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New approach for petroleum hydrocarbon degradation using bacterial spores entrapped in chitosan beads

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ABSTRACT

Spores of *Bacillus subtilis* LAMI008 were entrapped in 3-mm chitosan beads and cross-linked with 0.3% glutaraldehyde for *n*-hexadecane biodegradation and biosurfactant recovery. When exposed to nutrients, the spores generated vegetative cells without morphological alterations as revealed by atomic force microscopy. The entrapped cells degraded almost 100% of 1% of *n*-hexadecane in medium supplemented with 1% glucose and produce biosurfactant within 48 h, as well as free cells. The number of viable cells inside the beads was maintained throughout the *n*-hexadecane degradation process and the released biosurfactant was not used as a carbon source. Entrapment of bacterial spores in chitosan beads overcomes problems with stability, storage, and long term cell viability encountered with vegetative cells. This approach can potentially be utilized for biodegradation of complex compounds by entrapping spores of different species of bacteria.

1. Introduction

Bioremediation is an eco-friendly application for clean-up of oil spills since it allows complete *in situ* mineralization of pollutants (Ward, 2004). Among the recent methods employed to improve bioremediation, the use of immobilized microorganisms presents several advantages over the use of free cells. For instance, the use of immobilized cells can overcome adverse environmental conditions that threaten microbial survival and it can also prevent the direct contact of the introduced microorganisms with the autochthonous microbial community. In addition, immobilization facilitates the monitoring of the microbial metabolism and offers the possibility of repeated use of the cells (Cassidy et al., 1996). One of the most widely used techniques for cell immobilization is entrapment, in which the microbial cells are enclosed in a polymeric matrix which is porous enough to allow the diffusion of substrate to cells and of product away from the cells.

A wide range of polymers has been tested in the attempt to develop supports for cell immobilization (Cassidy et al., 1996). Chitosan, a copolymer of β-1,4-linked D-glucosamine and *N*-acetyl-D-glucosamine residues derivated by the deacetylation of chitin, presents several advantages over other polymers (Vorlop and Klein, 1987). The amino groups of the polymer, under acidic conditions, can interact with polyanionic counterions to form gels which can be appropriately managed for cell entrapment. Besides, the use of chitosan as matrix for cells immobilization might contribute to recycle the chitin waste originated from shrimp farming and it could become a profitable income source especially in regions where aquaculture is prevalent. However, only a few studies on cell entrapment in chitosan have been reported (Chen et al., 2007; Hsieh et al., 2008; Jobin et al., 2005), likely due to the known antimicrobial activity of chitosan and poor solubility above pH 6.5 (Kumar, 2000).

Bacillus is a heterogeneous group of rod-shaped bacteria, that can occur both in isolated or in chain forms and are able to produce dormant spores. Bacterial spores are formed when the cellular reproduction becomes threatened by drastic environmental

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conditions. Therefore, sporulation is understood as a defense form, allowing the maintenance of microbial viability (Driks, 2002). As bacterial spores are much more resistant than vegetative cells to physical and chemical environmental aggressions they represent a better option for immobilization. Once immobilized and in ideal conditions, spores can germinate and originate metabolically active vegetative cells. Using this approach, bacterial cells are protected from the harmful effects of the chitosan during the immobilization process, besides it can be stored for long periods.

Therefore, bacterial spores were entrapped in chitosan beads and the ability of germinated cells to degrade *n*-hexadecane was evaluated with the goal of developing of an innovative approach for bioremediation.

2. Methods

2.1. Microorganism and molecular identification

The strain LAMI008 used in this work was isolated from the Campus do Pici Wastewater Treatment Station, at the Federal University of Ceará, Brazil. It was characterized as Gram-positive, spore producer, and able to grow under the following conditions: temperature range of 10–50 °C, pH of 5.0–12.0 and NaCl concentrations of 2–10%. This strain is stored in the bacterial collection of the Microbial Ecology and Biotechnology Laboratory (LEMBiotech) of the Biology Department, Federal University of Ceará, Brazil.

