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Bioremediation of crude oil polluted seawater by a hydrocarbon-degrading bacterial strain immobilized on chitin and chitosan flakes

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Abstract

In this laboratory-scale study, we examined the potential of chitin and chitosan flakes obtained from shrimp wastes as carrier material for a hydrocarbon-degrading bacterial strain. Flakes decontamination, immobilization conditions and the survival of the immobilized bacterial strain under different storage temperatures were evaluated. The potential of immobilized hydrocarbon-degrading bacterial strain for crude oil polluted seawater bioremediation was tested in seawater microcosms. In terms of removal percentage of crude oil after 15 days, the microcosms treated with the immobilized inoculants proved to be the most successful. The inoculants formulated with chitin and chitosan as carrier materials improved the survival and the activity of the immobilized strain. It is important to emphasize that the inoculants formulated with chitin showed the best performance during storage and seawater bioremediation.
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Keywords: Bioaugmentation; Hydrocarbon-degrading bacterial strain; Chitin; Chitosan; Immobilization; Bioremediation

1. Introduction

Petroleum hydrocarbons are major pollutants of marine environments as a result of terrestrial and freshwater run-off, refuse from coastal oil refineries, offshore oil production, shipping activities and accidental spills. Although evaporation and photo-oxidation play an important role in crude oil detoxification, ultimate and complete degradation is accomplished mainly by microorganisms (Atlas, 1981; Oudot, 1984; Cerniglia, 1984; Bartha, 1986; Yakimov et al., 1998).

A popular option to favor the clean-up of hydrocarbons polluted environments has involved biostimulation. However, for more recalcitrants compounds or if the biodegradable pollutant is introduced to the environment at high concentrations (e.g. spills) and a rapid detoxification of the chemical is required, it may not be appropriate to rely on the natural response of members of the native microbial community. For example, a slow biodegradation in an accidental oil spill in coastal seawater may result in the movement of spilled crude oil to other coastal sites and probably its accumulation in the sediments, so the possibility of undesirable effects on the ecosystem is increased (Bartha, 1986; Alexander, 1999). Bioaugmentation to enhance natural biodegradation is a useful alternative (Vogel, 1996; Jansson et al., 2000; Cunningham et al., 2004).

Bioaugmentation has met with varying degrees of success (Crawford and Mohn, 1985; Brodkorb and Legge, 1992; Leavitt and Brown, 1994; Vogel, 1996; Atlas and Bartha, 1992) and there has been a considerable debate over the efficacy of this methodology. The fact is that the use of selected bacterial strains, with broad substrate range and high metabolic rates, frequently has failed in natural environments. The selected microorganisms that have beneficial traits for biodegradation must also be able to overcome biotic and abiotic stresses in the environment in which they are introduced (Alexander, 1999). Macnaughton et al. (1999) have demonstrated the early disappearance of the components of a microbial consortia introduced in a natural environment polluted with hydrocarbons. Hence, the maintenance of sufficient activity of an inoculant population over a prolonged period after release, often

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represents the main hurdle in the successful use of inoculants in bioremediation (Sanjeet et al., 2001).

The use of inoculant formulations involving carrier materials for the delivery of microbial cells to natural ecosystems, is an attractive option. Carrier materials are generally intended to provide protective niche to microbial inoculants, either physically, via the provision of a protective surface or pore space, or nutritionally, via the provision of a specific substrate. An optimal carrier should provide favorable conditions for survival as well as functioning of the inoculant cells, resulting in a sufficiently long shelf life as well as improved survival and activity (van Veen et al., 1997). The carrier should, furthermore, be nontoxic, nonpolluting, have a constant quality and be locally available at low price (Ronchi and Ballatti, 1996; Leenen et al., 1996). In the bioremediation of naturals environments the destruction of the carrier material once the introduced microorganisms have carried out their objective is desirable, so the materials should be biodegradable (Pometto et al., 1998). A wide range of carriers prepared from natural materials, e.g. peat, clay and plant-derived compounds, have been tested and used specially in Rhizobium inoculants industry, but a little information has been reported about the development of inoculants with biodegradable carrier materials for seawater bioremediation.

