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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.

2.5. Bacterial adhesion to hydrocarbons (BATH) assay

Bacterial adhesion to hydrocarbons was measured as described by Rosenberg et al. (1980). Spore and vegetative cell suspensions were washed twice in 100 mM sodium phosphate buffer, pH 6.8 under centrifugation at 10000*g* for 5 min. Spores and cells were suspended in the same buffer to an O.D.440 nm about 0.8–1.0. Volumes of 0.1, 0.2, 0.6, and 1.0 mL of *n*-hexadecane (Fisher Scientific Co., Dallas, Texas) were added to 3.0 mL of each spore or cell suspension. The mixture was vortexed (Vortex Genie 2; Fisher Scientific Co.; setting 5) for 1 min in round-bottom test tubes (15 mm × 100 mm) and the *n*-hexadecane and aqueous phases were allowed to partition for 15 min. The aqueous phase was carefully removed by using a Pasteur pipette, and O.D. was then measured. The percentage of hydrophobicity (%H) was calculated as follows: $\%H = 100(OD_i - OD_f)/OD_i$, where OD_i and OD_f were the initial and final optical densities, respectively.

2.6. Atomic force microscopy (AFM)

Samples of spores- or cells entrapped chitosan beads were deposited on circular 13 mm diameter glass and then characterized by AFM. All topography images were performed with a Nanoscope IIIa controller and Nanoscope software, (Digital Instruments – California, USA) at room temperature. The tapping mode (TM®) or intermittent mode was used to avoid lateral forces which can damage the sample. A 12 μm *xy* range scanner with a 5 Hz scan rate and resolutions of 512 × 512 was used to obtain the high resolution images. Pyramidal antimony-doped Si tips on rectangular cantilevers with approximately 30 N/m of spring constant and 125 μm of length (TESP, Veeco, California, USA) were used.

2.7. n-Hexadecane degradation

Biodegradation assays were performed in 50 mL of mineral medium (Sar and Rosenberg, 1983) containing 1% *n*-hexadecane (v/v) supplemented with 1% glucose (w/v) and inoculated either with 10% of spore suspension (v/v) or 10% of spore-entrapped chitosan beads (w/v), both adjusted to contain 10^7 CFU mL^{-1}. The cultures were maintained at 160 rev min^{-1} for 96 h. Aliquots were analyzed each 24 h for *n*-hexadecane biodegradation, biosurfactant production, pH measurements and viable cells counting.

The quantitative analysis of biodegradation was conducted by the external standard method using 99.9% *n*-hexadecane and performed on a Shimadzu GC17A gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector (GC-FID), column OV-5 (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Hydrogen was used as carrier gas at a flow rate of 1.0 mL min^{-1}; split mode (ratio 1:10); the column temperature was programmed from 100 °C to 250 °C and heated at a rate of 10 °C min^{-1}; both injector and detector temperatures were 250 °C.

2.8. Biosurfactant production

The surfactant production was monitored by the emulsification assay proposed by Iqbal et al. (1995). Briefly, 2.0 mL of cell-free supernatant, and 2.0 mL of kerosene were vortexed for 2 min. After 24 h, the height of emulsion layer was measured. The emulsification index (EI24) was calculated using the following equation: $EI24\ (\%) = H_{EL}/H_S \times 100$, where H_{EL} is the height of the emulsion layer and H_S is the height of total solution.All tests were run in triplicate and repeated twice.

3. Results and discussion

3.1. Molecular identification

The 16S rRNA gene sequence of LAMI008 was determined (1.481 bp) and deposited in the GenBank (accession number EU082292). A BLAST search revealed that the determined sequence had an identity of 99% or greater to 16S sequences from different strains of *B. subtilis*. The highest identity (99.7%) was found with the 16S RNA gene sequence from *B. subtilis* subsp. *subtilis* strain 168. Therefore, the strain LAMI008 was identified as *B. subtilis*.

3.2. Spore production and germination

The growth of *B. subtilis* LAMI008 in the sporogenic medium promoted 100% of sporulating cells. The staining technique proposed by Collins et al. (1995) allowed the distinction between spores and vegetative cells (data not shown). The spores did not germinate in mineral medium containing only *n*-hexadecane as carbon source. However, the supplementation with 1% glucose promoted 100% of their germination as observed by microscopy analysis. This result showed that sugars act as a signal promoting the breakdown of the spore dormancy (Setlow, 2003). Glucose might also modulate the early hours of growth and stimulate biosurfactant production, which facilitate the *n*-hexadecane capture and use. From a practical point of view, glucose can be provided to spore-entrapped chitosan beads by soaking the beads in a glucose solution and afterwards the beads must be dried or freeze dried. This strategy can be used for co-immobilization of nutrient or cofactor required for spore germination and/or cell metabolism.

3.3. In vitro growth inhibition

The spores of LAMI008 showed resistance to 4% chitosan solution while the vegetative cells were completely inhibited after 1 h of contact. The viability of the spores was not affected even after 24 h of incubation in chitosan solution.

