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Innovative, ecofriendly biosorbent-biodegrading biofilms for bioremediation of oil- contaminated water

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ABSTRACT

Immobilization of microorganisms capable of degrading specific contaminants significantly promotes bioremediation processes. In this study, innovative and ecofriendly biosorbent-biodegrading biofilms have been developed in order to remediate oil-contaminated water. This was achieved by immobilizing hydrocarbon-degrading gammaproteobacteria and actinobacteria on biodegradable oil-adsorbing carriers, based on polylactic acid and polycaprolactone electrospun membranes. High capacities for adhesion and proliferation of bacterial cells were observed by scanning electron microscopy. The bioremediation efficiency of the systems, tested on crude oil and quantified by gas chromatography, showed that immobilization increased hydrocarbon biodegradation by up to 23 % compared with free living bacteria. The resulting biosorbent biodegrading biofilms simultaneously adsorbed 100 % of spilled oil and biodegraded more than 66 % over 10 days, with limited environmental dispersion of cells. Biofilm-mediated bioremediation, using eco-friendly supports, is a low-cost, low-impact, versatile tool for bioremediation of aquatic systems.

Introduction

Petroleum and its derivatives are among the most serious environmental threat for the oceans [1]. New mitigation measures are urgently needed for the remediation of marine contaminated areas and various physical, chemical and biological methods have been proposed [2]. Bioremediation represents a promising, non-invasive and low cost technology that could provide a more effective and sustainable restoration of contaminated water and sediments [3,4]. Bioremediation exploits the ability of microorganisms to degrade and metabolize different environmental pollutants by assimilating organic molecules into cell biomass and converting them to other products such as carbon dioxide and water [4]. Biostimulation and bioaugmentation are the approaches most often applied. Biostimulation uses nutrients to stimulate the growth of autochthonous hydrocarbon (HC)-degrading microorganisms, while bioaugmentation introduces more efficient allochthonous microorganisms to improve biodegradation at a certain site. HC-degrading bacteria isolated from water, sediment and soil with high biodegradation capacities have been characterized [5–9]. In soil, actinobacteria, well known degraders of recalcitrant biomolecules, are generally abundant [10]; they are resistant to drought and good colonizers of organic and inorganic surfaces [11]. In marine environments, the most actively oil-degrading microorganisms are hydrocarbonoclastic bacteria that live almost exclusively on HC [12].

Environmental microbiological resources can be exploited into biotechnological tools to treat pollution caused by oil HC and its derivatives; an example is the immobilization of HC-degrading bacteria, on different supports, that has been used for the bioremediation of environmental pollutants [13–15]. Immobilization of bacteria on carriers preserves their viability and catalytic functions, as well as providing resistance to unfavorable environmental conditions and high concentrations of pollutants, while displaying a longer half-life [15]. Immobilization processes reduce bioremediation costs and eliminate dispersion and dilution of cells in the environment [14].

Abbreviations: HC, hydrocarbon; PLA, polylactic acid; PCL, polycaprolactone; TCM, chloroform; Ac, acetone; DCM, dichloromethane; LB, Luria-Bertani broth; BHSM, Bushnell Haas mineral salts medium; SEM, scanning electron microscopy; GC-FID, Gas Chromatography Flame Ionization Detection; TERHCs, total extracted resolved hydrocarbons; CMN, corynebacterium/mycobacterium/nocardia

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Oil removal by adsorption is a widely used approach, with polyethylene being the most used polymer sorbent [16], although inorganic materials, such as titania [17], have also been proposed. A superhydrophobic polyethylene-based shish-kebab membrane has been prepared with self-cleaning and oil/water separation properties [16]. Its membrane adsorption capacity ranged from 15 to 32 g/g, depending on viscosity and density of the organic liquids. Porous polyethylene bundles have been proposed with enhanced hydrophobicity and pumping oil-recovery ability via skin-peeling, able to adsorb up to 3 g/g of oil [18]. These products are quite efficient, but one of the main disadvantages is their non-biodegradability.

