Marine Pollution Bulletin 67 (2013) 146–151

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Enhancement of the diesel oil degradation ability of a marine bacterial strain by immobilization on a novel compound carrier material

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ARTICLE INFO

Keywords:
Carrier material
Immobilization
Enhance
Biodegradation ability

ABSTRACT

A novel floatable and biodegradable carrier material was made by coating puffed foxtail millet (PFM) with a calcium alginate (CA)-chitosan compound membrane. A diesel oil-degrading marine bacterial strain, *Acinetobacter* sp. F9, was immobilized on the carrier material. The number of viable F9 cells immobilized on the carrier material reached approximately 5×10^9 CFU/g. This formulation could be stored at −20 °C and 4 °C for 10 weeks without a significant decrease in the number of viable immobilized cells. SEM results showed that the coating membrane was porous and that F9 cells were immobilized on the walls of the pores. The immobilized F9 cells were able to remove more than 90% of the diesel oil by the second day, while free F9 cells did not remove 90% of the diesel oil until the seventh day. GC–MS analysis indicated that the immobilized F9 cells could remove diesel oil more completely than free cells. The immobilization of the F9 cells enhanced their ability to biodegrade diesel oil.

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1. Introduction

Bioremediation is an important means for treating marine pollution by petroleum and its derivatives. There are two approaches to bioremediation. One approach is biostimulation, and the other is bioaugmentation. In bioaugmentation, a cultured microorganism with degradability is applied to the polluted site to clean up the pollutant. Using bioaugmentation to enhance natural biodegradation is a useful alternative (Vogel, 1996; Jansson et al., 2000; Cunningham et al., 2004). Bioaugmentation has been attempted with varying degrees of success (Crawford and Mohn, 1985; Brodkorb and Legge, 1992; Leavitt and Brown, 1994; Vogel, 1996; Atlas and Bartha, 1992). However, there still are challenges to be overcome in the application of bioaugmentation. One challenge is the maintenance of sufficient activity of an inoculant population over a prolonged period after release (Sanjeet et al., 2001). A microorganism used for bioaugmentation in bioremediation may show a broad substrate range and high degradation rate in the laboratory, but when introduced into a natural environment, the organism frequently fails due to harsh environmental conditions. A previous study has shown that when a microbial consortium was introduced into a natural environment polluted with hydrocarbons, the components of the consortium disappeared after a short period of time (Macnaughton et al., 1999). If bioremediation is performed in an open-water system, such as a marine environment, the dilution of the bioremediating microorganisms is also a problem that remains to be solved (Tagger et al., 1983).

Immobilizing microbial cells with contaminant-degrading abilities on a carrier material to generate ready-to-use seeds or formulations may help to solve such problems. Immobilization prevents microbial cells from being diluted in an open-water system. Carrier materials could provide a protective surface or pore spaces for the bioremediating microbial cells, creating favorable conditions for the survival and functional output of the microbial cells. This results in a sufficiently long shelf life and improved survival and activity of the inoculant cells (van Veen et al., 1997). Ideally, the carrier material would be biodegradable, available in large quantities, low cost, and have the appropriate physical properties to allow for sufficient aggregation of specific microorganisms useful for bioremediation (Elliott et al., 2007). The carrier materials that have been studied include sawdust, wheat bran, calcium alginate, potato starch fiber, Styrofoam and polyurethane foams (OReilly and Crawford, 1989; Li et al., 1994; Resnick, 1998; Wang and Qian, 1999; Oh et al., 2000; Diaz et al., 2002; Obuekwa and Muttawa, 2001; Quek et al., 2006; Gentili et al., 2006; Elliott et al., 2007). To be applied in an oil-polluted marine environment, the carrier material should float. A floatable formulation makes it easier for degraders to have contact with the oil pollutant. However, it is not easy to find a floatable and biodegradable carrier material, and there are few reports of such a carrier material.

In this study, a compound carrier material that was floatable and biodegradable was produced. This carrier material was studied by Fourier transform infrared spectroscopy (FT-IR). A diesel oil-degrading marine bacterial strain (*Acinetobacter* sp. F9) was immobilized

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0025-326X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marpolbul.2012.11.021

on the carrier material. The diesel oil-degrading ability of this formulation was measured and compared with that of the F9 cells in free form. The viable cells immobilized on the formulation were quantified. The morphology of the formulation was observed by SEM.

2. Materials and methods

2.1. Development of the carrier material

Puffed foxtail millet (PFM) was purchased from a market. The density of the PFM was approximately 120 kg/m^3. Sodium alginate (SA) and chitosan were purchased from Guoyao Company (China). All other inorganic and organic chemicals were of analytical reagent grade. PFM was decontaminated by ultraviolet irradiation, and other sterile solutions were autoclaved at 121 °C for 15 min.

PFM was immersed in a stirred 1% solution of $CaCl_2$ for 5 min. Then, wet PFM removed and immersed in a stirred 1% solution of SA for 5 min to generate the calcium alginate (CA) membrane coating on the PFM. CA membrane-coated PFM was immersed in a stirred 1% solution of chitosan containing 1% $CaCl_2$ for 5 min to transform the CA membrane into a CA–chitosan compound membrane.

