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PAH removal by immobilized bacterial cells-support systems using low-cost culture media for biomass production

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ABSTRACT

The use of immobilized biomass for polycyclic aromatic hydrocarbon (PAH) removal from a liquid phase is a very promising technology. For this technology to be economically viable, the biomass production and immobilization should be as cost-effective as possible. The aim of this work was to produce a low-cost PAH removal system consisting of an immobilized biomass on different supports. A co-culture formed by two PAH-degrading bacteria (*Pseudomonas monteilii* P26 and *Gordonia* sp. H19, autochthonous Patagonian strains) was used. Industrial by-products such as sugar cane molasses, corn steep liquor, whey permeate and crude glycerol were used in combination to formulate different low-cost growth media. Naphthalene, phenanthrene and pyrene removal was evaluated using immobilized bacteria in polyurethane foam cubes, in sand and in calcium alginate beads. The growth of *P. monteilii* P26 and *Gordonia* sp. H19 was satisfactory in the different media assayed. The culture medium containing 1% corn steep liquor-2% molasses was selected for biomass production for subsequent immobilization. The removal of PAHs in different immobilization systems could be attributed to both the supports and the bacteria (up to 88.9% pyrene removal in 12 days); thus, immobilized cell systems for removing PAHs from an aqueous phase are proposed.

1. Introduction

Pollution caused by hydrocarbons is a matter of great concern worldwide because of its toxic effects on the environment (Pinedo et al., 2013; Zhou et al., 2014; Mohebbi-Nozar et al., 2015). Specially, polycyclic aromatic hydrocarbons (PAH) are considered very dangerous since they are carcinogenic, mutagenic and recalcitrant (Patel et al., 2012; Ortega-Calvo et al., 2013; Chang et al., 2014). Many studies reflect the efforts to mitigate the damage caused by PAH once they are introduced in soil and water (Lei et al., 2005; Augulyte et al., 2009; Seo et al., 2009). The major strategies for PAH removal are bioremediation (either by microbes or plants), extraction with solvents or surfactants, chemical oxidation and/or physical methods such as electrokinetic remediation and thermal technologies (Khodadoust et al., 2000; Gan et al., 2009; Lau et al., 2014).

Microbial biodegradation presents advantages compared with the other methods since it is economic and environmentally friendly, and it can be applied easily under different conditions (Moscoso et al., 2012; Patel et al., 2012). When using this strategy, the microbial biomass is usually immobilized in different support materials either by biofilm formation or entrapment in polymers. It has been proved that microorganism immobilization enhances PAH biodegradation, and it facilitates the handling of the biomass and its application on the field (Singh et al., 2006; Shimada et al., 2012; Mangwani et al., 2014). In addition, the support material itself can have properties that enhance PAH removal (Lamichhane et al., 2016).

To produce the PAH-degrading biomass, economic and sustainable bioprocesses should be used. The use of waste or industrial by-products as carbon, nitrogen and energy sources for biomass production is a way to reduce costs and provides an ecological

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alternative for waste management. In the literature, there are many examples of such bioprocesses (Aruldass et al., 2014; Guerra-Rodríguez and Vázquez, 2014; Tsakona et al., 2014; Gutiérrez-Rivera et al., 2015; Marone et al., 2015), but, to the best of our knowledge, this is the first report in which low-cost culture media are used to produce PAH-degrading biomass.

In this study, four industrial by-products were used in combination to produce low-cost culture media for bacterial growth of a PAH-degrading co-culture composed of two strains: *Pseudomonas monteilii* P26 and *Gordonia* sp. H19. Previous studies already demonstrated the capability of these strains to degrade PAH using planktonic cells (Isaac et al., 2015). This study assayed the immobilization of the PAH-degrading bacteria in co-culture in different economical supports using alternative culture media for microbial growth, thus providing an economically viable process that could be used for bioremediation of environments contaminated with PAH as well as treating the wastewaters.

2. Materials and methods

2.1. Microorganisms

Pseudomonas monteilii P26 (GenBank Acc. Num. HE798531) and *Gordonia* sp. H19 (GenBank Acc. Num. LN680636) were previously isolated from Patagonian contaminated sediments and selected for their ability to degrade polycyclic aromatic hydrocarbons. *P. monteilii* P26 was able to remove 100% of naphthalene and 65% of phenanthrene (initial concentration: 0.2 mM each). On the other hand, *Gordonia* sp. H19 was able to degrade relatively efficiently high-molecular-weight PAH, but it could not remove low molecular weight PAH from culture medium. In addition, *P. monteilii* P26 showed bioemulsifying activities in the presence of the tested hydrocarbons (Isaac et al., 2013, 2015). According to its PAH-degrading capabilities, emulsifying activity and negative antagonism (Isaac et al., 2015), the strains *P. monteilii* P26 and *Gordonia* sp. H19 were selected to formulate a co-culture for PAH removal.

Microorganisms were stored at −20 °C in JPP broth (% m v^{-1}: NaCl, 2; yeast extract, 0.1; meat peptone, 0.2; pH = 7.0) (Riva Mercadal et al., 2010) with 20% (v v^{-1}) glycerol.