Chitin (1–4 linked 2-acetamido-2-deoxy-β-D-glucan) is the main component in the cuticles of crustaceans, insects, and mollusks and in the cell walls of fungi, is the second most abundant polysaccharide found on earth next to cellulose (Muzzarelli, 1973). The exoskeletons of shrimps have long since attracted attention as a source of raw material for chitin production as the dry arthropod exoskeletons contain from 20% to 50% chitin (natural chelating polymers). This biopolymer is produced commercially from crab and shrimp exoeskeletons by treatment with dilute NaOH solution for deproteinization, followed by treatment with dilute HCL solution for demineralization. Chitosan, the deacetylated derivative of chitin, as a natural renewable resource, has numerous applications, which attract scientific and industrial interests (Li et al., 1997).

Bahía Blanca is a port city located at south of Buenos Aires province (Argentina). It has an important crude oil refinery and port facilities, destined to charge and discharge crude oil and its by-products. These activities generate hydrocarbon rich wastes and sometimes, accidental oil spills in seawater. Several ports are located at the Estuary of Bahía Blanca, which is a complex system of tidal channels, islands and extended tidal flats. The last ones delimit the main channel of the estuary, which constitute the access to one of the most important harbor complexes of Argentina.

Since the exoskeletons of shrimps are an abundant residue of the local fishing industry, chitin and chitosan obtaining technology is available in our university, both materials are nontoxic, nonpolluting and biodegradable, the flakes of chitin and chitosan could be considered an alternative carrier material for immobilizing microorganisms for bioremediation purposes. Therefore, the first aim of the present study was to formulate inoculants with chitin and chitosan flakes as carrier of a hydrocarbon-degrading bacterial strain isolated from Bahía Blanca coastal soils. The second aim was to evaluate the potential of the strain immobilized on chitin and chitosan flakes for the clean-up of crude oil-contaminated seawater.

2. Materials and methods

2.1. Obtaining of chitin and chitosan flakes

All reagents were of either analytical grade or the highest purity grade available. Chitin was obtained from shrimp (*Pleoticus mülleri*) wastes at the Laboratorio de Investigaciones Básicas y Aplicadas en Quitina (LIBAQ). Raw material was homogenized and triturated in an industrial triturator (Westinghouse model DASO6). The product was rinsed with water at room temperature in order to remove organic materials. The cleaned residue was treated with 9% (w/w) NaOH at 65 °C for 90 min to remove proteins, then demineralized by treatment with 10% (v/v) HCl at 20 °C for 15 min. Finally the obtained chitin was washed with water at room temperature, and then air-dried. Chitosan was obtained from chitin by heterogeneous deacetylation at 136 °C with 50% (w/w) NaOH for 1 h. The characteristics of chitosan used in this study were: deacetylation degree: 95, moisture: 6.0%, ash content: 0.55%.

2.2. Culture media

The culture media used in this study were: (a) SW: minimal salt medium of Winogradsky (Pochon, 1962), amended with 0.1% (w/v) NH_4NO_3 and 0.1% (w/v) KH_2PO_4, pH: 7.2; (b) ASW: SW with 1.2% (w/v) ultra pure agar–agar; (c) Locke solution: 1.5% (w/v) NaCl, 0.04% (w/v) $MgCl_2$, 0.01% (w/v) KCl, pH: 7 (Verna, 1945). All the media and solutions were prepared with distilled water and autoclaved at 1 atm for 15 min.

2.3. Decontamination treatments

To determine a suitable method for reducing the microbial load of the carrier material, 1 g of chitin and chitosan flakes were put into respective glass Petri dishes and treated with wet heat in a Chamberland autoclave under different conditions: (a) fluent steam for 15 min, (b) fluent steam for 30 min, (c) pressure saturated steam at 1 atm for 15 min and (d) pressure saturated steam at 1 atm for 30 min. The number of total viable heterotrophic aerobic bacteria was determined before and after each treatment. Samples of 0.1 g of flakes were taken out from each Petri dish. The flakes were suspended in 9.9 mL of sterile Locke solution. The flakes suspensions were disintegrated with a mixer at 10,000 rpm for 1 min. Successive decimal dilutions were prepared in Locke solution from the supernatant of the obtained suspension. From the dilutions, 0.1 mL portions were spread on Plate Count Agar (PCA). The plates were incubated at 28 °C for 5 days. At the end of incubation the colonies were counted and the results expressed as CFU per gram of dry chitin or chitosan.