For microbial approaches, the creation of a strong and reliable biofilm-based remediation technology remains challenging. The carrier material should be biodegradable, insoluble, non-toxic for the immobilized cells and environment, easily accessible, low cost, available in large quantities, stable and suitable for regeneration.

Recently, the properties of biodegradable polymers from either natural or synthetic sources have aroused great interest by finding applications in various technologically advanced fields [19–21]. The success of synthetic biopolymers such as polylactic acid (PLA), and polycaprolactone (PCL) is due to diverse characteristics, including the relative ease of production and low costs. For instance, a super light 3D hierarchical nanocellulose aerogel foam was prepared with low density (1.50 mg/cm^3) and high adsorption capacity (145.2–206.8 g/g for different oils) [22].

Combining technological properties of ecofriendly sorbents with their capacity to host a biodegrading biofilm, allows biodegrading biosorbent biofilms to be obtained which can remove oil from water with high efficiency and economically. Among the different approaches proposed to produce porous biopolymeric structures, electrospinning is one of the most investigated, enabling the production of fibers with diameters potentially ranging from nano- to micro-scale [23,24]. Compared to other porous structures, electrospun nanofiber mats show a higher specific surface area and greater porosity [24,25]. Thus, a wide range of electrospun polymers has been extensively studied for application in oil spill remediation [26] or removal of toxic metal ions from wastewater [27], as well as in the biomedical [23,28–31], catalysis [32] and electronic [33] fields. Here, the bioremediation efficiency of membrane-bacterial systems was analyzed on crude oil using four high performance HC-degrading bacterial strains isolated from different environments: two marine hydrocarbonoclastic gammaproteobacteria *Alcanivorax borkumensis* SK2 [34] and *Oleibacter marinus* [5], and two soil, long-chain n-alkane degrading actinobacteria, *Gordonia* sp. SoCg [35] and *Nocardia* sp. SoB [9]. The crude oil degrading ability of these formulations was measured and compared with that of the bacterial cells in planktonic form.

Materials and methods

Development of the biodegradable membranes

Commercial grade of amorphous PLA (PLA 2002D, Natureworks® LLC, Minnetonka, MN 55345, USA,) was used. PCL (Mw = 80 kDa), chloroform (TCM), acetone (Ac), dichloromethane (DCM) and absolute ethanol (EtOH) were from Sigma-Aldrich (Milan, Italy). All solvents were ACS grade (purity > 99 %) and were used as received without further purification. Double distilled water (DDQW) was obtained from MilliQ Plus systems (Millipore, Germany). The polymeric solutions and the electrospun membranes were prepared as previously described [24,30]. In brief, PLA or PCL solution was fed in a 10 mL glass syringe fitted with a 19-gauge stainless steel needle. A conventional electrospinning equipment (Linari Engineering-Biomedical Division, Pisa, Italy) was used to prepare the nanofiber mats working at the following constant parameters: distance between the needle tips and the collector, 15 cm; supplied high voltage, +15 kV.

Sterilization of PCL and PLA membranes

PCL and PLA nanofiber membranes were cut into sections of 15 mm × 10 mm. The membranes were treated with 70 % ethanol for 30 min and washed three times with sterile distilled water. The residual ethanol was dried overnight at 25 ± 1 °C in Petri dishes. The membranes were further treated by germicidal UV-C light (wavelength 253.7 nm) for 30 min. To check sterility, the membranes were placed in tubes containing 3 mL of Luria-Bertani broth (LB) (Laboratorios Conda, S.A, Pronadisa Madrid, Spain) and incubated at 30 ± 1 °C with shaking (200 rpm) for 7 d. One ml of LB broth and PCL/PLA nanofiber membranes were transferred onto agar plate LB medium and incubated at 30 ± 1 °C to observe microbial growth.

