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Pre-exposition to polycyclic aromatic hydrocarbons (PAHs) enhance biofilm formation and hydrocarbon removal by native multi-species consortium

Paula Isaac [a,b], Mauricio Javier Alessandrello [a,*], Alexandre José Macedo [c,d], María Cristina Estévez [a,b], Marcela Alejandra Ferrero [a,b]

[a] Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), Av. Belgrano y Pje, Caseros T4001MVB, Argentina
[b] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), Ayacucho 471, San Miguel de Tucumán T4000INI, Argentina
[c] Facultade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Porto Alegre/RS 90610-000, Brazil
[d] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre/RS 91501-970, Brazil

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ABSTRACT

In this work, a biofilm-mediated removal of polyaromatic hydrocarbons (PAHs) was proposed. PAHs are toxic and persistent contaminants from petroleum industry, dispersed on water, soils, air and sediments. The removal capabilities of C15 mixed culture of four indigenous strains of *Pseudomonas* and actinobacteria were successfully corroborated in a previous work. Results showed an interesting removal behavior of planktonic C15 consortium, with degradation capabilities notably enhanced when comparing with pure cultures. In the work reported in this paper, biofilm formation was evaluated in pure cultures and C15 defined consortium in order to propose a biofilm mediated immobilization strategy for bioremediation. Interestingly, a notable enhancement (approx. 180%) in biofilm formation was observed after mixing the four species of C15 compared with pure cultures. Phenanthrene and pyrene also stimulated biofilm arrangement and biofilm-immobilized microorganisms demonstrated a hydrocarbon removal significantly higher than planktonic cells. Maximal removal values were 100% for phenanthrene and 78% for pyrene, after 7 days incubation. The biodegradation assay was divided into two stages: biofilm formation and hydrocarbon removal. The importance of PAHs presence during biofilm formation was evaluated. Although no differences in biofilm structure were observed, bioemulsifier production was enhanced and removal was accelerated when phenanthrene and pyrene were added during biofilm formation stage. Even though more experiments must be conducted, considering the coexistence of *Pseudomonas* and actinobacteria groups during biodegradation and the improvement of removal performance, interspecies beneficial interactions could be suspected in this native multi-species biofilm.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules formed by two or more fused benzenic rings, consisting only in carbon and hydrogen atoms [1]. The toxic properties of PAHs have been largely investigated and their carcinogenic, mutagenic and teratogenic acute properties were corroborated [2,3]. Polyaromatic hydrocarbons are synthesized during thermal decomposition of organic matter. Some of their physical characteristics are low water solubility, high fusion and boiling points and low vapor pressure. According to these properties, PAHs persist in the environment for years and are highly resistant to biodegradation [4]. Bioremediation is an emerging and eco-friendly biotechnology resource for cleaning up polluted environments using living organisms. In terms of efficiency and economics, this strategy for treating contaminated sites gives better results than chemical and physical methods [5].

Biofilm lifestyle protects organisms against environmental stressing conditions, improves cellular communication and facilitates genetic material exchange [6,7]. Different genera of bacteria,

* Corresponding author.
E-mail address: mauricio.alessandrello@gmail.com (M.J. Alessandrello).

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fungi, algae and archaea are capable of adhering to diverse surfaces and develop stable biofilm structures [8]. The biofilm formation depends on particular environmental conditions, presence of acceptors/donors of electrons and nutrients concentration [9,10]. Biofilm-immobilized cultures represent an efficient biological tool for pollutants removal in bioremediation and biotransformation protocols [8]. These structures present high tolerance to toxic compounds even when substrate concentration results lethal for planktonic cultures. They also accumulate microbial biomass and immobilize environmental pollutants promoting their removal and subsequent biodegradation [11,12]. Furthermore, exopolymeric substances (EPS) present in biofilms contain molecules with surfactant or emulsifier properties which enhance bioavailability of hydrophobic compounds like hydrocarbons [13]. Biofilms have been widely used in bioremediation protocols in the last years to solve limiting natural detoxification. Methodologies were evaluated and applied in both, *in situ* or *ex situ* conditions [14–18].