2.2. Growth optimization of polycyclic aromatic hydrocarbons-degrading bacteria (PAH-DB) using low-cost substrates

2.2.1. Culture conditions and biomass measurements

Before growth experiments, each microorganism was propagated from the frozen stock cultures in JPP broth at 30 °C in an orbital shaker at 180 rpm for 24 h or 48 h for *P. monteilii* P26 or *Gordonia* sp. H19, respectively. Optical density at 600 nm ($OD_{600\ nm}$) of inoculum was adjusted to 2.5 and 0.7 for *P. monteilii* P26 and *Gordonia* sp. H19, respectively. Each strain was inoculated (5% v v^{-1}) into 10 ml of each growth medium assayed and incubated at 30 °C in an orbital shaker at 180 rpm. For each strain, control cultures in JPP broth were performed in all assays. Samples were taken at 24 and 48 h for further analysis.

Biomass was determined by $OD_{600\ nm}$ (measured range: 0.1–1.0. Samples that were out of range were diluted with the corresponding medium, which was also used as blank) or by viable cell counts, using the plate dilution method with saline (0.85% m v^{-1} NaCl) as the dilution medium and JPP agar (JPP-1.5% m v^{-1} agar) as the culture medium. Plates were incubated at 30 °C for 48 h (in the case of *P. monteilii* P26) or for 7 days (in the case of *Gordonia* sp. H19). *P. monteilii* P26 colonies were white, mucoid and round with regular borders. *Gordonia* sp. H19 colonies presented a dark orange color, dry aspect and irregular borders. The growth experiments were repeated at least twice on different days.

2.2.2. Effects of corn steep liquor and molasses concentrations on the growth of P. monteilii P26 and Gordonia sp. H19

For each strain, growth experiments were performed applying a 4^2 complete factorial design to evaluate the effects of the two low-cost nutrients (corn steep liquor and molasses) at four concentrations (0, 0.5, 1 and 2%; concentrations were expressed in % v v^{-1} for corn steep liquor and % m v^{-1} for molasses). Sixteen different culture media were assayed, and the complete factorial design was repeated at least twice on different days. The assayed culture media were prepared from concentrated solutions of the different components (4% v v^{-1} corn steep liquor and 4% m v^{-1} molasses in deionized water, pH adjusted to 7.0, both sterilized by heat at 121 °C for 15 min). Solids present in the molasses and corn steep liquor solutions were discarded; only the supernatants were used.

2.2.3. Effects of deionized and tap water on the growth of PAH-DB in the culture media

The use of tap water instead of deionized water as the aqueous solvent in the culture medium of PAH-DB was evaluated. Each microorganism was propagated in the culture medium formulated with 1% corn steep liquor and 2% molasses, which was prepared either in deionized or in tap water. When deionized water was used (pH = 6.5), a further pH adjustment of culture medium (up to an initial pH = 7.0) was necessary because of the acidity of substrates. In contrast, when preparing the culture medium with tap water (pH = 7.8), a subsequent pH adjustment was not required.

Only in the culture medium formulated with 1% corn steep liquor and 2% molasses (prepared with tap water) were the kinetics of bacterial growth determined by taking samples at specific time intervals. Growth parameters were estimated applying the Gompertz model (Zwietering et al., 1990; Fan et al., 2004), according to the following function:

$$X = Ae^{-e\mu e/A(\lambda - t)+1}$$

where X is the biomass concentration (expressed as $OD_{600\ nm}$ or log CFU ml^{-1} values), A is the maximum biomass concentration, μ is the maximum specific growth rate (h^{-1}), λ is the lag phase time (h) and t is the growth time (h). This model was selected because it performs better than other models for describing bacterial growth. The use of a model to estimate growth parameters is preferred over the use of a linear regression using a subset of the data because it reduces the amount of measured data (Zwietering et al., 1990). However, care should be taken since not all bacterial growth curves can be described by this model. The data fit was performed using nonlinear minimization of the sum of squared residuals (SSR) with respect to the parameters, using Matlab R2012a (MathWorks Inc., USA, 2012) with the EzyFit 2.42 application.

2.2.4. Evaluation of whey permeate and crude glycerol as alternative low-cost nutrients for PAH-DB growth

Whey permeate as the nitrogen source and crude glycerol as the carbon source were combined with molasses and corn steep liquor, respectively, to evaluate alternative low-cost culture media. *P. monteilii* P26 and *Gordonia* sp. H19 were subcultured in media containing either 2% m v^{-1} molasses combined with 1 or 0.5% m v^{-1} whey permeate or 1% v v^{-1} corn steep liquor combined with 0.5 or 0.1% v v^{-1} crude glycerol. All media were prepared with tap water.

2.3. P. monteilii P26-Gordonia sp. H19 co-culture immobilization

2.3.1. Entrapment in calcium alginate

For all immobilization experiments, each microorganism was subcultured in the culture medium containing 1% corn steep liquor and 2% molasses (in tap water for 48 h at 30 °C and 180 rpm). Co-

cultures of *P. monteilii* P26 and *Gordonia* sp. H19 (5% v v^{-1} of each strain) were performed in 400 ml of the same medium and incubated in the same condition as the subcultures. Then, the cells were harvested by centrifugation (6300 *g* for 10 min), washed twice and suspended in 40 ml tap water. The bacterial suspension (8 g $_{\text{cell dry weight}}$ l^{-1}) was homogeneously mixed with 40 ml of sodium alginate (1% m v^{-1}). Aliquots of bacterial cells and sodium alginate mix were dripped (using automatic pipettes with tips loaded with 10 ml of mixture) in 10 ml of $CaCl_2$ 0.05 M (100 ml flasks). Beads were hardened in $CaCl_2$ for 15 min at 4 °C and washed twice with deionized water. The wet mass of the calcium alginate beads in each flask was 4 g.

