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Petroleum oil removal by immobilized bacterial cells on polyurethane foam under different temperature conditions

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ABSTRACT

In this work, a mixed biofilm composed by *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 was formed using polyurethane foam (PUF) as immobilization support, for crude oil removal from artificial sea water. Fresh immobilized cells and immobilized cells that were stored at 4 °C for two months before use were assessed. The oil removal assays were carried out at microcosm scale at 4, 15 and 30 °C. A viability loss of *P. monteilii* P26 was observed after the storage. The highest removal value (75%) was obtained at 30 °C after 7 days using fresh immobilized cells on PUF. Enhanced oil bioremoval was obtained at 4 °C and 15 °C with the previously stored immobilized cells compared to the fresh immobilized cells. Crude oil sorption on the different systems was responsible for the removal of 22–33% oil at the different temperatures. In conclusion, an economic tool for petroleum bioremediation is proposed.

1. Introduction

Oil spills at sea or land produce serious environmental and economic damage. Studies about the Deepwater Horizon oil spill, occurred in 2010, revealed the harmful effects that suffered the ecosystem due to this accident (Beyer et al., 2016). In an economic point of view, oil spills are very costly. The Deepwater Horizon spill clean-up cost US$ 10 billion (Lim et al., 2016). Therefore, the development of both economic and efficient remediation processes is needed. Bioremediation is a promising methodology because it could fulfill these requirements (Bayat et al., 2015). There are many bioremediation processes reviewed in literature (Dzionek et al., 2016). One of them, bioaugmentation, consists in the introduction of organisms able to degrade the target pollutant directly in the contaminated site. To avoid the risk of human or animal infection, non-pathogens environmentally isolated bacterial strains should be used and safety tests should be done prior to the employment of this technology. In general, the added microorganisms have to be immobilized on a carrier to enhance biodegradation (Nuñal et al., 2014; Wang et al., 2015; Dellagnezze et al., 2016). Biofilm formation or entrapment and encapsulation using polymeric gels are the most common immobilization techniques. They were deeply described by Dzionek et al. (2016) and Bayat et al. (2015). In this work, a co-culture of *Pseudomonas monteilii* P26 (Genbank Access Number HE798531) and *Gordonia* sp. H19 (Genbank Access Number LN680636) was immobilized by biofilm formation on polyurethane foam (PUF). These strains were previously isolated from oil polluted sediments of the Patagonian coast, Argentina (Isaac et al., 2013) and showed polycyclic aromatic hydrocarbons degradation ability (Isaac et al., 2015). The immobilized cells were used for the removal of petroleum oil from artificial seawater (ASW) at different temperatures in a first approach towards the development of an efficient and economic bioremediation technology. While many studies already dealt with oil removal from artificial seawater using immobilized cells (Hou et al., 2013; Nuñal et al., 2014; Liu et al., 2015; Wang et al., 2015), to the best of our knowledge, this is the first study in which a single co-culture is tested at different temperatures for the bioremediation of oil polluted water. The aim of this work was to assess the effect of different temperatures on the oil removal capacity from ASW of a bacterial co-culture immobilized on PUF. This carrier was selected for being economic, readily available and for presenting good buoyancy in ASW and oleophilic properties (Li et al., 2012). Besides, microbial immobilization in oil sorbents as PUF can produce a synergistic sorption-biodegradation reaction as described by Wang et al. (2015). In addition, to make this technology as economic as possible, microbial immobilization was carried out using only corn steep liquor as culture medium. Corn steep liquor is in the best case scenario a low value by-product of the food industry, but is mostly

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discarded as a waste since it is produced in higher amounts than demanded by the market.