Multi-species biofilms are different and expectedly more complex than monospecies biofilms. Interactions between biofilms members often cause changes in culture behaviors where some functions and products could be enhanced or modified [19]. Generally, microorganism viability depends on metabolites and other chemical compounds from other organisms living in ecosystems and communities [20,21].

In a previous work we have reported a synthetic consortium (C15) with promising capabilities on hydrocarbon bioremediation [22]. C15 mixed culture was formulated combining *Pseudomonas* and actinobacteria and showed high removal levels of low- and high-molecular weight polyaromatic hydrocarbons in planktonic conditions. Removal capabilities of C15 were significantly higher than pure cultures suggesting beneficial interactions between members. The aim of this work was to improve PAHs degradation capabilities of C15 using a biofilm mediated immobilization strategy and determine the importance of the substrates addition during the biofilm formation stage.

2. Materials and methods

2.1. Microorganisms and growth conditions

According to PAHs degrading capabilities, emulsifying activity and negative antagonism, the strains *Pseudomonas monteilii* P26 (HE798531), *Pseudomonas* sp. N3 (LN680634), *Rhodococcus* sp. F27 (LN680637) and *Gordonia* sp. H19 (LN680636), recovered from chronically hydrocarbon-contaminated soil and sediment in Patagonia Argentina, these four strains formed so-called defined mixed culture C15 for PAHs removal in biofilm [22,23].

Microorganisms were kept individually at −20 °C in JPP broth (% m v^{-1}: NaCl, 2; yeast extract, 0.1; meat peptone, 0.2; pH 7.0) [24] added with 20% (v v^{-1}) glycerol.

Before experiments, each microorganism was propagated in JPP broth, at 30 °C in an orbital shaker at 180 rpm for 24 or 48 h, for pseudomonadales and actinobacteria, respectively. C15 defined mixed culture was further composed by the same proportion of each individual strain.

2.2. Biofilm formation assay

Sterile 96-well polystyrene microtiter plates and glass tubes, as model of hydrophobic and hydrophilic material, were used to evaluate biofilm formation according to the crystal violet staining assay [25]. Assay was performed with pure and C15 mixed cultures. Inoculums were prepared according to growth conditions described above and a bacterial suspension of each strain containing 3×10^8 CFU/mL in 0.9% NaCl was used. Biofilms of C15 were formed in JPP medium with KNO_3 addition (1 g L^{-1}). Nitrate was added to stimulate cellular metabolism in anoxic regions inside the biofilm structure [26]. In microtiter plates, 100 μL of the bacterial suspension and 100 μL of JPP-NO_3 medium were added. In the case of glass tubes, 1 mL of the bacterial suspension and 1 mL of JPP-NO_3 were mixed. Same proportion of the bacterial suspension of each four species was used when mixed culture C15 was evaluated. In order to determine stimulating effect of hydrocarbons on biofilm formation, bacterial cultures were evaluated after incubation in absence or presence of PAHs (a mix of phenanthrene and pyrene, 0.2 mM each). Sterile JPP medium was used as negative control. Microtiter plates and tubes were incubated at 30 °C during 3 days without shaking. After incubation, the content of the wells/tubes was removed and optical density was measured at 600 nm ($OD_{600\ nm}$). The remaining attached bacteria were washed three times with sterile saline and heat-fixed at 60 °C for 1 h. The formed biofilms were stained with 0.4% crystal violet for 15 min at room temperature. The stain bound to the cells was solubilized with absolute ethanol and optical density was measured at 570 nm ($OD_{570\ nm}$) (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA).

2.3. PAH removal by C15 in biofilms

All PAHs removal experiments were carried out in 15 mL glass tubes containing 2 mL of JPP-NO_3 medium. Formation of C15 biofilms was assayed in two different conditions: without hydrocarbon or supplemented with 0.05 mM of each PAH assayed (phenanthrene and pyrene) in acetone, incubated in darkness at 30 °C for 3 days without shaking. The acetone was allowed to evaporate for 15 min before adding the cells (7.5×10^7 CFU/mL each pure culture).