2.3.2. Biofilm formation on polyurethane foam (PUF) and coarse sand

P. monteilii P26 and *Gordonia* sp. H19 were subcultured as described in 2.3.1. PUF cubes (5 mm size) and coarse sand (2 mm > particle size > 0.5 mm) were washed with distilled water and dried at 105 °C. 250 ml flasks containing, respectively, 2.69 g PUF cubes and 100 g sand were filled with the same medium used for subcultures, until the content was entirely submerged (150 ml and 100 ml in flasks containing PUF cubes and sand, respectively). These flasks were inoculated with 5% v v^{-1} of each strain, at 30 °C without agitation. An air pump supplied air to the flasks through a 0.22 μm nylon filter. Every 48 h, the medium with free cells was removed, and the flasks were filled again with fresh medium. This procedure was repeated for 14 days.

2.4. PAH removal

2.4.1. PAH removal by P. monteilii P26-Gordonia sp. H19-support systems and free cells

All PAH removal experiments were carried out in 100 ml flasks containing 5 ml of JPP medium spiked with 0.2 mM of each PAH assayed (naphthalene, phenanthrene and pyrene) in acetone and incubated in darkness at 30 °C and 180 rpm agitation. The acetone was allowed to evaporate for 15 min before adding the biomass.

To evaluate the PAH removal by free cells, 250 μl of a subculture of each strain previously incubated in 2% m v^{-1} molasses and 1% v v^{-1} corn steep liquor, as described in 2.3.1, were inoculated in the flasks after acetone evaporation. Uninoculated flasks were used as abiotic controls. In addition, a control comprising free heat-killed cells was assayed. For this, a 5 ml culture of P26-H19 co-culture in the same medium was incubated for 48 h at 30 °C. Then, the cells were harvested and suspended in the same volume of saline. The cell suspension was heated at 100 °C for 10 min in a boiling bath. Afterwards, the cells were harvested and suspended in the same volume of JPP spiked with 0.2 mM of each PAH. The suspension of heat-killed cells was incubated at 30 °C and 180 rpm agitation for 12 days.

To assay the PAH removal by the different systems, approximately 4 g of calcium alginate beads, 5 g of PUF cubes or 15 g of sand (wet weight in all cases), either with immobilized cells or sterile, were added to the PAH containing flasks.

For sample taking, the whole content of the triplicate flasks was extracted by adding 10 ml acetone. After vigorous homogenization, a sample of the extract was taken and centrifuged at 6300 *g* for 10 min, and the supernatant was filtered through a 0.22 μm nylon filter. The filtrate was kept at −20 °C until analysis.

2.4.2. PAH analysis

PAH concentration was determined by RP-HPLC applying an external standard calibration curve. The RP-HPLC equipment was a Waters e2695 model coupled to a PDA detector (Waters 2998, Waters Corporation, MA, USA). The samples were injected in a Phenomenex type Spherisorb 5 ODS (2) column (size 250 × 4.60 mm). Two mobile phases were used: 9:1 water:-methanol (solvent A) and methanol (solvent B). Each sample run was carried out by the following solvents program: at first, 20% solvent A and 80% solvent B for 10 min; then, 10% solvent A and 90% solvent B for 3 min; and finally, 100% solvent B for 7 min. For each PAH, percentage of removal was calculated by the following expression:

PAH removal (%) $= 100 \times [(Ci - Ct)/Ci]$, where C_i is the PAH concentration at the initial time of removal assay, and C_t is the PAH concentration at incubation time "t".

2.5. 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).

3. Results

3.1. Growth optimization of PAH-DB using low-cost substrates

Results obtained in different growth experiments were statistically analyzed for each strain. When analyzing growth data obtained from the 4^2 complete factorial design, significant positive

Table 1
Statistical evaluation (ANOVA test) of the effects produced by different low-cost substrates and incubation time on the growth of polycyclic aromatic hydrocarbons-degrading bacteria.

Source of variation	Polycyclic aromatic hydrocarbons-degrading bacteria							
	Pseudomonas monteilii P26[a]				*Gordonia* sp. H19[b]			
	SS	DF	MS	F-ratio	SS	DF	MS	F-ratio
Corn steep liquor (CSL)	133.76	3	44.58	501.11**	143.36	3	47.79	65.22**
Molasses (M)	19.88	3	6.63	74.50**	91.51	3	30.50	41.63**
Time (T)	2.35	1	2.35	26.45**	36.49	1	36.49	49.80**
CSL x M	11.09	9	1.23	13.85**	20.48	9	2.28	3.11**
CLS x T	0.58	3	0.19	2.18	8.68	3	2.89	3.95**
M x T	6.90	3	2.30	25.86**	18.95	3	6.32	8.62**
CLS x M x T	2.16	9	0.23	2.64**	9.00	9	1.00	1.37
Residuals	2.85	32	0.09		23.45	32	0.73	

SS: Sum of squares; DF: Degrees of freedom; MS: Mean squares.
** $P < 0.05$.
[a] $R^2 = 98.41\%$.
[b] $R^2 = 93.34\%$.

effects of the culture time and corn steep liquor and molasses concentrations on the $OD_{600\ nm}$ were observed for the two PAH-DB (Table 1 and Fig. 1a–b). This means that the bacterial growth was favored by the increase in the concentration of corn steep liquor (from 0 to 2%) and molasses (from 0 to 2%). A significant positive interaction effect between corn steep liquor and molasses was observed, i.e., the positive effect of molasses was higher at higher corn steep liquor concentrations (Table 1 and Fig. 1a–b).