2. Methods

2.1. Bacterial immobilization

Frozen stock cultures of *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 were thawed and inoculated separately in fresh corn steep liquor medium (10% v/v, pH 7 and sterilized by heat at 121 °C for 15 min). The inoculum concentration was 5% v/v. Both cultures were incubated for 48 h at 30 °C and 180 rpm in an orbital shaker. After incubation, 5 ml of each culture were inoculated in 100 ml of 10% (v/v) corn steep liquor with 1.5 g of PUF rectangular pieces (1 × 1 × 0.5 cm) submerged in it. This co-culture was incubated at 30 °C for 120 h under static conditions to allow biofilm development over the PUF surface. Sterile air was supplied by an air pump through a 0.22 μm nylon filter. Every 48 h, the spent medium was removed and replaced with fresh medium. The immobilized cells were used immediately or stored at 4 °C for 2 months before use to evaluate its storage resistance.

2.2. Mixed biofilm formation quantification

For biofilm quantification, the method described by Quek et al. (2006) was used with modifications. Briefly, PUF pieces with immobilized cells were taken (fresh or after storage), their wet weight determined, washed twice with 5 ml saline solution (NaCl 0.9% w/v) to remove the planktonic cells and cut in small pieces. The pieces were suspended in 5 ml saline and vigorously vortexed for 10 min to detach the cells. This suspension was diluted (10-fold serial dilutions), and dilutions were plated in JPP-agar medium (% m/v: NaCl, 2; yeast extract, 0.1; meat peptone, 0.2; agar, 1.5 pH = 7). The plates were incubated at 30 °C for 5 days when the colonies of P26 and H19 were clearly distinguished from each other and could be counted. *P. monteilii* P26 colonies were white and mucoid, while *Gordonia* sp. H19 colonies were dark orange color and showed a dry aspect. The CFU/ml of the suspension was determined and the CFU/g $_{support}$ of both P26 and H19 were calculated.

2.3. Petroleum oil removal by immobilized cells on PUF

Approximately 4 g (wet weight) of PUF-immobilized cells (six pieces), fresh or after 2 months of storage, were placed over 10 ml artificial sea water (ASW) (for ASW composition see Quek et al. (2006)) spiked with 0.1 g crude oil in 100 ml glass flasks with cotton stoppers to allow volatilization of oil as would occur in a natural environment. The removal assay lasted for 7 days at 4, 15 and 30 °C. The flasks were gently agitated twice a day to aerate the system. After incubation, PUF pieces were removed from the liquid. Two samples were taken separately: 1) the oil remaining in the liquid, and 2) the sorbed oil on PUF and bacterial biofilm. The two samples were extracted with pentane until no color was observed in the organic phase. The pentane was then allowed to evaporate from the extract at room temperature and the residual oil was determined gravimetrically. The same procedure was followed using a sterile system (sterile PUF submerged in ASW spiked with 0.1 g crude oil) as abiotic control for each temperature. Representative samples for each condition were analyzed by gas chromatography. The equipment used was an Agilent 6890N with a flame ionization detector in splitless mode. The injector temperature was set at 285 °C. The chromatographic run time was 30 min. The oven program was as follows: the initial temperature was 30 °C and was maintained for the first 3 min of the run. Then the temperature increased by 15 °C/min until reaching 300 °C and it was held for 5 min. Finally, the temperature was increased by 15 °C/min until reaching 325 °C and held for the rest of the run. The column used was a HP-5 (5% Phenyl Methyl Syloxane). The carrier and make up gas was nitrogen. The carrier gas flow was constant, 3.0 ml/min. Prior to injection, petroleum oil samples were suspended in 100 ml pentane (HPLC grade) and filtered through a 45 μm pore size nylon membrane (Whatman) to remove insoluble particles.

In a parallel experiment, biofilm formation after 7 days of incubation in ASM with crude oil was quantified as described in the previous section.

Overall Petroleum oil removal was calculated according to the following expression:

$$\text{Overall oil removal (\%)} = (100/\text{ROav}) \times (\text{ROav} - \text{RO})$$

Petroleum oil removal attributed only to biological activity was calculated according to the following expression:

$$\text{Oil bioremoval (\%)} = (100/\text{ROav}) \times [\text{ROav} - (\text{RO} + \text{SO})]$$

where ROav is the remaining oil after volatilization, RO is the remaining oil in the liquid after the removal assay and SO is the sorbed oil on the biofilm/carrier system.