Microorganisms and culture conditions

Four highly efficient oil degrading bacterial strains were selected from existing collections for development of bioremediation devices: two marine gammaproteobacteria, *Alcanivorax borkumensis* SK2 [34] and *Oleibacter marinus* 5 [5], and two soil actinobacteria, *Nocardia* sp. SoB and *Gordonia* sp. SoCg [9] which are able to degrade medium and long-chain *n*-alkanes up to C_{36} [10,35]. Marine and soil HC-degrading bacteria were cultivated in mineral medium ONR7a [36] and Bushnell Haas Mineral Salts Medium (BHSM) (Difco, Milan, Italy) respectively, containing 0.1 % of crude oil (v/v) as unique carbon source. Plate cultures were prepared on agar mineral medium, with crude oil (1% Arabian Light Crude Oil, ENI S.p.A.) supplied on a filter paper on the lid of the Petri dish. The cultures were incubated at 30 ± 1 °C until visible growth.

Immobilization of bacterial cells

Bacteria were cultivated in 3 mL mineral medium containing PLA or PCL nanofiber membrane (15 mm × 10 mm) previously soaked in 0.1 % of crude oil (v/v). The cultures were incubated for 48, 120 and 240 h at 30 ± 1 °C with shaking (200 rpm). Abiotic controls (without bacterial inoculation) were prepared under the same conditions. Immobilization by adhesion of bacterial cells on PCL or PLA membranes at the three different times was confirmed by scanning electron microscopy (SEM) by preventive processing of the samples as described in [25]. To evaluate the viability of cells immobilized on PCL or PLA at the three times, PLA and PCL membranes were suspended in 5 mL of mineral medium. The suspension was agitated vigorously using a vortex mixer for 3 min to dislodge the immobilized cells and aliquots of 0.1 mL were spread on mineral medium plates prepared as described above and incubated until colonies appeared.

Petroleum degradation in microcosm

Set-up of microcosms

A. borkumensis sp. SK2, *Oleibacter marinus* sp. 5, *Gordonia* sp. SoCg and *Nocardia* sp. SoB were inoculated into 30 mL of the appropriate mineral medium (ONR7a and BHSM, for marine and terrestrial strains, respectively) containing 0.1 % (v/v) of crude oil (Arabian Light Crude Oil, ENI S.p.A.) as unique carbon source. The cultures were incubated at 30 ± 1 °C with shaking (200 rpm) for 7 d. In order to remove crude oil, cells were centrifuged at 9000 × g for 10 min, washed in mineral medium and recentrifuged. The procedure was repeated three times. Clean cells were suspended in mineral medium to an OD_{600} of 0.1. 1 mL of each bacterial suspension was then inoculated into 100 mL vials containing 30 mL of mineral medium and 0.1 % (v/v) of crude oil. One of the sterilized PLA or PCL membranes (15 mm x 10 mm) was placed into the vial and the microcosms were incubated at 30 ± 1 °C with shaking (200 rpm) for 48, 120 and 240 h. Abiotic controls and planktonic cell controls (without membranes) were prepared in order to evaluate the degradation of HC due to physico-chemical processes, and

the biodegradation by free cell cultures compared to immobilized cells. Each treatment was performed in triplicate and data were analysed by one-way ANOVA.

Gas Chromatography – Flame Ionization Detection (GC-FID) analysis

Total extracted and resolved hydrocarbons and their derivates (TERHCs) from microcosms, both in liquid medium and adsorbed onto PCL/PLA membranes, were analyzed by high resolution GC-FID using the 3510 EPA (Environmental Protection Agency) and 3550C EPA (US Environmental protection Agency) procedures respectively [37]. The samples were acidified and the extraction was performed at room temperature using 10 % (v/v) of dichloromethane or hexane as extraction solvent from liquid medium or PCL/PLA membranes respectively. The extraction procedure was performed as described in [25].

Fiber diameter analysis

Fiber diameter distribution of electrospun membranes was determined using ImageJ as image processing software [29,30,38]. The plugin DiameterJ is able to analyze an image obtained by SEM and determine the diameter of nanofibers at every pixel along a fiber axis [39].