The highest $OD_{600\ nm}$ values of *P. monteilii* P26 and *Gordonia* sp. H19 (6.58 ± 0.15 and 9.80 ± 0.23, respectively) were obtained in the presence of 2% corn steep liquor and 2% molasses (2%CSL-2%M) at 48 h of incubation. For the two strains, high $OD_{600\ nm}$ values were also reached in 1% corn steep liquor-2% molasses (1%CSL-2%M) and 2% corn steep liquor-1% molasses (2%CSL-1%M) culture media. These values were significantly higher than those obtained in the control JPP broth (2.29 ± 0.30 and 1.20 ± 0.55 for *P. monteilii* P26 and *Gordonia* sp. H19, respectively) (data not shown). The numbers of viable cells were determined in these culture media (2%CSL-2%M, 1%CSL-2%M, 2%CSL-1%M), and for each strain, the CFU ml^{-1} values obtained were similar (approximately 10^9 CFU ml^{-1}; data not shown). The media containing 2% corn steep liquor were too acidic, and a high amount of NaOH was required to adjust the

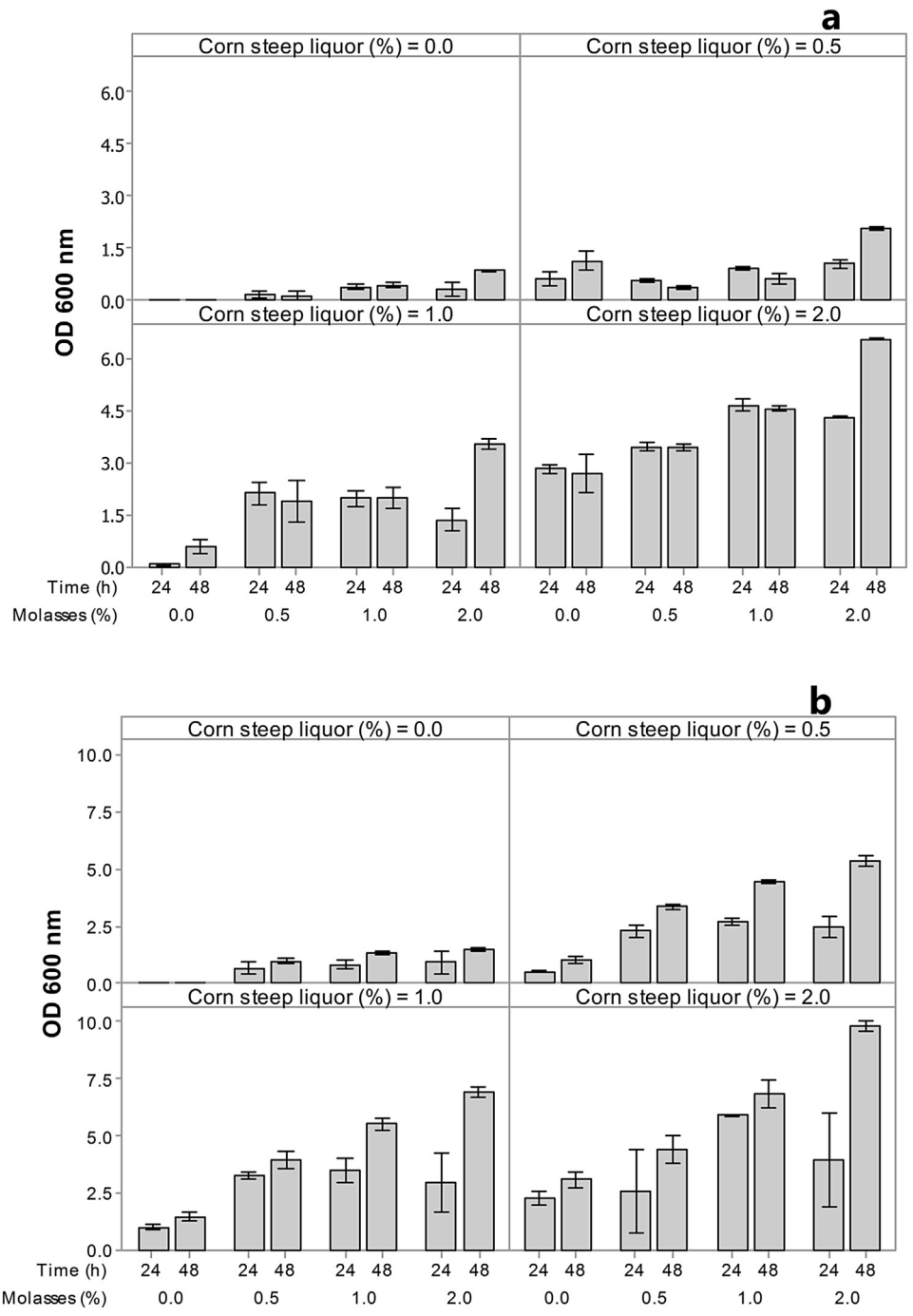

Fig. 1. Microbial growth of a) *Pseudomonas monteilii* P26 and b) *Gordonia* sp. H19 on different culture media containing molasses and corn steep liquor. The data express the mean $OD_{600\ nm}$ values ± standard error from at least two independent experiments.

appropriate pH for the bacterial growth, increasing the culture medium cost. Therefore, 1% corn steep liquor-2% molasses culture medium was selected for further assays.