The antimicrobial activity of chitosan solution is attributed to the great number of cationized amines that can potentially interact with the negatively charged residues of macromolecules on microbial cell surface causing the formation of an impermeable layer around the cell, and disrupting the normal functions of the membrane, e.g. by promoting the leakage of intracellular components and also by inhibiting the transport of nutrients into the cells (Rabea et al., 2003; Raafat et al., 2008). It is noteworthy that this effect is achieved only at acid pH in which chitosan is completely protonated (Rabea et al., 2003). Based on the presumed mechanism of chitosan action, the resistance of the spores can be explained by the difficulty of chitosan to penetrate the spores due to the several protective coats that surround the spore that protect the underlying cortex peptidoglycan layer and prevent damage to the cell membrane. Besides, the fact that spores are physiologically inert contributes to the decrease of the effects from any chemical.

3.4. Hydrophobicity of LAMI008 spores and cells

The hydrocarbon adhesion data showed that the spores of LAMI008 are more hydrophobic than its vegetative cells, in agreement with the observation of Wiencek et al. (1990) with other *Bacillus* species. This difference is attributed to the high abundance of protein found on the external layers of spores. The hydrophobicity of LAMI008 spores and vegetative cells increased with the in-

crease of the *n*-hexadecane concentration (Table 1) presumably by exposing more hydrophobic residues of their surfaces.

Bacteria with hydrophobic surfaces may achieve intimate contact with substrates by attaching to the oil–water interface and may take up the substrate directly from oil droplets.

3.5. Spores immobilization

Spores of LAMI008 were encapsulated in chitosan beads with an average diameter of 3 mm. All produced beads had good spherical geometry (Fig. 1). Aiming to detect possible cell damage during the immobilization process, entrapped spores and cells were visualized by AFM. There was no evidence for cell wall lysis or apparent morphological alteration of the spores or germinated cells (data not shown). In fact, LAMI008 spores inside chitosan beads remained viable for more than one year.

Table 1
Hydrophobicity of spores and vegetative cells of *B. subtilis* LAMI008.

% of *n*-hexadecane concentration	Hydrophobicity (%)	
	Spores	Vegetative cells
0.0	0.5 ± 0.02	0.3 ± 0.02
0.2	17.0 ± 0.20	0.8 ± 0.02
0.6	22.0 ± 0.50	2.0 ± 0.01
1.0	25.0 ± 0.30	6.0 ± 0.04

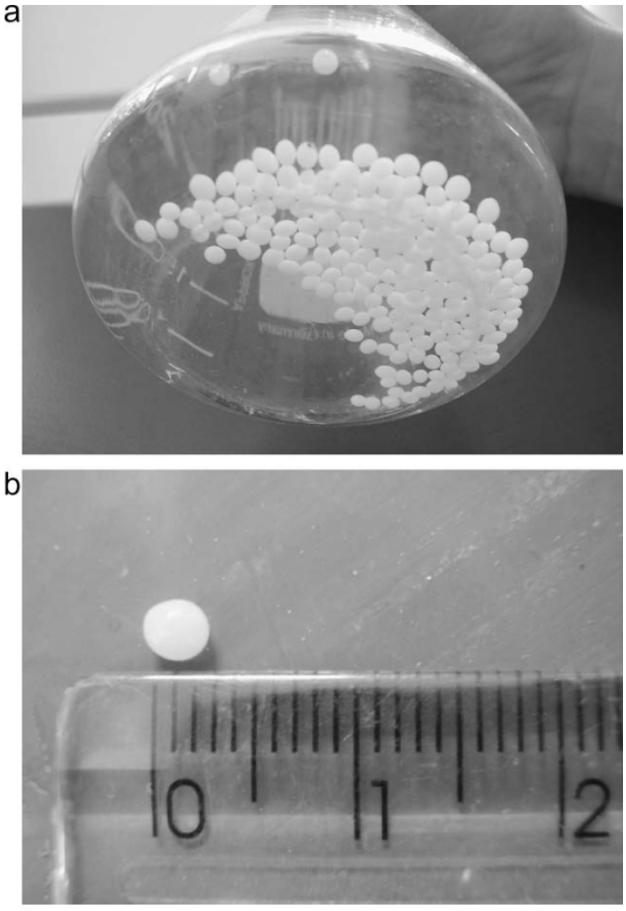

Fig. 1. (a) Chitosan beads containing immobilized spores of *B. subtilis* LAMI008 and (b) diameter of the bead.

3.6. Biodegradation of n-hexadecane

The degradation of *n*-hexadecane by LAMI008 entrapped in chitosan beads was compared with that by free cells under similar conditions. *n*-Hexadecane was chosen because it is one of the most studied hydrocarbons in biodegradation experiments and represents the largest aliphatic fraction of diesel. *n*-Hexadecane can be found in rivers, as a result of routine petroleum activities, released from storage tanks or even by accidental oil spills (Chénier et al., 2003).