2.4. Dry weight

Viable counts were referred to the dry weight of flakes. From each sample a portion of chitin and chitosan flakes were dried at 105 °C overnight in aluminum boxes until constant weight. The flakes were weighed on a precision balance (AND ER-180 A) and water content of the flakes was calculated.

2.5. Microorganisms

The hydrocarbon-degrading bacterial strain (QBTo) used in this study was isolated in a previous research (results not shown), from coastal soils of Bahía Blanca Estuary influenced by port and petrochemical industry activities. This strain has been characterized by 16S rRNA gene sequence and proposed as *Rhodococcus corynebacterioides* (Barengo et al., 2002; NCBI GenBank accession number AY157677).

2.6. Production of inoculants

To immobilize the strain QBTo on the carrier material, the cells were cultured together with chitin and chitosan flakes in WS with kerosene as the sole source of carbon and energy. Previously, to determine the minimal suitable concentration of kerosene and the incubation time that could improve the strain QBTo attachment and the biofilm formation on the flakes, combination of three different kerosene concentration and three incubation times were tried. Eighteen 250 mL sterile erlenmeyers flasks containing 100 mL of SW were prepared. Nine flasks were added with 0.25 g (w/v) of chitin flakes and nine with 0.25 g (w/v) of chitosan flakes. The flakes had been autoclaved at 1 atm for 15 min. 0.5% (v/v) of kerosene were added to three flasks of each group, 0.37% (v/v) to the other three and 0.25% (v/v) to the last three ones. The kerosene had been autoclaved at 1.5 atm for 20 min. All flasks were inoculated with 1 mL (10^8 CFU mL^{-1}) of 72 h strain QBTo culture in SW with crude oil as sole source of carbon and energy, incubated at 28 °C. The growth conditions of the strain were established in a previous study (Gentili and Cubitto, 2004). All flasks were incubated at 28 °C and 150 rpm in a rotary shaker. At 72, 96 and 120 h of incubation, samples of chitin and chitosan flakes were taken from the flasks. Each sample was divided into three 0.01 g portions, which were assigned to scanning electron microscopy (SEM), viable count and dry weight determinations, respectively. For SEM, the flakes were suspended in 10 mL of Locke solution and stirred at 500 rpm during 1 min in order to wash the non-attached cells. Washed flakes were taken with sterile forceps and placed in fixative (buffer phosphate, pH 7.2 and 1% v/v glutaraldehyde) overnight. The flakes were prepared as was described by Lozano (1990) and finally were observed in scanning electron microscope (JEOL 35).

To assess the number of attached viable bacteria, 0.01 g of flakes sample were washed as was indicated above and then suspended in 9.99 mL of Locke solution. The flakes in suspension were disintegrated with a mixer at 10,000 rpm for 1 min, successive decimal dilution were made from the supernatant and 0.1 mL from dilutions were spread on ASW. Filter papers saturated with crude oil were placed in the covers of Petri dishes to supply the carbon and energy source without affecting the isolation of the colonies (Robertson et al., 1973). The plates were incubated at 28 °C for 5 days.

Once the more suitable conditions for the formation of an abundant biofilm on the flakes were established, the inoculant was produced in a larger scale in a bioreactor with two glass tanks (New Brunswick Scientific Co., USA) with a working volume of 3 L in each tank. SW medium with 0.25% (v/v) of kerosene as the sole source of carbon and energy was used in the bioreactor. A 72 h QBTo culture in SW with 0.5% (v/v) crude oil was used as inoculum and 7.5 g of chitin or chitosan flakes were added to each tank. The conditions in the bioreactor were as follows: temperature, 28 °C; aeration, 0.60 volume of air/volume of medium/min; agitation, 150 rpm; incubation time, 5 days. After this time, the flakes were filtered aseptically through a sterile sieve, put on sterile metal trays and covered with sterile gauzes. The carrier-based culture was air dried at 30 °C for 24 h and then distributed into sterile polyethylene bags in portions of 2.5 g in each bag.