After incubation, free planktonic cells were carefully removed and the biofilm formed on the inner walls of the tube was washed twice with sterile water. Fresh JPP-KNO_3 medium (2 mL) plus PAH 0.2 mM each, was added. Rubber stoppers were used to tightly seal glass tubes and incubation was performed under the same conditions described for previous biofilm formation. Control tubes with sterile JPP-KNO_3-PAH medium were used to determine PAH abiotic loss. PAH removal was evaluated at different times during 10 days. Samples were withdrawn by sacrificing cultures at 0, 1, 3, 7 and 10 days and residual PAH was quantified according to [27]. Equal volume of hexane (2 mL) was added to the tubes and vigorously vortexed for 2 min. Aqueous and organic phases were further separated by centrifugation at $6000 \times g$ for 10 min. Hexanic phase was separated and PAH concentration was determined on a UV/vis Spectrophotometer at 292 and 335 nm, respectively, according to a standard curve.

For quantification of biofilm, tubes were washed twice with 2 mL saline solution (0.9% w/v) to remove the planktonic cells. Biofilm was removed from the glass tubes with 2 mL saline using a sterile spatula and vortexed for 10 min to detach the immobilized cells. The CFU/mL of this suspension was determined. Different colony morphology allowed us to distinguish the four strains. Plates were in triplicate.

2.4. Bioemulsifier activity

The emulsification index (EI %) of supernatants from the PAHs removal assay by C15 biofilm was determined using the kerosene test [28] with modifications. Biofilm was mechanical disrupted and centrifuged to separate bacterial cells. Aqueous phase was mixed with equal volume of kerosene and vigorously vortexed during 2 min. Mixture was left to settle 24 h and EI was calculated as a fraction of the height of the emulsified layer (mm) considering 100% the total height of the liquid column (mm). Sterile JPP culture medium and Tween 20 were used as negative and positive controls, respectively.

2.5. Microscopic analysis

An evaluation of the biofilm structure was performed using confocal laser scanning microscopy (CLSM). Microscopic study was carried out during biofilm formation (initial 3 days) and during hydrocarbon biodegradation (final 10 days). Differences according to PAHs addition were also considered in this assay.

C15 biofilm were cultured on coverglass-bottom dishes during the corresponding times. Immediately, samples were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, USA) for 20 min in darkness (room temperature), and the dish contents were gently replaced with water according to manufacturer's instructions. Samples were then directly observed using a confocal laser scanning microscope (Leica DM 6000 CS) with a 63× upright objective. For each sample, a sequential scan in two channels was carried out and corresponding *xy* optical sections and 4 random images were acquired. Overlapping images and orthogonal cuts were obtained by using Olympus FV 1000 software.

2.6. Statistical analysis

A general linear model of analysis of variance (ANOVA) was applied to study the main and interaction effects of factors assayed in the different experiments. In each analysis, Tukey's test was used to determine significant differences ($P < 0.05$) between mean values (MINITAB statistical software, version 15 for Windows).

A schematic representation of the entire methodology can be seen in Fig. 1.

3. Results

3.1. Pure and mixed cultures biofilm formation

Pure and mixed cultures were capable to adhere and develop biofilm only on hydrophilic glass surface. The assays permitted to compare individual *Pseudomonas* and actinobacteria strains and C15 mixed culture behaviors.

No significant differences ($P < 0.05$) were observed in growth values ($OD_{600\ nm}$) for *Pseudomonas* and actinobacteria strains and for C15 mixed culture in both treatments.

Biofilm formation was quantified through crystal violet staining and a biofilm index ($OD_{570\ nm}/OD_{600\ nm}$) was calculated for each experiment (Fig. 2). *Pseudomonas* showed limited biofilm formation capabilities in pure culture. Although scarce, this biofilm lifestyle was detected only when phenanthrene and pyrene were added to culture medium. On the other hand, actinobacteria strains *Rhodococcus* sp. F27 and *Gordonia* sp. H19 did not show a strong adhesion on glass, even after hydrocarbon exposure.