When using tap water as the aqueous solvent for the 1% corn steep liquor-2% molasses culture medium, viable cell counts were slightly but not significantly higher than those obtained when using deionized water (8.80 ± 0.41 vs 8.27 ± 0.16 log CFU ml^{-1} for *P. monteilii* P26; 9.00 ± 0.16 vs 8.40 ± 0.79 log CFU ml^{-1} for *Gordonia* sp. H19) (Fig. 2a–b). Fig. 2 also shows the numbers of viable cells obtained when using whey permeate as the nitrogen source in combination with molasses and when using glycerol as the carbon source in combination with corn steep liquor. In the case of *P. monteilii* P26, viable cell counts in media containing whey permeate (8.93 ± 0.16 log CFU ml^{-1}) were slightly but not significantly higher than those reached in media with glycerol (8.44 ± 0.09 log CFU ml^{-1}). In the case of *Gordonia* sp. H19, CFU ml^{-1} values in 1% corn steep liquor and 0.5% glycerol (8.80 ± 0.09 log CFU ml^{-1}) were slightly higher than in the presence of whey permeate (8.35 ± 0.36 log CFU ml^{-1}).

In demonstrating that the biomasses of the two PAH-DB assayed were similar (Fig. 2a–b) and since corn steep liquor and molasses were more readily available than whey permeate and glycerol, 1% corn steep liquor-2% molasses medium in tap water was selected for PAH-DB biomass production for subsequent immobilization. In the selected culture medium, growth kinetics were evaluated and growth parameters, both from $OD_{600\ nm}$ and log CFU ml^{-1} values, were estimated using the Gompertz model. Growth curves from $OD_{600\ nm}$ data showed a better fit to the model than growth curves

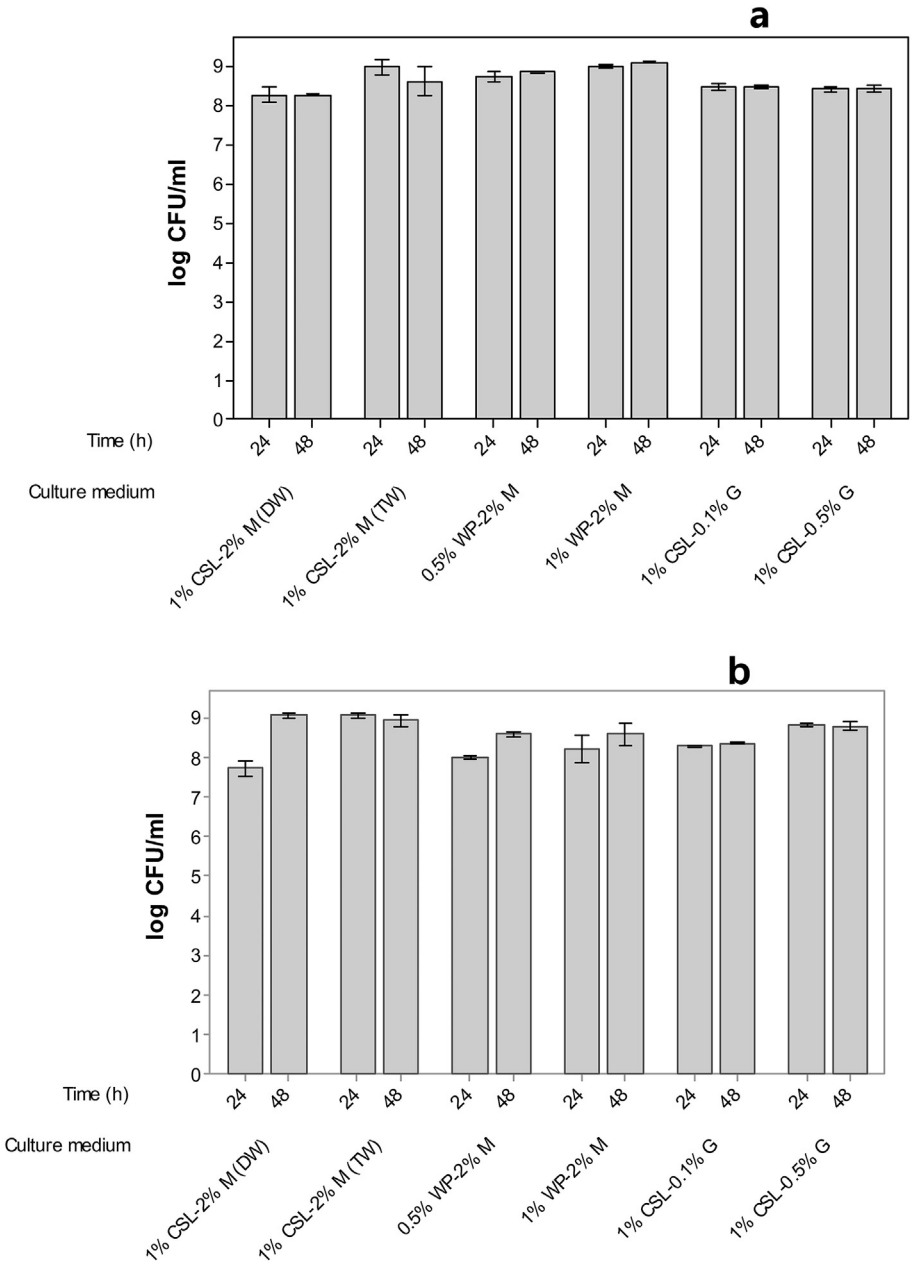

Fig. 2. Microbial growth of a) *Pseudomonas monteilii* P26 and b) *Gordonia* sp. H19 on several culture media containing different carbon and nitrogen sources (CSL: corn steep liquor. DW: deionized water. TW: tap water. M: molasses. WP: whey permeate. G: glycerol). The data express the mean log CFU ml^{-1} values ± standard error from at least two independent experiments.