2.7. Storage conditions and viability assessment

Polyethylene bags with 2.5 g of inoculants obtained as was described above, were stored at three different temperatures: (a) room temperature (25 °C $\pm$ 2), (b) 4 °C, and (c) 20 °C. Each condition was tested by triplicate. Periodically, 0.1 g flakes samples were taken from each bag to establish the number of viable cells immobilized on each carrier material. Viable counts on ASW were carried out as was described in 2.6 and the counts were referred to the flakes dry weight. To monitor the staying of the strain QBTo hydrocarbon degrading activity along the storage time, 0.01 g of inoculants from each bag were put into tubes with 5 mL of SW amended with 25 μL of crude oil, the tubes were incubated at 28 °C for 96 h. Tubes showing turbidity and visible changes in crude oil compared with controls prepared with sterile chitin and chitosan flakes were recorded as positive.

2.8. Seawater bioremediation

To evaluate the effectiveness of inoculants addition in a bioremediation process, seawater microcosms simulating a crude oil spill were prepared. Seawater was collected in sterile glass bottles from the Main Channel of Bahía Blanca Estuary (38° 44′–39° 27′ S; 61° 45′–62° 30′ W). The bottles were transported to the laboratory at 10 °C and stored at 4 °C for no more than 24 h. Twelve sterile 250 mL Erlenmeyer flasks were prepared with 150 mL of seawater and 0.75 mL of autoclaved crude oil (type: "Medanito"; origin: Neuquén, Argentina; density: 0.84), provided by a local refinery. Small volumes of crude oil were autoclaved in 10 mL tubes at 1.5 atm for 30 min. Three flasks were inoculated with 0.2 g of the QBTo inoculant formulated with chitin, three with the QBTo inoculant formulated with chitosan and three with 1 mL of 72 h QBTo culture (10^8 CFU mL^{-1}) in seawater with crude oil as sole source of carbon and energy. The inoculants were produced as was indicated in 2.7. The last three flasks were prepared without inoculum to assess the activity of the native microbial community alone (positive control). Three control flasks of the abiotic changes in the crude oil were prepared with autoclaved seawater and autoclaved crude oil (negative control). In order to obtain semi-continuous culture conditions, each flask was supplemented with a glass device, designed to replace 25 mL of seawater in the flasks with fresh seawater without affecting the crude oil concentration (Cubitto and Cabezalí, 2001). All microcosms were incubated for 15 days at 25 °C on a rotary shaker set at 100 rpm. Every 72 h, 25 mL of seawater from the flasks were replaced by 25 mL of seawater recently collected. In the case of the negative controls, the seawater was previously autoclaved.

To monitor the survival and prevalence of the introduced bacterial strain, at the end of the experiment, 1 mL water samples were taken from all flasks and decimal successive dilutions were made in Locke solution. Viable counts were made on ASW plates with crude oil, prepared as was described in Section 2.6. The plates were incubated at 28 °C for 5 days. The number of the strain QBTo characteristics colonies (smooth, red-orange) was recorded. All colonies grown on agar plate were harvested, resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]) and subjected to extraction of DNA for further DGGE analysis. DNA was extracted using BACTOZOL TM KIT Cat. No: BA 154. The extracted genomic DNA was used as target in the PCR to amplify 16S rRNA genes. Bacterial fragments suitable for subsequent denaturing gradient gel electrophoresis (DGGE) were amplified with the primer combinations 341fGC-534r as was described by Rölleke et al. (1996). We used 6% polyacrylamide gel with empirically determined gradient of DNA-denaturant by mixing two stock solutions of acrylamide containing 30% and 70% denaturant (100% denaturant is defined as 7 M urea and 40% deionized formamide). About 800 ng of PCR product was loaded for most of the samples and the gels were run at 120 V, 60 °C for 4.5 h using 1 × TAE buffer (40 mM Tris base [pH 7.4], 20 mM sodium acetate, 1 mM EDTA) in a CBS (Scientifc Co., USA). The gels were stained with the nucleic acid stain SybrGold for 45 min, rinsed with TAE buffer, removed from the glassplate to a UV transparent gel scoop and visualized with UV radiation in a GelDoc 2000 Image Analyzer with the Quantity One software (Bio-Rad).