Interestingly, all four species in C15 mixed culture showed a biofilm notably enhanced. C15 mixed culture showed a 179.5% increase on biofilm index compared with the major biofilm former pure culture (*Pseudomonas* sp. N3). On the other hand, PAHs also stimulated biofilm formation in C15, increasing the biofilm index values obtained without hydrocarbon addition from 1.23 ± 0.09 to 5.59 ± 0.23.

3.2. Hydrocarbon removal and emulsifying activity by C15 biofilm

Residual PAHs were quantified during 10 days to evaluate hydrocarbon removal efficiency of biofilm-immobilized cells. An incomplete removal was observed for both phenanthrene ($65 \pm 5.93\%$ of removal) and pyrene ($28 \pm 2.31\%$ of removal) when bacterial biofilm was developed in absence of PAH (Fig. 3A). PAHs removal was notably accelerated when biofilm was previously formed in presence of PAHs (Fig. 3B). Under this condition, phenanthrene was completely removed, with $69 \pm 3.91\%$ of pyrene removal being achieved after 3 days of incubation by the C15 mixed bacterial biofilms. It is worth highlighting that there was no hydrocarbon removal during biofilm formation (data not shown).

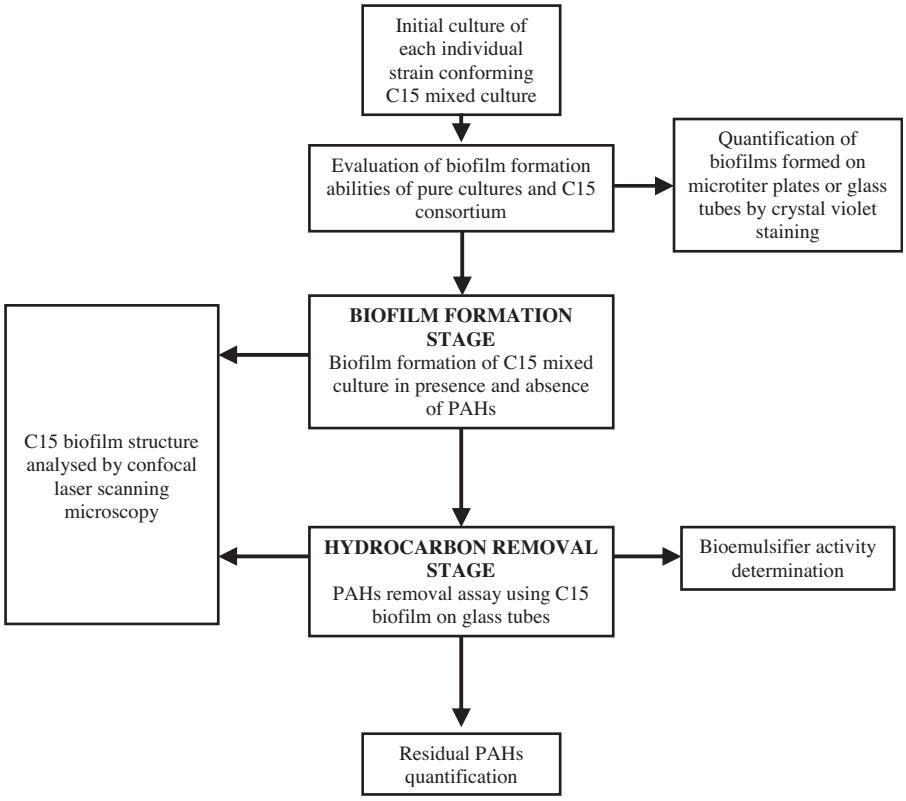

Fig. 1. Schematic representation of the methodology used in this study.

