells and peanut shells. The mechanism by which immobilized bacteria degrade diesel was studied by investigating removal pathways and through computational modeling. From our results, we conclude the following:

1. The main removal pathways used by immobilized bacteria included surface adsorption of diesel, internal uptake of diesel, and biodegradation. Over time, the amount of diesel adsorbed on the surface of the immobilized bacteria increased rapidly and then decreased before stabilizing. In contrast, the rate of internal uptake of diesel increased slowly, and the value was low.
2. The adsorption of diesel by the immobilized bacteria fit the pseudo second-order kinetic model and was mediated by chemical interactions.
3. Sodium alginate interacted with benzoic acid and dimethyl carbonate largely through hydrogen bonds, and interacted with pentadecane and phenylthiol through weak bonds. Altogether, we conclude that the immobilized carrier sodium alginate interacts with diesel through hydrogen bonds to promote the adsorption of diesel components, representing a chemical adsorption process. Next, residual diesel is decomposed by microorganisms into small molecular substances, which are then slowly degraded into CO₂ and H₂O.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the scientific research fund project of the National Natural Science Foundation of China (grant numbers 51408347), the SDUST Graduate Technology Innovation Project (SDKDYC190321), the Major Science and Technology Innovation Projects in Shandong Province (2019JZZY020808), and the Open Research Fund Program of Shandong Key Laboratory of Eco-Environmental Science for Yellow River Delta (Binzhou University) (2019KFJ02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jese.2020.100028.

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