Oil uptake analysis

As standard oily waste, a commercial 10W-40 motor oil was chosen, provided by Total S.A. The chemical composition consists of hydrocarbons with between 18 and 34 C atoms per molecule (density = 0.87 g/cm^3, kinematic viscosity = 97.7 mm^2/s at 40 °C). The adsorption capacity (q) was calculated according to Eqn. (1):

$$q\ (g/g) = \left(\frac{m_{wet} - m_{dry}}{m_{dry}} \right) \quad (1)$$

where m_{wet} and m_{dry} respectively indicate the weight of the electrospun mats before and after oil adsorption. These measurements were obtained with a precision balance (Sartorius AX224) with a resolution of ± 0.1 mg.

Results and discussion

PCL and PLA nanofiber membranes

SEM images of the electrospun PCL and PLA are presented in Fig. 1A and B respectively. The images show that the fibers are in the nanoscale for both systems and randomly oriented, even though a slight, non-significant, orientation along the hoop direction could be observed, in particular for the PLA electrospun mats. PCL fibers presented larger diameters and a broader average size distribution (average diameter ϕ: 1.71 ± 0.69 μm) compared to PLA (average diameter ϕ: 1.21 ± 0.48 μm), as quantitatively reported in Fig. 1A' and B'. In membranes, PLA fibers presented a more homogeneous diameter all over the surface compared to PCL fibers. The oil removal efficiency (q) of both types of mat were evaluated as gm of bound oil per gm of membrane as a function of its thickness (Fig. 2). The adsorption capacity of PLA electrospun membranes (q ~ 30–40 g/g) was approximately twice that of PCL (q ~ 9–20 g/g) and decreased slightly upon increasing the membrane thickness. These results can be likely explained by considering that oil adsorption capacity mainly depends on two factors: superficial adsorption and capillary forces. By increasing membrane thickness, the superficial adsorption is less intense and oil removal efficiency decreases. On the other hand, while the smaller dimensions of PLA fibers can facilitate oil adsorption by capillarity, the larger dimensions of PCL fibers can also reduce the void volume available for oil uptake. According to the literature, the removal efficiency of PLA and PCL electrospun membranes was comparable to some non-biodegradable polymers such as polyethylene-based shish-kebab membrane (q = ~ 15–32 g/g) [16] and was higher than that observed for mineral-based or other biodegradable materials investigated, such as perlite (q = 6 g/g) [40], wool (q = 16 g/g) [41], barley straw (q = 7 g/g), cotton (q = 20 g/g), kapok (q = 25 g/g) [42] and biopolymeric sponges produced by salt leaching (q = 5 g/g) [25]. The best removal efficiency was achieved by 3D hierarchical nanocellulose aerogel foam (q = 145.2–206.8 g/g) [22] and by polypyrrole-encapsulated melamine formaldehyde superhydrophobic sponge (q = ~ 60–104 g/g) [43]. For the following bioremediation tests, a membrane thickness of 100 μm was selected for ease of handling.

Bacterial immobilization on the membranes

The immobilization of oil-degrading bacteria on carriers was studied by SEM. Immobilization was performed in BHSM or ONR7a containing PLA or PCL nanofiber membranes presoaked in 0.1 % crude oil (v/v), and observed after 48, 120 and 240 h. Fig. 3A and B show that the PCL and PLA carriers were partially or completely covered by bacterial cells at various time of incubation, demonstrating the ability of both marine and soil degrading bacteria to adhere to, and colonize, the entire membrane surface.