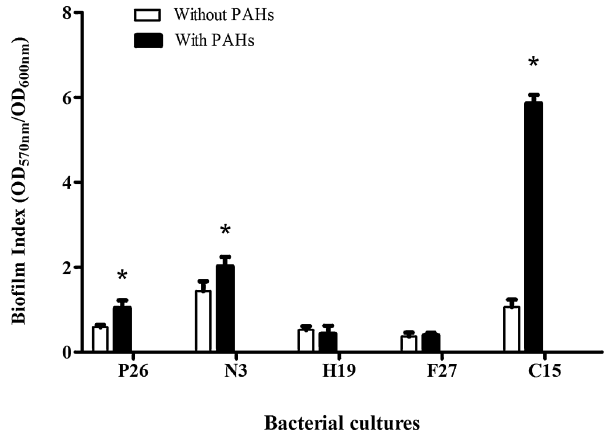

Fig. 2. Biofilm index of both pure and mixed cultures. Biofilm formation during PAHs exposition (filled bars) was compared with the condition without PAHs (empty bars). Asterisks in bars denote values significantly different to control. Values are the average of triplicate samples ± standard deviation. P26: *Pseudomonas monteilii* P26, N3: *Pseudomonas* sp. N3, H19: *Gordonia* sp. H19, F27: *Rhodococcus* sp. F27 and C15: mixed culture C15.

Polyaromatic hydrocarbons notably enhanced bioemulsifier production (maximum EI = 89.2 ± 5.09%) (Fig. 3). In both evaluated biofilm conditions (Fig. 3A and B), maximum EI (%) values were obtained after 7 days coinciding with maximum hydrocarbons removal.

Different colony morphology allowed us to distinguish the strains, and these four different colony morphologies were observed in every plating confirming *Pseudomonas monteilii* P26, *Pseudomonas* sp. N3, *Rhodococcus* sp. F27 and *Gordonia* sp. H19

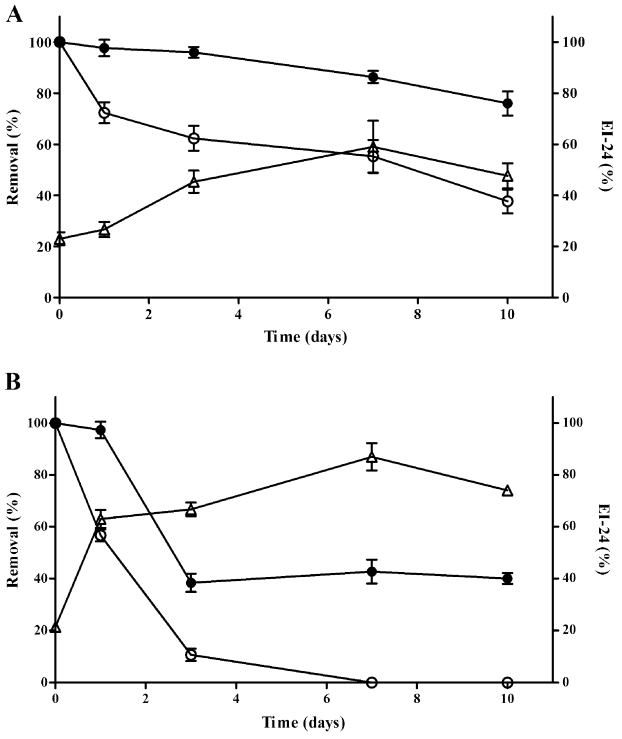

Fig. 3. Kinetics of PAHs removal and bioemulsifier production by biofilm-immobilized mixed culture C15. Biofilms formed in absence (A) or presence (B) of hydrocarbons. Residual phenanthrene (○) and pyrene (●) values were quantified considering hydrocarbon abiotic loss in all cases, in triplicate assays and average values ± standard deviation were plotted. Bioemulsifier was determined according to emulsification index at 24 h (EI-24(%)) (△).

presence. Each C15 members were all detected at 0, 1, 3, 7 and 10 days during biodegradation assay, confirming that the bacterial cultures used for the study remained along assays.