A schematic representation of the entire methodology can be seen in Fig. S1.

2.4. Statistical analysis

One-way analysis of the variance (ANOVA) with Fisher test was performed over the data collected to determine significant differences ($p < 0.05$) between the means obtained from triplicate values. The software used was MINITAB 17 (PA, USA).

3. Results and discussion

3.1. Mixed biofilm formation quantification

Biofilm formation on PUF was quantified before and after the crude oil removal assay using fresh or 2-months stored immobilized cells. To assess the storage resistance of the immobilized cells is very important because the immobilization procedure usually takes days. In case of emergency, it would be desirable that the biocatalyst be stored and ready to use, allowing a rapid response and minimizing the damage produced by an accidental oil spill. Fig. 1 shows the logarithmic CFU/g values of each strain present in the biofilm. It was observed that, while *Gordonia* sp. H19 seemed not to be affected by the 2-months storage, *P. monteilii* P26 in the mixed biofilm showed a decrease in the cell viability of approx. 3.93 log units compared with the fresh immobilized cells. After 7 days of incubation at 4 °C in the presence of crude oil, the P26 viability from the stored biofilm decreased even further 1.16 log units; however, after incubation at 15 °C and 30 °C, the viability of P26 increased approx. 2.27 log units. Nuñal et al. (2014) reported a decrease in viable counts of 3–4 orders of magnitude after storing an immobilized bacterial consortium for 6 months at 0 °C, − 30 °C and room temperature. The bacterial consortium was formed by two γ-*Proteobacteria*, one α-*Proteobacteria* and one *Cytophaga-Flavobacterium-Bacteroides*, and was immobilized on rice hull, cocopeat and calcium alginate. Gentili et al. (2006) used a *Rhodococcus* strain immobilized on chitin and chitosan for removal of oil. Storage experiments revealed a viable counts decrease of 1 order of magnitude when storing chitin immobilized cells at 4 and − 20 °C for 185 days. In this work, *Gordonia* sp. H19, showed a similar resilience when storing it at 4 °C for 2 months immobilized on PUF.

3.2. Petroleum oil removal by immobilized cells on PUF

Immobilized cells on PUF were evaluated for its capacity to remove crude oil at different temperatures. Maximum oil removal was obtained at 30 °C with fresh immobilized cells (75%) (Fig. 2). At 4 and 15 °C the removed oil by the fresh immobilized cells was similar to the removed oil by the sterile PUF at the same temperature ($p > 0.05$). However,