2.9. Hydrocarbon concentration

At the end of the incubation time, all microcosms were acidified at pH 2 with sulphuric acid. The residual hydrocarbons were recovered by five

successive liquid–liquid extractions with 5 mL of *n*-hexane each time. The extraction was exhaustive including the devices and flakes. Solvent was evaporated at room temperature (25 °C ± 2) during a normalized time of 72 h to avoid variations in evaporations induced by differential volatilization of the naphtha (Oudot, 1984). The amount of residual hydrocarbons recovered was determined gravimetrically in an analytical electronic balance (AND, model ER-180 A). After gravimetric quantification, 1-eicoseno (Alltech Co., USA, 4174) was added as internal standard to the obtained extract. The extracts were suspended with 5 mL of hexane (Chromatographic quality) and resolved aliphatic hydrocarbons (RAH) were quantified by gas chromatography. The chromatograph (Shimadzu GL-14A) was equipped with a flame ionization detector (FID), split injection system and a capillary column (Shimadzu, S 50 0.50 CBP1). During analysis, the injector temperature was set at 170 °C, the detector temperature at 310 °C and the oven temperature was programmed to rise from 100 °C (3 min) to 300 °C (10 min) in 3 °C min^{-1} increments (Oudot, 1984, UNEP, 1992). The RAH fraction was analyzed for individual *n*-alkanes, pristine, and phytane isoprenoids.

3. Results

The results obtained from the treatments to reduce the microbial load of chitin and chitosan flakes are shown in Table 1. The fluent steam produced a 99.9% reduction in the viable counts and the pressure saturated steam caused a reduction greater than the 99.99%. No visible modification in the flakes was observed after any treatments.

During the production of the inoculant, neither kerosene concentration nor incubation time produced significant differences ($p < 0.05$) in the viable counts obtained from the flakes incubated with *R. corynebacteriorides* strain QBTo. Despite the viable counts results, SEM showed that flakes incubated with 0.37% and 0.50% (v/v) kerosene formed more compact bacterial aggregates than those incubated with 0.25% during 72 h. The flakes incubated with 0.25% (v/v) of kerosene did not show the compact cell aggregates until 120 h of incubation.

The images obtained from the culture with 0.25% (v/v) kerosene after 5 days of incubation (Fig. 1), decided us to choose these culture conditions that involved a lower concentration of kerosene and allowed a suitable colonization on the flakes surfaces. A low concentration of kerosene residues in the inoculant is desirable for bioremediation purposes.

Figs. 2 and 3 show the survival of strain QTBo cells immobilized on chitin and chitosan flakes during the storage at three different temperatures. All the inoculants maintained their initial viable counts for 45 days at the three temperatures tested. At 90 days, a decrease about 2 orders of magnitude was detected in the inoculants stored at room temperature. The inoculants stored at 4 °C and −20 °C showed a decrease only about one order of magnitude and yielded stable CFU counts (about 10^8 CFU g^{-1} of carrier material) for a period of 135 days. After 6 months of storage, chitosan flakes yielded counts about 10^8 CFU g^{-1} only at −20 °C (Fig. 5). Chitin flakes maintained the QBTo viable count about 10^8 CFU g^{-1} at 4 °C and at −20 °C (Fig. 2). The strain QBTo immobilized on the both materials preserved its crude oil—degrading capacity at the three storage temperatures tested.

Table 1
Decontamination treatments applied to chitin and chitosan flakes

Treatment	Chitin flakes (CFU g^{-1})	Chitosan flakes (CFU g^{-1})
No treated	6.6×10	5.0×10^7
Fluent steam, 15 min	1.3×10^3	7.0×10^2
Fluent steam, 30 min	2.5×10^2	1.0×10^2
Pressure-saturated steam 1 atm, 15 min	50	20
Pressure-saturated steam at 1 atm, 30 min	< 10[a]	< 10[a]

The viable counts are expressed as CFU per gram of chitin/chitosan dry weight.
[a]Below detection level.