Table 2
Growth parameters of polycyclic aromatic hydrocarbons-degrading bacteria obtained from optical density measurements and viable cell counts, using the Gompertz model.

Growth parameters	Polycyclic aromatic hydrocarbons-degrading bacteria							
	Pseudomonas monteilii P26				*Gordonia* sp. H19			
	$OD_{600\ nm}$		Log CFU ml^{-1}		$OD_{600\ nm}$		Log CFU ml^{-1}	
	JPP[b]	1% CSL-2% M[c]	JPP	1% CSL-2% M	JPP	1% CSL-2% M	JPP	1% CSL-2% M
A[a]	2.08	3.19	9.36	8.97	1.88	8.31	ND	9.17
μ[a]	0.40	0.27	0.74	0.59	0.05	0.34	ND	0.19
λ[a]	0.77	1.34	0.00	0.00	6.25	9.10	ND	0.00
R^2	0.99	0.99	0.86	0.74	0.92	0.99	ND	0.84

ND: not determined (lack of fit).
[a] Parameters of the Gompertz model: A, maximum biomass concentration, μ, maximum specific growth rate (h^{-1}), λ, lag phase time (h).
[b] Control culture medium.
[c] 1% corn steep liquor-2% molasses culture medium.

from log CFU ml^{-1} data, as indicated by the R^2 values (Table 2). When analyzing growth kinetics from $OD_{600\ nm}$ data, maximum biomass concentrations (A) of the two strains were higher in 1% corn steep liquor-2% molasses than in standard JPP broth (Table 2 and Fig. 3). In the case of *Gordonia* sp. H19, maximum specific growth rate (μ) was markedly higher in the selected culture medium than in the control JPP broth.

When analyzing growth kinetics from viable cell data, maximum biomass concentration (A) of *P. monteilii* P26 was similar in 1% corn steep liquor-2% molasses and standard JPP broth (Table 2); maximal log CFU ml^{-1} values were reached at 8 h of culture (Fig. 3). In the case of *Gordonia* sp. H19, maximal log CFU ml^{-1} values in the selected medium were observed at 24 h of culture; these values were approximately 1.5 log units higher than those reached in control JPP (Fig. 3).

3.2. PAH removal by P. monteilii P26-Gordonia sp. H19-support systems and free cells

Bacterial immobilization was carried out by biofilm formation or entrapment using the selected medium (1% corn steep liquor-2% molasses medium in tap water) for microbial growth. The co-culture free cells and the remotion systems comprising the supports and the immobilized cells were tested for PAH removal in JPP medium. In the experiments with free cells of P26-H19 co-culture, a removal of approximately 100% naphthalene and phenanthrene after 3 days of incubation was observed, taking into account that 87% of naphthalene and 53% of phenanthrene was loss by volatilization (Fig. 4a). The removal of pyrene by this system was 16.2%. No pyrene volatilization was observed. PAH removal by free heat-killed cells showed no statistically significant difference with the results obtained with sterile JPP without supports (Fig. S1).

When using PUF as support for biofilm formation, the system showed a higher PAH removal than the support without cells (60% more for naphthalene, 28.9% more for phenanthrene and 30.5% more for pyrene) (Fig. 4b). The removal of phenanthrene and naphthalene observed by the sand and alginate systems was high even in the absence of immobilized cells (approx. 100%, adding volatilization) (Fig. 4c and d). The system P26-H19-sand removed approximately 79% of pyrene. The P26-H19-alginate system showed a removal of pyrene of 88.9% after 12 days, 27.4% more than the sterile alginate (Fig. 4d). All sterile supports removed statistically similar percentages of pyrene (approximately 55%) while the systems with immobilized bacteria removed approximately 30% more in 12 days.

4. Discussion

The use of low-cost culture media is a requisite for developing feasible and economically viable biotechnologies, as shown in recent articles. Elain et al. (2016) used leguminous and fruit processing waters as growth media for the production of polyhydroxyalkanoates. In that study, high bacterial growth levels were obtained. Michailides et al. (2014) used molasses as carbon source to produce Cr(VI)-reducing bacteria for a biological Cr(VI)-