2.2. Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR spectra were obtained using a NEXUS 470 FTIR spectrometer (NICOLET, USA) with a resolution of 4 cm^{-1}. The lyophilized samples were crushed into a powder, and the powder was milled with KBr. Then, this mixture was pressed into a disk for analysis. The absorption (or transmission) spectra of the samples were recorded in the 400–4000 cm^{-1} range.

2.3. Culture of the marine diesel oil-degrading bacterial strain

The marine diesel oil-degrading bacterial strain (named F9) was isolated from diesel oil-polluted seawater and identified as *Acinetobacter SP* in our lab (data not shown). The F9 cells were inoculated into 200 mL of nutrient broth (LB medium) in a 500 mL flask. LB medium was prepared as follows (per liter): 10 g NaCl, 10 g tryptone, 5 g yeast extract. The pH of the LB medium was adjusted to 7.5 with either HCl or NaOH. The flask was incubated for 24 h at 30 °C on a rotary shaker operated at 180 rpm. The resulting culture was used in subsequent experiments.

2.4. Immobilization of F9 cells

The prepared wet carrier material was placed into a 500 mL flask containing 200 mL of an F9 culture. Then, this mixture was incubated at 30 °C with shaking at 100 rpm to allow for the F9 cells to attach to the carrier material (wet formulation). The wet formulation was removed from the flask and rinsed with sterile LB to remove any F9 cells that were not sufficiently adhered to the carrier material. Then, the wet formulation was pre-frozen at −70 °C for 4 h with sterile 0.5% mannitol solution as its protectant. Finally, the pre-frozen wet formulation was moved to the freeze-drier to be lyophilized at −45 °C for 72 h.

2.5. Viable cell enumeration

To count the attached viable cells, 0.1 g of the formulation sample was suspended in 5 mL LB medium. The suspension was agitated vigorously using a vortex mixer for 3 min to dislodge the immobilized cells. Serial dilutions were made from the supernatant, and aliquots of 0.2 mL were spread on LB plates. The plates were incubated at 30 °C until colonies appeared (24–48 h). The colonies were counted to assess the number of viable F9 cells immobilized on the carrier material.

2.6. Examination of the formulation using scanning electron microscopy (SEM)

The lyophilized formulation sample was crushed and sputter-coated with gold (Bal-Tec SCD005 Sputter Coater). Observations were performed using a JEOL JSM 6390LV scanning electron microscope.

2.7. Diesel degradation experiments

Degradation experiments were performed in artificial seawater (ASW) supplemented with 2% (v/v) trace elements solution (Liu and Shao, 2005) to simulate bioremediation in a marine environment. ASW contained the following (per liter): 24 g NaCl, 2.0 g KH_2PO_4, 1.0 g NH_4NO_3, 7.0 g $MgSO_4{\cdot}7H_2O$, 0.7 g KCl, 3.0 g $Na_2HPO_4{\cdot}2H_2O$. The pH of the ASW medium was adjusted to 7.5 with either HCl or NaOH. The trace element solution contained the following (per liter): 2.0 mg $CaCl_2$, 50 mg $FeCl_3{\cdot}6H_2O$, 0.5 mg $CuSO_4$, 0.5 mg $MnCl_2{\cdot}4H_2O$, 10 mg $ZnSO_4{\cdot}7H_2O$. The ASW and the trace element solution were prepared separately and autoclaved at 121 °C for 15 min. The trace element solution was added to the ASW immediately before use.

F9 cells were first inoculated into LB liquid medium and cultured for 24 h. Then, approximately 2.5×10^9 cells were collected, washed twice with sterilized ASW culture and inoculated into 50 mL ASW containing 1% (v/v) diesel oil as the sole carbon source in a 150 mL Erlenmeyer flask. The flasks were incubated at 30 °C with shaking (180 rpm) for 7 days.

The F9 formulation was inoculated (1%, w/v) into 50 mL ASW containing 1% (v/v) diesel oil in 150 mL Erlenmeyer flasks. The flasks were incubated at 30 °C with shaking (180 rpm) for 7 days. Aliquots of 50 mL ASW containing 1% (v/v) diesel oil in 150 ml Erlenmeyer flasks were used as a control. After every 24 h interval, one flask for each test condition was removed, and the entire contents were used to determine the extent of degradation. All experiments were performed in triplicate.

The diesel oil remaining in each sample was extracted with ligarine, and the concentration of diesel oil in the ligarine was measured by UV absorbance at 255 nm. The diesel oil degradation rate was determined according to the following equation:

$$\mathbf{R_d} = \left(1 - \frac{\mathbf{X_c} - \mathbf{X_s}}{\mathbf{X_c}}\right) \times 100\%$$

where $\mathbf{R_d}$ is the diesel oil-degradation rate, $\boldsymbol{X_c}$ is the diesel oil remaining in the control culture, and $\boldsymbol{X_s}$ is the diesel oil remaining in the sample culture.

2.8. Gas chromatography–mass spectrometry (GC–MS) analysis of diesel degradation

Residual diesel oil was extracted using dichloromethane. The extract was analyzed by GC–MS. GC–MS analysis was performed on a *Focus DSQ* GC–MS instrument (Thermo, USA). An HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used to separate the hydrocarbons. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector and detector temperatures were set at 250 °C and 300 °C, respectively. The temperature program was as follows: 2-min hold at 60 °C, ramp to 300 °C at 20 °C/min and 5-min hold at 300 °C.

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