3.3. Structural characterization of degrader biofilms

Fig. 3 shows biofilms structures of C15 during biofilm formation and PAHs degradation stages. According to our results, mature condition of biofilms and maximum biomass accumulation was observed after 72 h when no PAHs were added, reaching around 100 μm of thickness (Fig. 4A). When biofilm was constructed under PAHs exposition, thickness of biofilm structures reached a maximum of 150 μm after 24 h (Fig. 4B). Although PAHs clearly accelerated biofilm formation, no structural differences were observed when comparing to those formed without hydrocarbon addition. No structural changes were observed after planktonic cells removal and hydrocarbons addition, corroborating biofilm resistance to PAHs toxicity (Fig. 4C and D). Biofilm architecture was also stable during the complete biodegradation process (data not shown).

4. Discussion

Numerous reports have corroborated biofilm lifestyle advantages compared with their free-living planktonic counterparts in bioremediation [29–33]. The biodegradation capabilities of C15 mixed culture of indigenous *Pseudomonas* and actinobacteria were successfully corroborated in planktonic condition [22] and a biofilm-mediated removal was proposed in this work.

Pavithra and Doble stated that bacterial biofilm formation depends on several factors, such as the microorganisms involved and the surface properties of the material used [34]. Hydrophobicity is largely accepted as a non-specific joining parameter in bacterial adhesion [35]. It is often assumed that adherence of cells to hydrocarbons and other hydrophobic surfaces is directly related to PAHs bioavailability and pollutant removal [36], but this does not really predict utilization [37]. Thereby, impossibility of all evaluated cultures to form biofilms in hydrophobic polystyrene microtiter plates does not reject their PAHs degradation capabilities. Using hydrophilic glass surface, *Pseudomonas* strains and C15 mixed culture, were capable of adhering and forming stable biofilms. On the other hand, nocardioform actinomycetes (including *Rhodococcus* and *Gordonia* genus) have a high proportion of mycolic acids on their cell envelope, which benefits the formation of stable biofilms [38]. However, pure actinobacteria strains composing C15, *Rhodococcus* sp. F27 and *Gordonia* sp. H19, were not capable of adhering to either glass or polystyrene surfaces. A significant enhancement of biofilm formation was obtained after mixing *Pseudomonas* and actinobacteria strains. This behavior seems to be the result of microbial interactions in mixed cultures. In these multi-species cultures, each member of microbial communities could employ a set of molecular interaction mechanisms to synchronize its individual behavior and achieve a collective one of the entire community [39]. Burmølle et al. [40] obtained similar results after mixing four epiphytic bacteria enhancing more than 160% levels of formed biofilms when comparing to the mono-species biofilms. Also, an enhancement of the protective effects of the biofilm structure has been previously demonstrated when multiple species are present [19].

In contaminated sites, biofilm lifestyle represents a natural strategy of microorganisms to survive and optimize life conditions [41,42], so it is not surprising to observe that phenanthrene and pyrene notably increased bacterial adhesion and biofilm formation in this work.

Edwards and Kjellerup showed that microbial closeness in biofilms structures is beneficial for cells interactions and,