The molecular identification of the LAMI008 strain was done using its 16S rRNA gene sequence. Bacterial genomic DNA was purified using a CTAB-based protocol (Warner, 1996) and the 16S rRNA was amplified by polymerase chain reaction (PCR) using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as the forward primer (Gürtler and Stanisich, 1996), and 1525R (5′-AAGGAGGTGATCCAGCC-3′) as the reverse primer (Lane, 1991). Amplification reactions were performed using a final volume of 25 μL, which contained 300 ng of genomic DNA (template), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (GE Healthcare Life Sciences, Buckinghamshire, UK), 12.5 pmol of each primer and 0.5 units of Taq DNA polymerase (Amersham Biosciences, São Paulo, Brazil). PCR reactions were carried out in a PTC-200 thermocycler (MJ-Research Inc., Maryland, USA) programmed for an initial denaturation step (4 min at 94 °C) followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The last cycle was followed by a single final extension of 10 min at 72 °C. The presence of PCR products with the expected size was confirmed by analyzing 2 μL of the PCR product on a 1.0% agarose gel electrophoresis and staining with ethidium bromide. PCR products were purified from the remaining reaction using GFX PCR DNA and Gel Band Purification kit (GE Healthcare). DNA sequencing was performed with the DYEnamic ET terminators cycle sequencing kit (GE Healthcare), following the protocol supplied by the manufacturer, and both strands were sequenced using the primers 27F, 1525R, 782R (5′-ACCAGGGTATCTAATCCTGT-3′) and 1100R (5′-AGGGTTGCGCTCGTTG-3′). Sequencing reactions were then analyzed in a MegaBACE 1000 automatic sequencer (GE Healthcare). High quality reads (phred >20) were used to generate full 16S sequences using the Phred/Phrap/Consed package. The determined sequence was then compared to those already deposited in the GenBank using BLAST.

2.2. Chitosan and spores immobilization

The chitosan used in this work was provided by the Parque de Desenvolvimento Tecnológico (PADETEC) of the Federal University of Ceará, Brazil. It was obtained from shrimp residues and has the following physicochemical characteristics: 117.0 kDa and 78.0% of deacetylation.

Bacterial spores were produced in a sporulation medium (SM) with the following composition (in grams per liter): 1.0 glucose, 1.0 sodium L-glutamate, 0.5 yeast extract, 5.0 KH_2PO_4, 1.0 $(NH_4)_2HPO_4$, 0.2 $MgSO_4 \times 7H_2O$, 0.1 NaCl, 0.05 $CaCl_2$, 0.007 $MnSO_4 \times 5H_2O$, 0.01 $ZnSO_4 \times 5H_2O$, 0.01 $FeSO_4$, and 20.0 agar. Five hundred microliters of bacterial cell suspension with turbidity of 1.0 at O.D.600 nm (Genesys 10UV–Vis Spectrophotometer, New York, USA) were spread on the SM and incubated for 72 h at 30 °C. After this period, spores were aseptically removed from the agar surface and washed three times with distilled water under centrifugation at 10000*g* for 15 min. These spores were visualized after staining (Collins et al., 1995) using light microscope and stored at 4 °C until use. An aliquot of 0.5 mL of the spore suspension (O.D. 600 nm 0.6) was inoculated in 5.0 mL of mineral medium (Sar and Rosenberg, 1983) supplemented with 1% *n*-hexadecane (Fisher Scientific Co., Dallas, USA) (v/v) used as carbon source. Control was performed in the same medium using 1% glucose solution (v/v). Cultures were grown under agitation at 160 rev min^{-1} and 30 °C, and the germination was monitored by O.D.600 nm. Successive fold-dilutions in 0.9% NaCl of bacterial suspension were plated in triplicate on nutritive broth (Merck, Darmstadt, Germany) for viable cells count.

Five hundred microliters of a spore suspension adjusted to 1.0 at O.D.600 nm (10^7 CFU mL^{-1}) were transferred to 30 mL of sterilized 4% chitosan solution prepared in 1% acetic acid, pH 6.0. This mixture was added drop-wise through a 1.0 mL plastic tip into an 8% NaOH solution for coagulation and formation of the beads. To control the size of the beads, a constant height from the solution to the plastic tip was maintained. After 30 min, the beads were separated from the solution and washed twice with 200 mL of sterile water for 15 min three times under agitation. The collected washing solutions were analyzed by viable cell count until no viable cell was detected. This procedure was important to ascertain that the only cells remaining were those entrapped inside the beads. For immobilized cell count, the beads were suspended in 0.9% NaCl solution (1:10, w/v), disrupted by using a sterilized blender and used for viable cell determination. The entire procedure was carried out under aseptic conditions in a laminar flow bench (Labconco, Kansas, USA). The spore-entrapped beads were stored in sterile water at room temperature until use.

2.3. Bacterial killing assay

A 100 μL volume of bacterial spores and cells suspension (10^7 CFU mL^{-1}) were incubated in the absence of 1.0 mL sterilized 4% chitosan solution in 1% acetic acid, pH 6.0 as control; and also in the presence of this solution. The tubes were incubated at 37 °C and after 1 and 24 h aliquots were plated on nutrient agar to obtain a viable cell count.

2.4. Chitosanase activity

Chitosanase activity was evaluated following the protocol described by Choi et al. (2004). Bacterium was initially cultivated on nutrient agar to obtain isolated colonies. One colony was transferred to nutrient broth and incubated for 24 h at 37 °C. One drop of that culture was inoculated on the surface of the 0.1% chitosan agar plate pH 6.0 and incubated for 24 h at 37 °C. The enzymatic activity was evaluated by the emergence of a colorless halo around the colony. A drop of a chitosanase-producer *Bacillus subtilis* ATCC 14579 was used as positive control.