Bacterial cells appear abundant throughout the structure after 48 h (data not shown) while after 240 h the PCL and PLA membranes surface appears almost completely covered by bacterial film. The two marine *Alcanivorax* and *Oleibacter* strains (Fig. 3A) showed a lower colonization capacity compared to the actinobacteria *Gordonia* and *Nocardia* (Fig. 3B). A complex three dimensional matrix due to a bacterial biofilm was observed for all the strains. In particular, the filamentous *Nocardia* SoB formed a well-structured biofilm, which was compact with the nanofibers completely incorporated into it after 240 h (Fig. 3B). No difference in adhesion capacity was observed between PCL and PLA membranes. Cell-surface hydrophobicity and biosurfactant production are generally recognized as the main factors regulating adhesion of microorganisms to interfaces. In the interaction of PLA and PCL nanofibers with actinobacteria, hydrophobic interactions should prevail due to highly hydrophobic mycolic acid-containing cell walls of *Gordonia* and *Nocardia* which belong to the *Corynebacterium/ Mycobacterium/ Nocardia* (CMN) complex [35].

Viability of the immobilized cells was examined after dislodging from carriers and spreading suspensions on mineral medium plates. All bacterial strains both in PCL and PLA membranes were viable and formed colonies at all stages of the experiment.

Biodegradation ability of the biosorbent biofilm

The degradation of crude oil in each microcosm inoculated with *A. borkumensis* strain SK2, *O. marinus* strain 5, *Gordonia* sp. strain SoCg, *Nocardia* sp. strain SoB, was analyzed by GC–FID after 120 and 240 h incubation and compared with the degradation capacity of free planktonic cells under the same conditions. Both free and immobilized systems demonstrated good degradative ability for petroleum hydrocarbons, with a degradation rate ranging from 30 % to 55 % for planktonic cells, which was increased from 51 % to 66 % for immobilized bacterial cells (Fig. 4). For each isolate, biodegradation in microcosms with immobilized cells was significantly higher than in microcosms with free living cells at 120 h and 140 h ($p < 0.05$, $n = 3$). Oil degradation increased during the whole incubation period confirming that the bacteria were catabolically active throughout the experiment.

Degradation by immobilized bacteria was more rapid during the first 120 h of incubation compared to degradation of microcosms without the membranes; enhancement of crude oil degradation ability between 7% and 23 % was observed in biosorbent-biofilm microcosms. The greatest enhancement in HC degradation ability, compared to the corresponding planktonic cells, was observed in *Gordonia* sp. SoCg (23 %) after 120 h immobilization on PCL carrier. The most efficient biodegrading-biosorbent biofilm was the combination of *Nocardia* sp. SoB