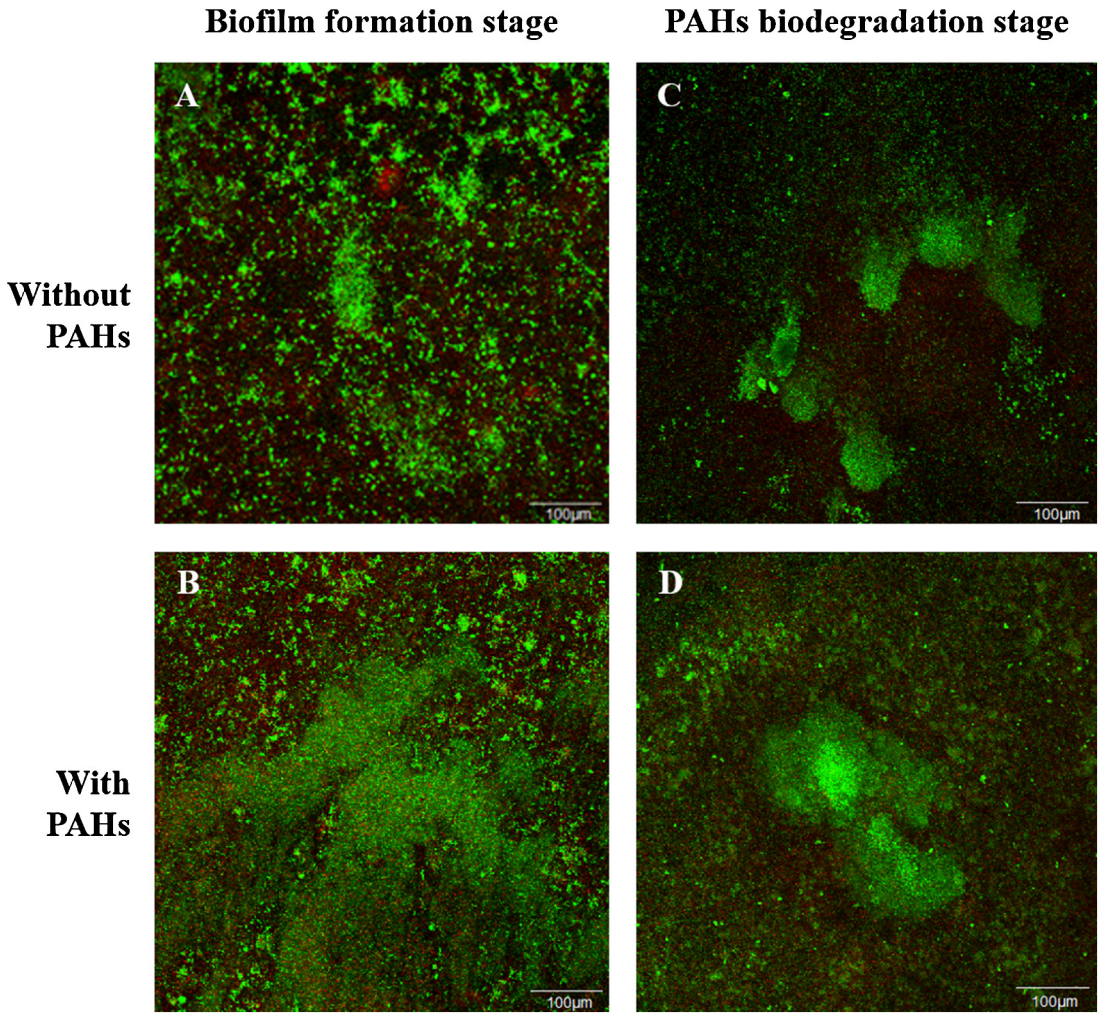

Fig. 4. Confocal laser scanning micrographs of C15 mixed culture biofilm during biodegradation assay. SYTO9 specifically stains intact cells, whereas propidium iodide stains bacteria with damaged membrane, distinguishing live and dead bacteria. Biofilm formed were observed in a first formation stage and a second stage during PAHs biodegradation. Two conditions were evaluated in absence or presence of PAHs. A: mature biofilm obtained without PAHs (72 h), B: mature biofilm obtained with PAHs (24 h), C: biofilm formed without PAHs during hydrocarbon removal, D: biofilm formed with PAHs during hydrocarbon removal.

consequently, degradation processes could be accelerated [8]. When C15 biofilm removal performance was compared with planktonic culture previously evaluated [22], the most interesting result was to note that pyrene degradation potential of biofilm-immobilized C15 was found to be notably higher than that reported in planktonic culture where a maximum of 52% of this contaminant was removed. Pyrene is a tetracyclic PAH considered recalcitrant, thermodynamically stable [43], highly resistant to microbial degradation and many efforts are being made to its remediation [44–47].