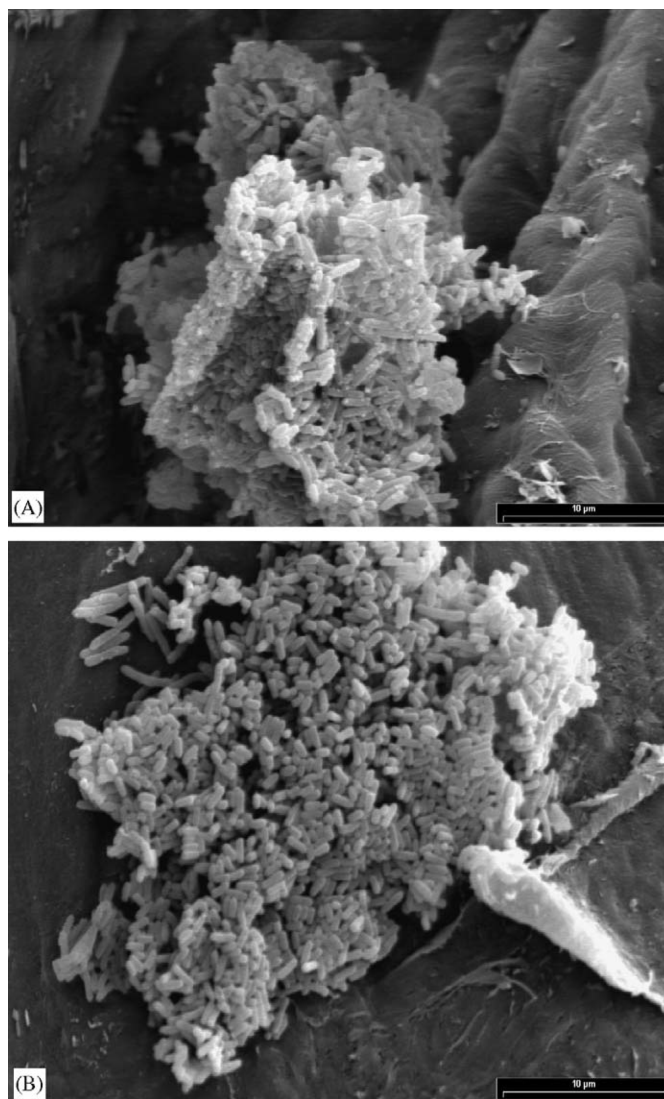

Fig. 1. SEM showing the *Rhodococcus corynebacterioides* QBTo biofilm on flakes incubated with 0.25% kerosene (v/v) for 120 h: (A) chitin; (B) chitosan. Magnification 3000 ×. Bars = 10 µm.

In the seawater bioremediation trials, the highest biodegradation rates were obtained in the microcosms inoculated with *R. corynebacteriorides* QBTo immobilized

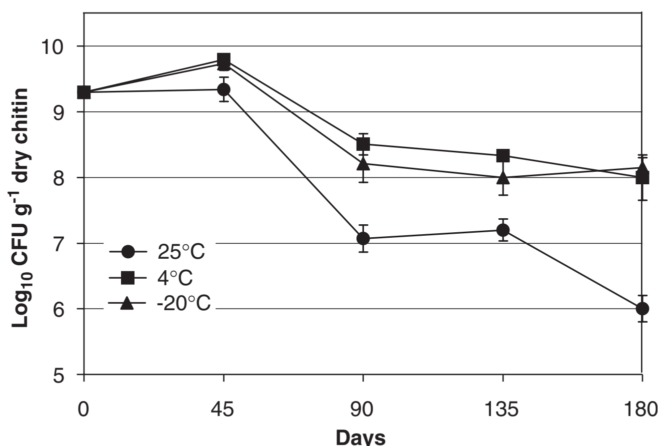

Fig. 2. Viable counts obtained from the inoculant formulated with chitin flakes at three storage temperatures along time. Values are arithmetic means for three bags per temperature. Bars represent standard deviations.

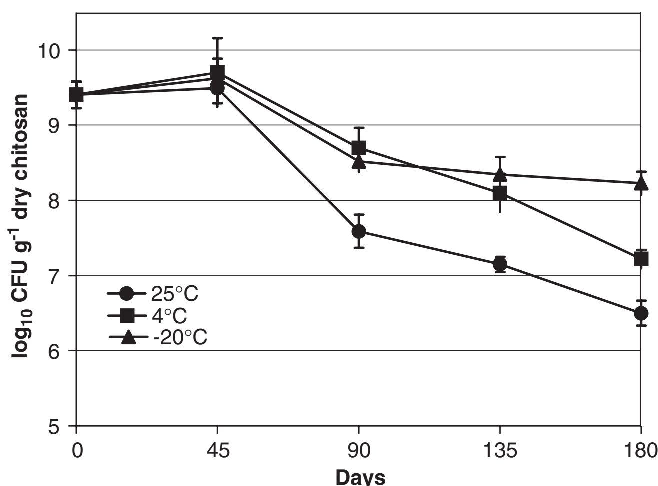

Fig. 3. *Rhodococcus corynebacterioides* QBTo viable counts obtained from the inoculant formulated with chitosan at three storage temperatures along time. Values are arithmetic means for three bags per temperature. Bars represent standard deviations.