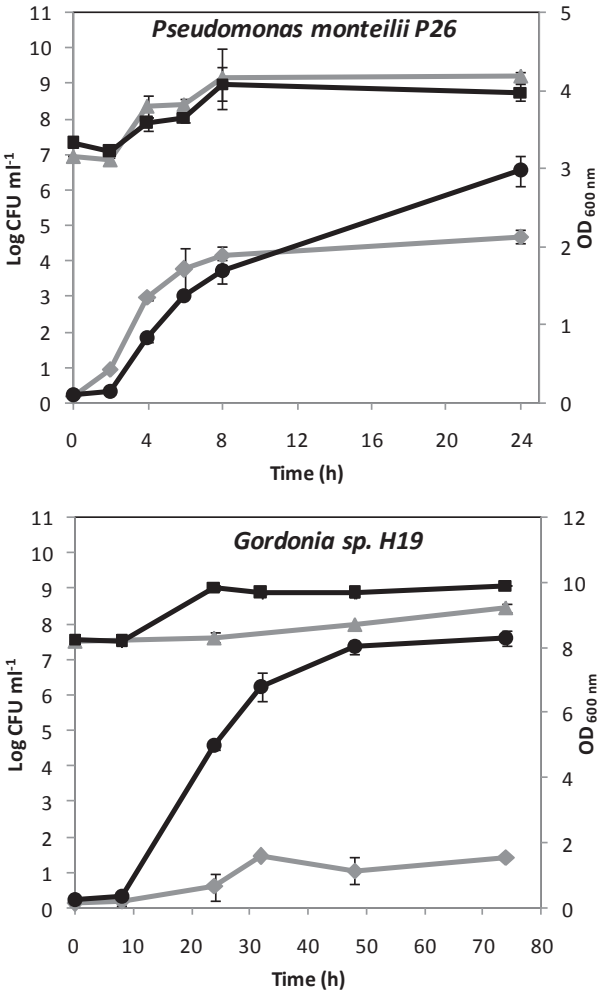

Fig. 3. Kinetics of growth of *Pseudomonas monteilii* P26 and *Gordonia* sp. H19 in 1% corn steep liquor-2% molasses (1% CSL-2% M) and control JPP broth (JPP). Bacterial growth was expressed as log CFU ml^{-1} in 1% CSL-2% M (■) and JPP (▲), and as $OD_{600\ nm}$ in 1% CSL-2% M (•) and JPP (◆). The data are plotted as the average values ± standard error from at least two independent experiments.

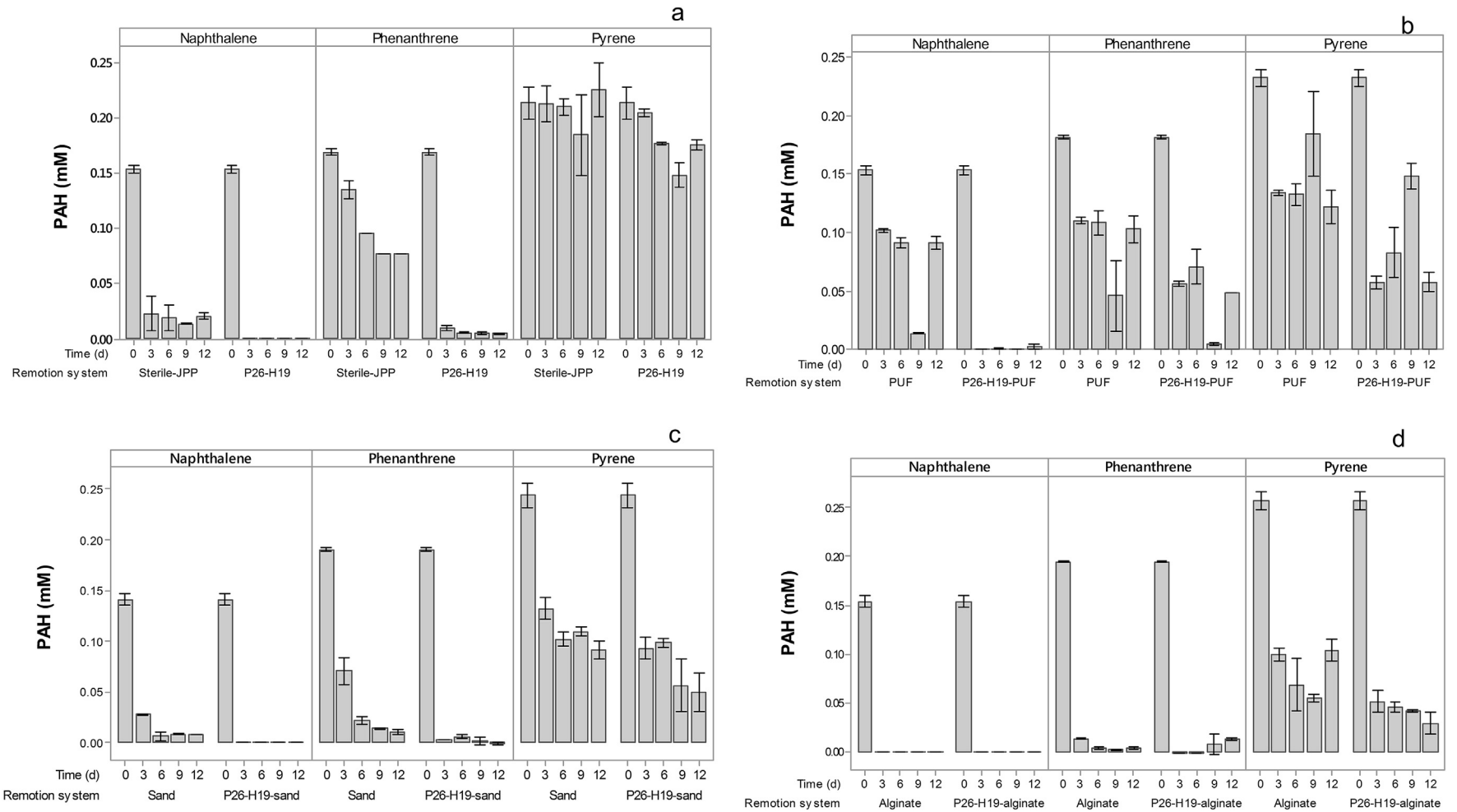

Fig. 4. Concentrations of individual polycyclic aromatic hydrocarbons (PAH: naphthalene, phenanthrene and pyrene) during removal process by different remotion systems with and without *Pseudomonas monteilii* P26-*Gordonia* sp. H19 (P26-H19) cells: a) free cells, b) PUF, c) sand and d) alginate. Values are the average ± standard error of triplicate samples.

containing wastewater treatment.