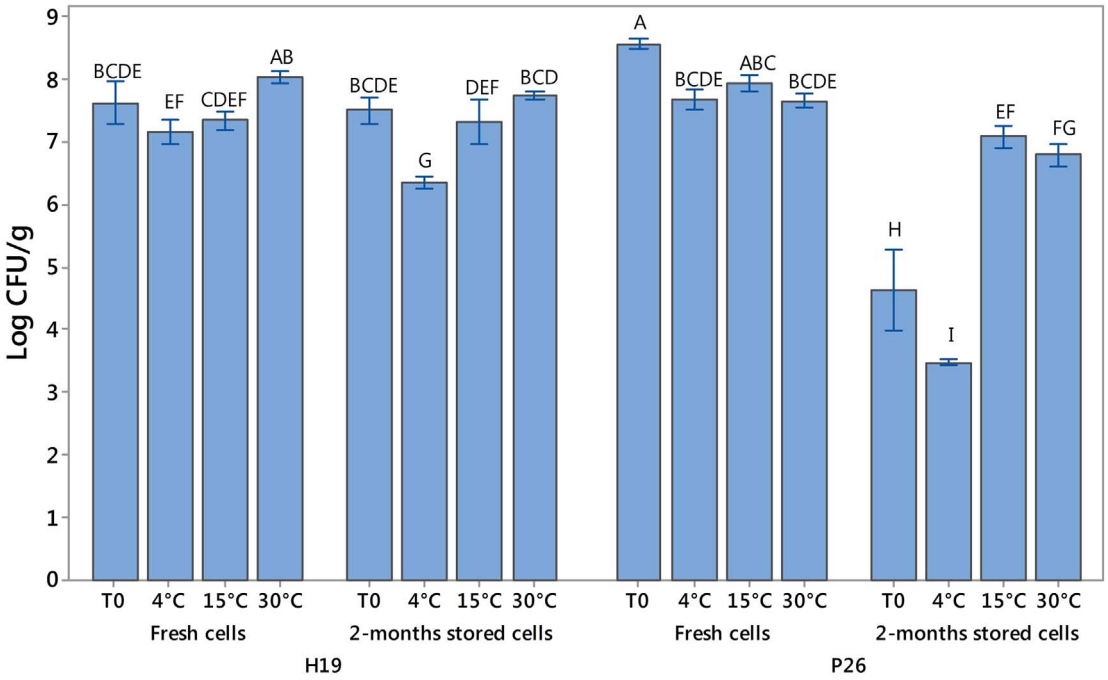

Fig. 1. Cell viability of the two strains (H19: *Gordonia* sp. H19; P26: *Pseudomonas monteilii* P26) that compose the biofilm formed in polyurethane foam. Viable cell counts were determined from fresh cells and 2-months stored cells, before (T0) and after 7 days incubation with petroleum oil at different temperatures. Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values which present the same letter are not statistically different ($p > 0.05$).

immobilized cells stored for 2 months, showed a significative difference in the removed oil comparing with the sterile PUF at those temperatures. In average, the overall removal of stored immobilized cells at 4 and 15 °C was 55.5% (22% more than oil removal by the sterile PUF). At 30 °C, the oil removal by stored immobilized systems did not differentiate from the sterile PUF. Oil removal by volatilization was 10% at 4 and 15 °C and 20% at 30 °C. Oil sorption accounted for a removal of 22–33% among treatments (Fig. 3). The chromatographic profiles of representative samples can be seen in Fig. S2.

When analyzing the petroleum oil removal attributed only to biological activity (Fig. 4), it becomes evident that the previous storage at 4 °C improved the removal ability of the immobilized cells at that temperature and at 15 °C. It is important to note that the two strains used are psychrotolerant, i.e. they can grow at low temperature, but the optimal grow rate is achieved at 30 °C. There was no increase in the cell density of the biofilm after storage, so that this removal improvement could be due to improved metabolic activity due to bacterial acclimatization to a low temperature. To the best of our knowledge, this finding wasn't reported for the removal of pollutants from petroleum at different temperatures using bacterial cells. Further studies will aim to determine if the bacterial acclimatization to different environmental factors could allow using the same immobilized cells for oil removal in different environments and year seasons.

Recent works used different immobilization carriers for hydrocarbon removal from water using immobilized cells. Liu et al. (2015) used calcium alginate entrapped cells for the removal of diesel oil (initial concentration: 2342 ppm) from ASW. They reported a removal of 78% in 16 days under optimal aeration conditions. Costa et al.