It has been largely studied that biofilms high resistance to environmental and chemical stresses are due to their static, dormant and inactive properties [48–50]. Furthermore, Shimada et al. confirmed that biofilm-associated cells exhibit less degradation activity than planktonic cells when comparing their removal performance in liquid culture [31]. A similar behavior was observed in C15 biofilm for phenanthrene removal. The degradation rate of this hydrocarbon was higher in planktonic condition where a complete removal was obtained after only 3 days of incubation [22]. We propose that the delay for hydrocarbon starting consumption observed in biofilms comparing to planktonic cells, could be explained as the time taken by the substrate to be uptake by the bacterial cell in the biofilm.

Considering a slower but complete phenanthrene removal and a remarkably accelerated pyrene degradation, biofilm mediated immobilization of C15 represents an improved strategy for bioremediation when comparing to planktonic cultures and even more with pure cultures. However, these interesting removal abilities of C15 biofilm were observed only when hydrocarbons were added during biofilm formation. Conversely, removal values of C15 biofilms formed without hydrocarbons were even lower than those obtained in planktonic condition. In this way, substrate presence during biofilm formation seems to be crucial for biodegradation process, inducing stress responses and maybe enhancing gene expression and preparing cells for biodegradation.

Already in 1987, Heitkamp and Cerniglia [51] considered that substrate bioavailability was the most important limitation in PAHs bioremediation. Surfactant or emulsifier addition results in an effective strategy for hydrocarbons biodegradation protocols [52–55]. The nature of emulsifiers could be diverse, like cellular structures or biological membranes [56]. In biofilm cultures, while surfaces properties of EPS could enhance solubility and bioavailability of hydrophobic compounds [57], some bacterial derived biosurfactants were confirmed to control the attachment and to disrupt formed biofilms of pure and mixed cultures [58,59]. Bioemulsifier production of planktonic C15 mixed culture was previously corroborated and the beneficial impact of emulsifier

release on removal activity was determined [22]. When C15 was biofilm-immobilized, incubation conditions used for development of mature biofilms were a determining factor in bioemulsifier production, as well as in removal assay. Exposure to PAHs during biofilm formation notably enhances bioemulsifier release. In accordance with this feature, Tribelli et al. confirmed that PAHs accumulation in the inoculums of biofilms caused a reduction of surface tension supernatants [32]. Results from the present work corroborated the importance of bioemulsifier production during bacterial PAHs-removal. In hydrocarbons remediation protocols, to reach high EI values without addition of exogenous synthetic surfactants represents economic advantages. Moreover, natural bioemulsifiers are biodegradable and harmless, and do not inhibit pollutant degradation [60].

Occurrence of *Pseudomonas* and actinobacteria seems to be crucial for enhancement in biofilms formation and improvement in biodegradation performances when comparing with pure cultures. The importance of the coexistence of diverse bacterial phenotypes in the physiochemical structure of biofilms cultures was already proposed by Ehlers and Turner, resulting in higher removal values [61]. Interactions suspected in multi-species biofilms change (qualitative or quantitative) the volume and function of the culture. In general, enhanced bacterial diversity and thereby also functionality of multi-species biofilms does generate an improvement in potential for biodegradation as was showed by Stach and Burns [62]. Relating presence of *Pseudomonas* and actinobacteria with obtained results in biofilm formation, removal performances, and bioemulsifier production, microbial interactions between members of C15 to reach a collective behavior could be hypothesized. Additional experiments must be performed to describe this behavior.

5. Conclusion

The advantages of C15 mixed culture compared with pure cultures were previously confirmed in our laboratory and a biofilm-immobilized approach was successfully proposed in this work. No beneficial behavior was observed in phenanthrene degradation after C15 was biofilm-immobilized. However, pyrene removal and bioemulsifier production were notably enhanced comparing with planktonic condition. Biofilm formation and preparation for biodegradation process was developed in two different conditions considering presence or absence of PAHs. The novelty of this research is that although no differences in biofilm structure were found, the desired enhancement on biodegradation abilities of C15 was observed only when hydrocarbon were added during biofilm formation stage. The results obtained make C15 biofilm, formed with hydrocarbon exposition, a suitable candidate for the development of biotechnological techniques for PAHs remediation.

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Conflict of interest

All authors declare no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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