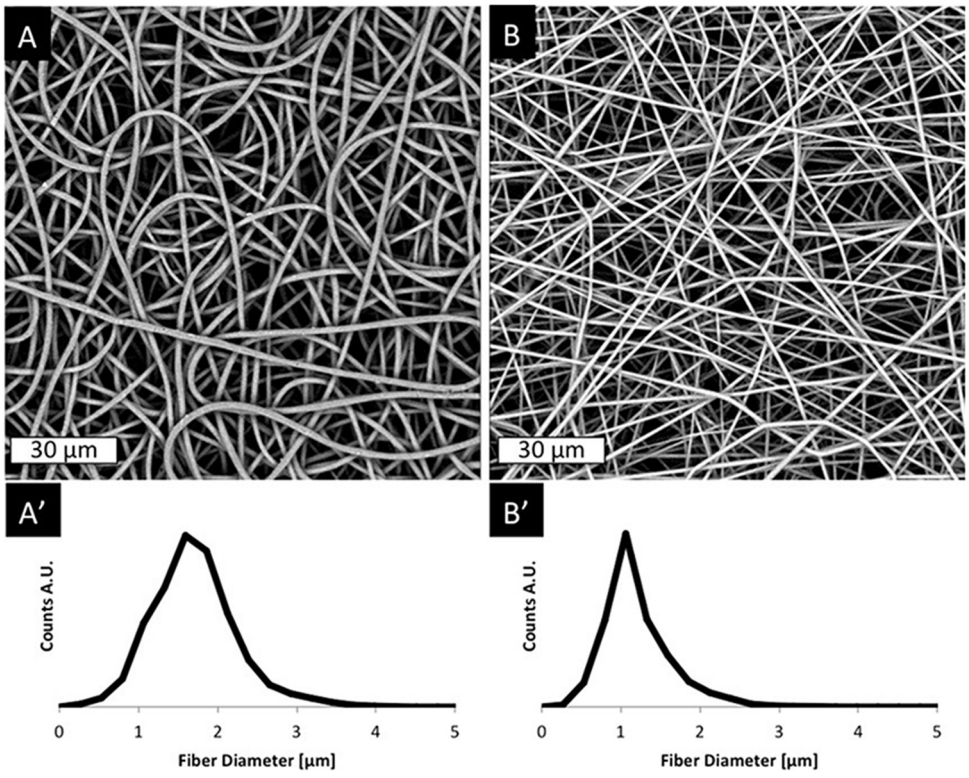

Fig. 1. SEM images of (A) PCL (10 wt% in DCM:EtOH 4:1 vol) electrospun mats and (B) PLA (10 wt% in TCM:Ac 2:1 vol) electrospun mats. (A') and (B') are the fiber diameter distribution of PCL (ϕ: 1.71 µm) and PLA (ϕ: 1.21 µm) membranes respectively.

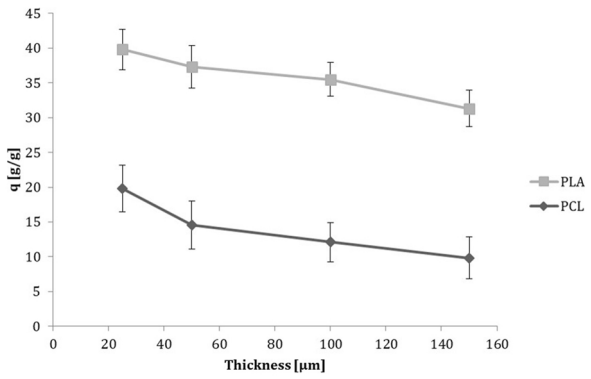

Fig. 2. Oil removal efficiency of electrospun PCL and PLA reported as gm of oil removed per gm of membrane (q) as a function of mat thickness, ranging from 20 µm to 150 µm.

and PLA membrane (although not significantly different from the *Nocardia*-PCL biofilm). The membrane adsorbed all the dispersed oil, while *Nocardia* SoB biodegraded approximately 66 % crude oil from the microcosm in 240 h.

An incubation period of 10 days is the most frequently used in studies on HC biodegradation by free and immobilized cell systems [13]. A few studies have analyzed biodegradation for a period greater than 10 days and up to 70 days incubation [13]. However, the results showed that, increasing the incubation time for longer periods generally did not increase the biodegradation rate of crude oil.

Differences between bacterial degradation performance on PLA compared to PCL carrier were not statistically significant, suggesting that both PLA and PCL biopolymers can be used as biosorbent-carriers for immobilization of HC-degrading bacteria. The adsorptive capacity of our biocompatible membranes is complementary to the oil biodegradation capacity of the bacteria which, when immobilized on those structures, have better conditions for their catabolic activity. The carrier provides stable microenvironments for bacteria, in which the immobilized cells are protected from adverse environmental factors. The affinity between bacterial cells and the hydrophobic substrate is improved, by promoting the mechanism of hydrocarbon uptake by the microorganisms at the water/oil interphase and solving the problem of low bioavailability of hydrocarbons [14,15]. Moreover, the static nature of the system, compared to free cells subjected to continuous agitation, favors hydrocarbon uptake with consequent higher degradation rate.

It has also been demonstrated that biofilm-associated cells show higher resilience and survival at low temperatures [44] and preserve degradative activity for longer periods than planktonic cells. Biofilms may thus provide a constant stream of super-active degrading bacteria, improving bioremediation performance [45].