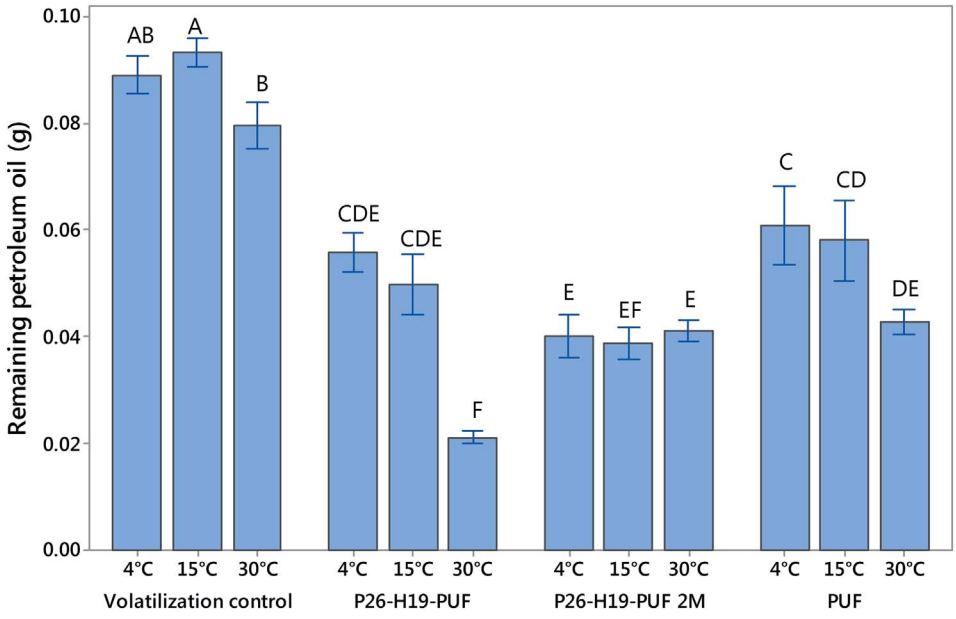

Fig. 2. Petroleum oil remaining in the artificial sea water after 7 days treatment with fresh immobilized *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 cells in polyurethane foam (P26-H19-PUF), 2-months stored immobilized cells (P26-H19-PUF 2M) and sterile PUF at different temperatures. Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values which present the same letter are not statistically different ($p > 0.05$).

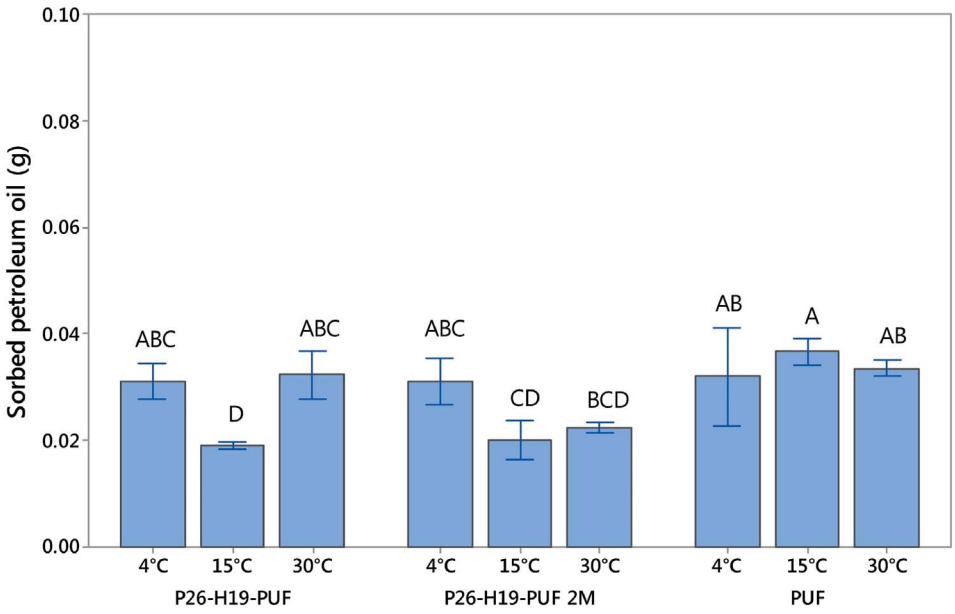

Fig. 3. Petroleum oil sorbed on the carrier and the biofilm after 7 days treatment with fresh immobilized *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 cells in polyurethane foam (P26-H19-PUF), 2-months stored immobilized cells (P26-H19-PUF 2M) and sterile PUF at different temperatures. Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values which present the same letter are not statistically different ($p > 0.05$).