The beads disintegrated completely within 48 h of *n*-hexadecane biodegradation. Since the pH did not decrease during the experiment, it was hypothesized that the production of chitosanase by LAMI008 could be responsible for the beads disintegration. This hypothesis was confirmed by the hydrolysis of chitosan on chitosan agar plate assay performed within 48 h. As chitosanase was only detected in control at 24 h, it was speculated that the production of this enzyme by LAMI008 is substrate-induced. To overcome this problem, immediately after the spore immobilization, the beads were treated with 0.3% glutaraldehyde. Cross-linking has been done to improve the stability of chitosan (Hsien and Rorrer, 1997; Oyrton and Monteiro, 1999). The covalent linkage between the amine group of chitosan and the aldehyde group of glutaraldehyde is stable in wide pH and temperature ranges and resistant to enzymatic hydrolysis. Glutaraldehyde does not prevent the biodegradation of chitosan since it does not affect enzymatic hydrolysis of β-1,4-linkages of the chitosan (McConnell et al., 2008). The concentration of glutaraldehyde used in this study did not affect the integrity of the LAMI008 germinating cells, as confirmed by AFM (data not shown), or their physiology and biodegradation capacity.

Table 2 presents an overview of *n*-hexadecane biodegradation by free and immobilized spores of LAMI008 in chitosan beads. The biodegradation of *n*-hexadecane by vegetative cells originating from free and immobilized spores was the same. Both types of cells degraded almost 100% of 1% *n*-hexadecane within 48 h. The free cells released a significant amount of surfactant at 48 h of biodegradation whereas the entrapped cells did not produce surfactant within the same time; nevertheless, for both types of cells, the maximum emulsification index was achieved at 72 h. The emulsification index of free cells decreased after 72 h by almost 50%, when the culture reaches the decline phase of growth, whereas the emulsification activity of entrapped cells remained unchanged until the end of experiment (96 h).

Most extracellular surface-active compounds are synthesized by microorganisms growing on poorly soluble substrates, especially *n*-alkanes (Hommel, 1990). Biosurfactant production is often associated with the capacity of microorganisms to utilize hydrocarbons as substrates (Oberbremer and Müller-Hurtig, 1989). According to Naitali-Bouchez et al. (1999) bacteria use *n*-hexadecane generally via two pathways. The first one involves the direct contact between the cells and non-emulsified drops of the hydrocarbon. The second combines the previous emulsification and solubilization of hydrocarbon through surfactant released by the cell. The pathway mediated by biosurfactants is prevalent among microorganisms that degrade *n*-hexadecane (Puntus et al., 2005), as observed with LAMI008.

Our results showed the advantages of using *B. subtilis* LAMI008 spores rather than its vegetative cells entrapped in chitosan beads for *n*-hexadecane biodegradation as well as for biosurfactant production and recovery. Although in this study this approach was tested only for bioremediation of water contaminated with *n*-hexadecane its application is not necessarily limited to this situation. This technology can be designed to entrap different species of bacterial spores providing a multi-use catalyst when the process requires sequential degradation of complex compounds and used

Table 2
Data of *n*-hexadecane biodegradation by free and immobilized cells of *B. subtilis* LAMI008 in chitosan beads.

	Time (h)	Colony-forming unit (CFU)/mL	pH	*n*-Hexadecane biodegradation (%)	Emulsification index (%) of supernatant
Free cells	0	$2.1 \times 10^7 \pm 2.7$	7.00 ± 0.02	0	0
	24	$3.5 \times 10^7 \pm 1.2$	6.96 ± 0.02	28.50	0
	48	$6.4 \times 10^8 \pm 2.7$	6.86 ± 0.04	98.74	34.0
	72	$5.2 \times 10^8 \pm 1.8$	6.73 ± 0.03	99.30	54.2
	96	$3.1 \times 10^8 \pm 2.3$	6.92 ± 0.03	99.97	22.3
		CFU/g of chitosan beads			
Immobilized cells	0	$2.3 \times 10^7 \pm 1.0$	7.00 ± 0.02	0	0
	24	$3.8 \times 10^7 \pm 0.9$	6.95 ± 0.01	18.00	0
	48	$6.0 \times 10^7 \pm 1.3$	6.96 ± 0.02	99.51	0
	72	$2.4 \times 10^9 \pm 1.8$	6.90 ± 0.03	100	53.1
	96	$7.5 \times 10^9 \pm 1.2$	6.87 ± 0.02	100	54.8

for *in situ* bioaugmentation (addition of microbial inocula). Besides, chitosan is readily degradable in marine, estuarine, soil and sand environments (Richmond et al., 2001; Setti et al., 1999) without negative biological impacts to the ecosystems.

4. Conclusion

The overall data of this work showed the potential to develop products based on bacterial spores entrapped in chitosan beads as an innovative approach for hydrocarbon biodegradation. Besides preserving the cells for long storage periods, the entrapment of suitable bacterium spores inside chitosan beads can be easily activated and still managed to degrade hydrocarbons and to produce surfactants. This technology could be an effective carrier of contaminant-degrading bacteria in environmental bioremediation.

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