Conclusion

Although many different mechanical, physical and biological remediation strategies have been applied to remove oil from the environment, the best approach is biodegradation by environmental microorganisms, which have been recognized as key players in cleanup events [46]. In this study, we propose a low cost bioremediation tool for sea and fresh water contaminated by crude oil. A multidisciplinary approach enabled synergistic exploitation of the enhanced sorbent capacity of reusable biodegradable nanofiber membranes and the HC degradative capacity of high performing bacteria to obtain 100 % oil

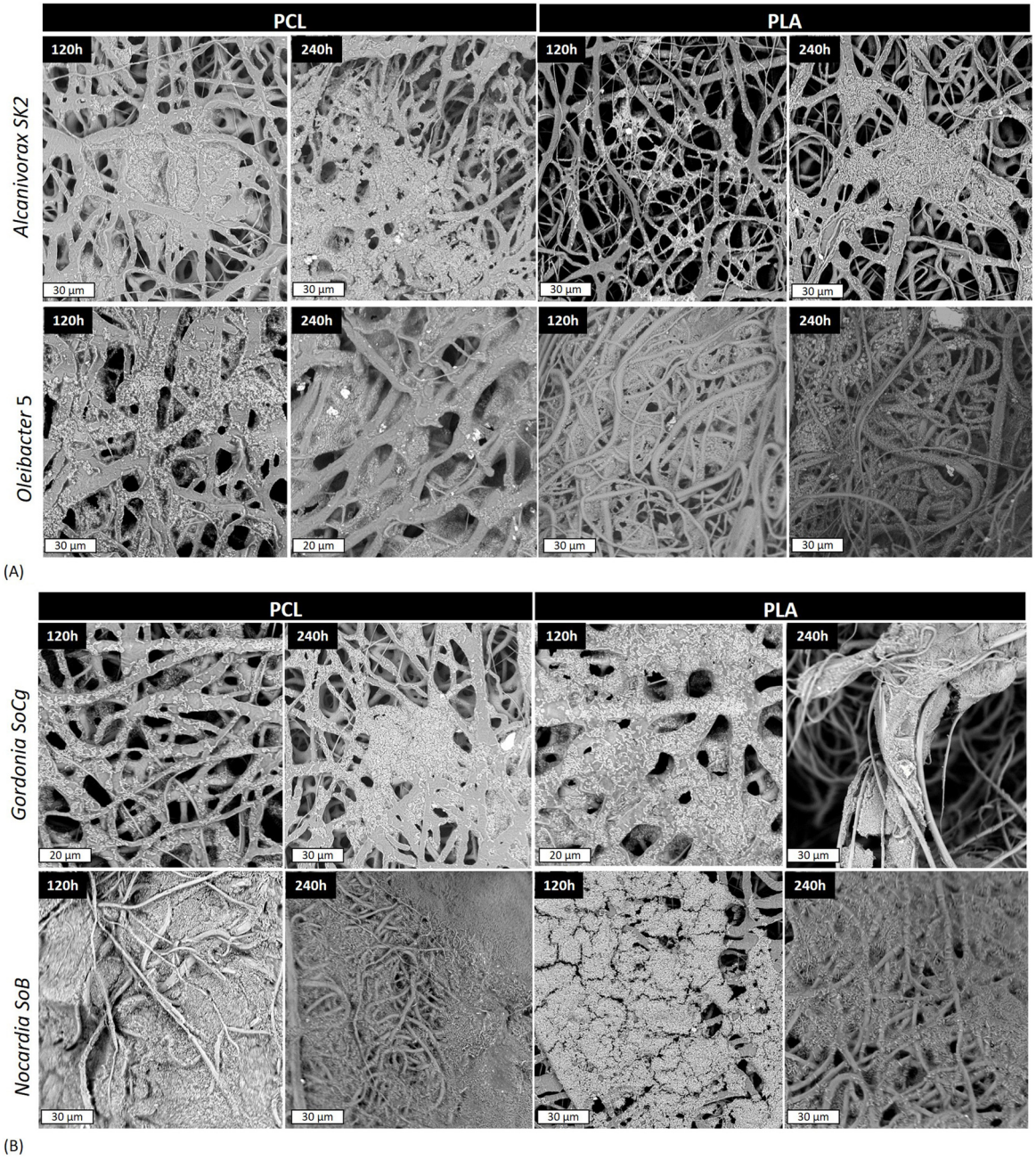

Fig. 3. SEM images of the marine (A) and soil (B) HC-degrading bacteria immobilized in PCL and PLA nanofiber membranes after 120 h and 240 h of incubation. The marine bacteria *A. borkumensis* strain SK2, *O. marinus* strain 5 (A) and the soil bacterium *Gordonia* sp. strain SoCg (B, above), have rod shaped cells that cover most PCL and PLA nanofibers at 120 h and form a three dimensional matrix after 240 h. *Nocardia* sp. strain SoB (B, below) is a filamentous bacterium that completely incorporates the nanofibers already after 120 h. No difference in adhesion capacity between PCL and PLA can be observed.