(2014) used chitosan for cell entrapment and removed 90% of hexadecane in 6 days (initial concentration: 10,000 ppm). Chen et al. (2016) investigated the use of modified bamboo charcoal as immobilization matrix. They obtained a diesel oil removal of 90% in 96 h (initial concentration: 200 ppm). Lin et al. (2015) immobilized *Acinetobacter venetianus* cells on sugar cane bagasse for the removal of tetradecane. They obtained approx. 93% removal in 96 h (initial concentration: 400 ppm). In this work, a high concentration of crude oil (10,000 ppm), which is a complex mixture of hydrocarbons, was used. It was the intention of this study to simulate natural conditions when it was possible and, therefore, the microcosms assays were carried out without optimal aeration and mixing. It could be expected that petroleum oil removal values obtained in this work would be improved under better aeration conditions. In addition, this study emphasized the economic nature of the immobilization process by using an agroindustrial waste as culture medium for biofilm formation. This point that very oft is ignored is very important for the final accomplishment, which is the development of new technologies to solve already existing and emerging problems.

4. Conclusion

In this study, an economical bioremediation tool for the restoration of crude oil contaminated sea water is proposed. The immobilized mixed biofilm on polyurethane foam showed satisfactory oil removal values in 7 days, mainly at 30 °C. Both oil removal by biological activity and sorption on the biofilm/carrier system was determined. Storage of the immobilized cells at 4 °C enhanced oil bioremoval at low temperature although bacterial viability of *P. monteilii* P26 in the biofilm decreased. This suggests that bacterial acclimatization occurred during the storage improving their metabolic activity at low temperature.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2017.06.040.

Conflict of interest

The authors declare that they have no conflict of interest.

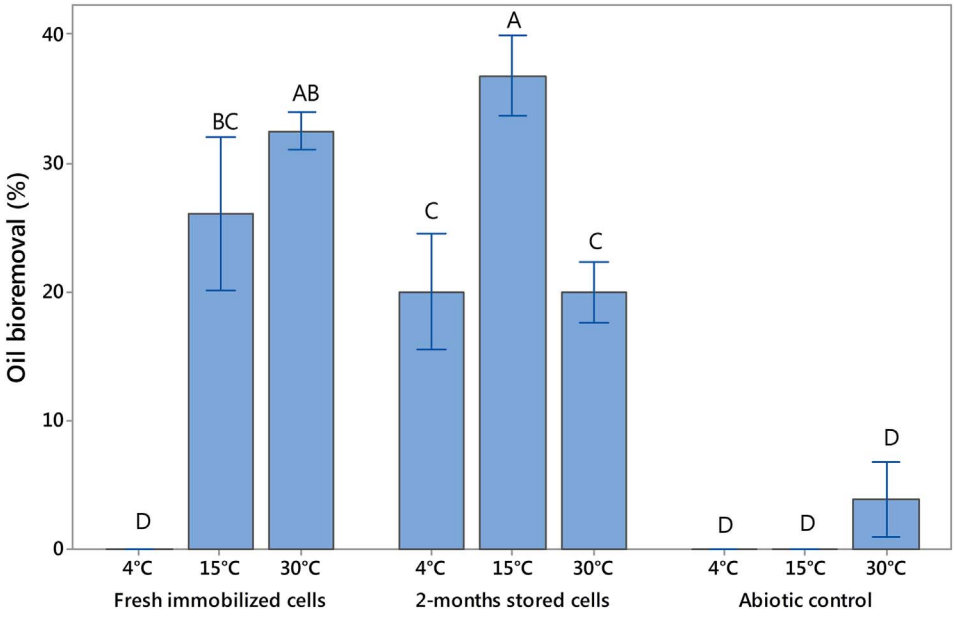

Fig. 4. Petroleum oil removal (%) attributed only to biological activity by fresh and 2-months at 4 °C stored immobilized *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 cells in polyurethane foam (PUF). As abiotic control, sterile PUF was used. Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values which present the same letter are not statistically different ($p > 0.05$).

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