onto chitin and chitosan flakes as carrier materials. In these microcosms, 60% of hydrocarbons in the hexanic extract were removed compared with controls (Fig. 4). In the seawater microcosms where the strain was inoculated without carrier, only a decrease about 30% of hexanic extract was obtained. The gas liquid chromatography of the hexanic extracts showed a higher degrading activity on RAH fraction in the microcosms where the inoculant formulated with the flakes, were applied (Fig. 5). The inoculants formulated with chitin showed the highest activity on this fraction. Changes in pristane and phytane isoprenoids concentrations were not observed in any microcosms during the experiment, probably because they are more resistant to bacterial degradation.

R. corynebacteriorides QBTo only was recovered from the microcosms seeded with the carrier-based inocula. DGGE analyses confirmed the presence of the inoculated strain.

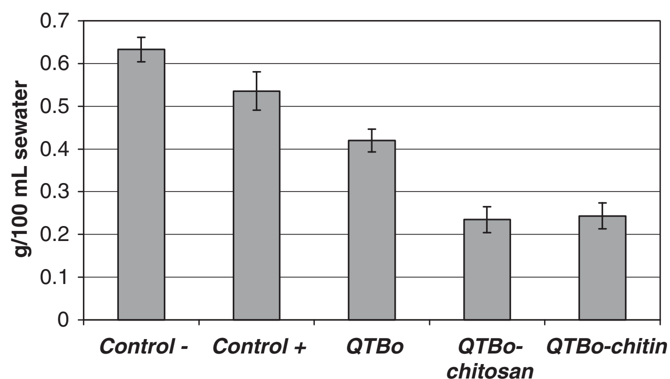

Fig. 4. Gravimetric determination of residual crude oil extracted with hexane from seawater microcosms after 15 days of incubation. Contol −: autoclaved seawater; Control +: seawater without inoculant; QTBo: seawater inoculated with the strain QTBo without carrier material; QTBO-chitin: seawater inoculated with the inoculant formulated with chitin flakes; QTBo-chitosan: seawater inoculated with the inoculant formulated with chitosan flakes. Values are arithmetic means for three microcosms per treatment. Bars represent standard deviations.

4. Discussion

The chitin and chitosan flakes are natural products obtained from an abundant waste of the fish industry of Bahía Blanca region. They are nontoxic, nonpollutant, have defined nature, and a constant quality can be obtained.

The flakes can be efficiently decontaminated by fluent steam and pressure saturated steam, which are common and available technologies in the industry.

The *R. corynebacteriorides* QBTo was cultured with the carrier materials in presence of kerosene as sole source of carbon and energy to favor the growth of the population on the flakes surfaces and the subsequent formation of a biofilm. We have previously observed that kerosene was rapidly adsorbed on the flakes surfaces. This situation favored the growth of the population on the flakes surfaces and inside its "pores" and "crevices". It is well known that in these surface-attached population bacteria are protected from environmental stresses and predation. Biofilm formation therefore emerges as an important process for microbial survival in the environment (Davey and O'Toole, 2000). On the other hand, the presence of hydrocarbons in the production culture medium could help to maintain a selective pressure and the hydrocarbon-degrading activity of the cells to be applied in a bioremediation process.

The differences observed between the viable counts and the SEM seem reasonable probably because many cells strongly attached could not be recovered from the supernatant of the flakes suspension.

A high number of strain QBTo cells survived immobilized on both carrier materials for 6 months. The storage at −20 °C was the most suitable storage temperature for the inoculants formulated with chitosan as carrier material. However, both 4 °C and −20 °C appeared as suitable for the inoculants formulated with chitin flakes.