removal and up to 66 % biodegradation in 10 days at low cost and without negative impacts on the environment.

Compliance with ethical standards

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Ethical approval

This article does not contain any studies with human participants or animals.

Declaration of Competing Interest

None.

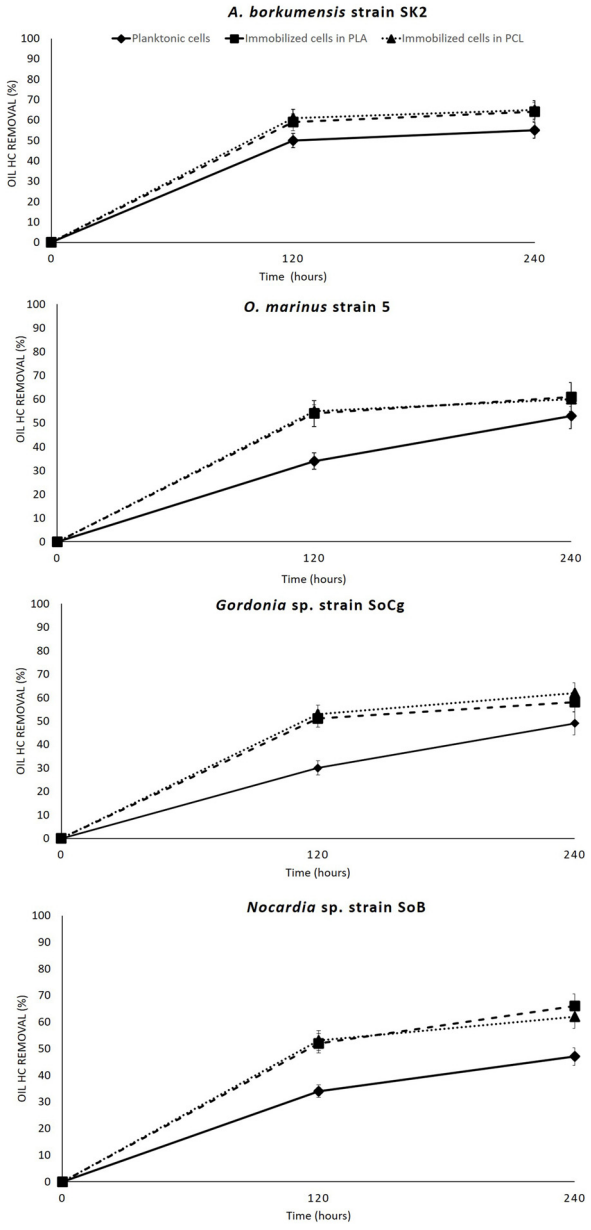

Fig. 4. HC removal by the biodegrading biofilms in microcosms containing 0.1 % crude oil. Oil biodegradation is expressed as percentage of crude oil removed in microcosms inoculated with *A. borkumensis* strain SK2, *O. marinus* strain 5, *Gordonia* sp. strain SoCg, *Nocardia* sp. strain SoB immobilized in PLA (dotted line, square) or PCL (dotted line, triangle) membranes or as free cells (continuous line). Biodegradation in microcosms with immobilized cells is significantly higher than biodegradation measured in microcosms with free living cells within the same species ($p < 0.05$; $n = 3$).

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