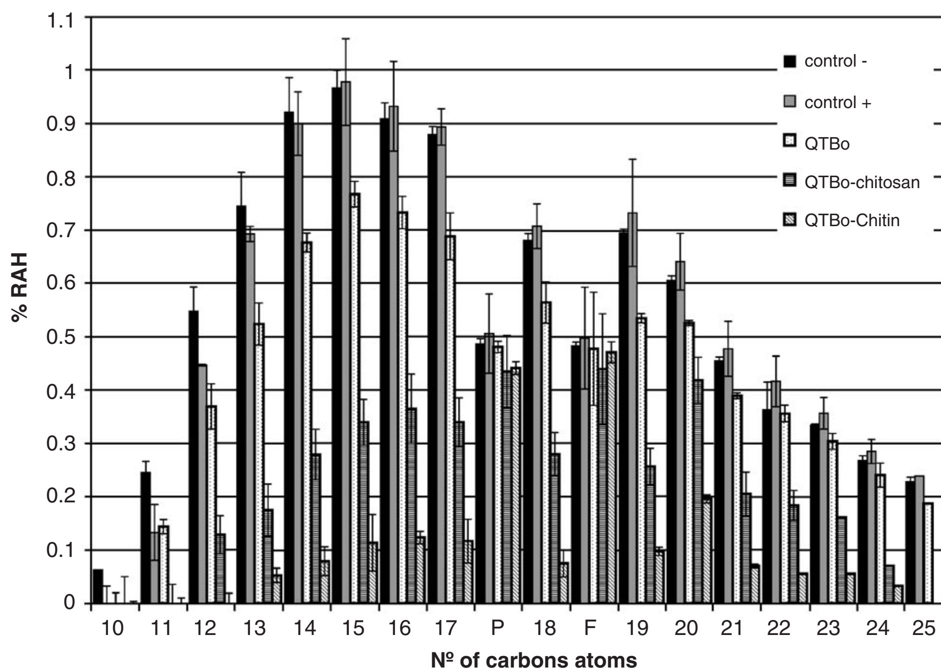

Fig. 5. Percentage of resolved aliphatic hydrocarbons (RAH) after 15 days of incubation. Contol −: autoclaved seawater; Control +: seawater without inoculant; QTBo: seawater inoculated with the strain QTBo without carrier material; QTBO-chitin: seawater with the inoculant formulated with chitin flakes; QTBo-chitosan: seawater with the inoculant formulated with chitosan flakes. Values are arithmetic means for three microcosms per treatment. Bars represent standard deviations.

The bioremediation experience showed that the native population in the positive control microcosms could not produce any significant reduction in the pollutants hydrocarbons concentrations. In the microcosms where the strain QBTo was inoculated without carrier, the low hydrocarbon removal would not justify the bioaugmentation. This result probably was due to the short survival of the strain in seawater, although it was originally isolated from Bahía Blanca Estuary coastal environments and that at the beginning of the experience, it outnumbered the native microbial community. The bioaugmentation with the strain immobilized on chitin and chitosan flakes enhanced significantly the crude oil biodegradation. We consider that the principal reason for this result was the strain survival due to protective effect of the carrier material and the biofilm structure that the cells have developed on it.

From the results obtained in this laboratory scale study, we did not find differences between chitin and chitosan flakes about their decontamination, the inoculants production and the inoculant shelf-life. However, the inoculants formulated with chitin yielded the highest number of cell survival at 4 °C during 6 months of storage and showed a higher degrading activity on RAH. One reason could be the crystalline structure of chitin that would provide more protective microhabitats to microbial inoculant.

Chitin production is cheaper than chitosan and since it is an abundant polysaccharide found in nature, there are several organisms that contain chitin-degrading enzymes in marine environments. These characteristics indicated the inoculant formulated with this carrier material as environmentally friendly, avoiding the potential problems that generate the synthetic supports materials (Leenen et al., 1996).

These results indicated that seawater represented a hostile environment to introduced microorganisms. The success of the application of a microbial inoculant depends to a large extent on how favorable to its survival the target environment is or can be made. In the present study a crude oil degrading bacterial strain was applied in form of carrier-based-inocula. The carrier material used, chitin and chitosan flaskes and the inoculant production method applied, allowed the development of a biofilm, providing a protective niche to the bacterial strain and resulting in a long shelf life and in a high crude oil degrading activity in natural seawater.

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