In this work, substrates that have little or no commercial value were used for biomass productions of PAH-degrading bacteria, *P. monteilii* P26 and *Gordonia* sp. H19, and high bacterial biomass concentrations were obtained in different culture media. The use of low-cost substrates in fermentation adds value to these products and alleviates industrial waste treatment. Biodiesel plants produce glycerol as a by-product, and for every 1 kg of biodiesel, 10 kg of crude glycerol are produced and many plants cannot afford its purification and further commercialization. Therefore, these industries must treat a high amount of crude glycerol before discharging it increasing the cost of biodiesel production (Pott et al., 2014; Ardi et al., 2015). Molasses, corn steep liquor and whey permeate are by-products of food industries, and although they have commercial value, they are always produced to a major extent as demanded and many times must be discarded. A culture medium formulated with 1% corn steep liquor and 2% molasses was selected on the basis of the results obtained in growth assays, in which a satisfactory growth of both *P. monteilii* P26 and *Gordonia* sp. H19 was observed. Moreover, corn steep liquor and molasses are readily available from the local industries of our region, such as sugar cane mills and food manufacturers.

Optimal culture conditions to produce microbial biomass for industrial applications are those in which the highest biomass concentration can be reached in the shortest time possible. In this study, the estimation of growth parameters using the Gompertz model supported the selection of 1% corn steep liquor and 2% molasses medium compared with standard JPP. In the case of *P. monteilii* P26, similar maximum biomass concentrations and maximum specific growth rates were observed in the selected low-cost medium and in JPP. However, in the case of *Gordonia* sp. H19, significant higher maximum biomass concentrations and growth rate were obtained in the selected medium than in JPP. Despite some differences between growth parameters estimated from absorbance measurements and from viable cell counts, similar and complementary information on the optimal culture conditions of the microbial population could be obtained.

PAH removal assays were carried out using *P. monteilii* P26-*Gordonia* sp. H19 free cells and different remotion systems that consisted of supports and the cells immobilized on them. When using free cells, the sterile JPP medium showed a high loss of naphthalene and phenanthrene but not of pyrene. Naphthalene loss was greater than phenanthrene loss. We suggest that this loss could be caused by the volatilization phenomena. In contrast, pyrene was not affected, probably because of its lower vapor pressure. This is in accordance with several works that reported PAH abiotic loss during biodegradation assays. Wu et al. (2013) reported a 30% of phenanthrene abiotic loss in the cell free controls after 10 days at 25 °C. Tao et al. (2009) observed an abiotic loss of phenanthrene between 12.4 and 15.1% in uninoculated flasks after 4 days at 30 °C.

The remotion systems using supports without cells (i.e., alginate, PUF and sand) also presented PAH removal. It is well known that PAH adsorb on many solid surfaces so we believe that there is an interaction between the PAH and the supports which could not be totally disrupted by the acetonic extraction. Interestingly, the abiotic loss of naphthalene in the free cells control was higher than in the PUF system. It seems that PUF prevents PAH volatilization either because the PAH are bound to the support or the PUF acts as a physical hindrance that prevents the vapor from reaching the atmosphere.

Recently, Lamichhane et al. (2016) reviewed PAH removal by sorption in activated carbon, such as biochar and modified clay minerals. Likewise, Biswas et al. (2015) studied the adsorption of PAH in clay minerals. PAH can also be adsorbed on exopolymeric substances (EPS) produced by bacteria (Liao et al., 2015). While the acetone extraction should be sufficient to solubilize the sorbed PAH on EPS in free cells experiments (since no PAH removal by heat-killed cells was observed), probably a harsher extraction method should be used to desorb the PAH bound to the high amounts of EPS that are present in biofilms. Therefore, in this work, we have cautiously not distinguished between biosorption and biodegradation in the removal experiments using biofilms; however, the results obtained in the experiments with free cells allow inferring that biodegradation is indeed occurring and that biosorption is a prior step in the process (Liao et al., 2015).

In this study, the pyrene removal of the systems P26-H19-PUF and P26-H19-sand was much higher than that of free cells alone. In addition to the sorption of pyrene on the supports, the nature of the biofilms could contribute to a higher pyrene removal. Zhang et al. (2015) found that biofilms degraded 32% more pyrene than cells with extracted EPS that could not form a biofilm. According to these authors, this degradation enhancement could be attributed to several factors: a) the bacterial cells in biofilms could be protected from hydrophobic damage caused by PAH; b) when forming biofilm, the cells are at high densities, and there might be a preferential gene expression of enzymes that are involved in PAH degradation; and c) the bioavailability of PAH could be increased for the biofilms by enlarging the area of contact between the hydrocarbons and the bacteria. In addition, the sorption of PAH degradation intermediates on the support surface could also enhance biodegradation by shifting the reaction towards more degradation (Biswas et al., 2015).

In this work, the P26-H19-alginate system showed a slightly higher removal of pyrene than the biofilm-based systems. When comparing the two immobilization methods used here, entrapment in calcium alginate is less time consuming than biofilm formation and enables the control of the bacterial concentration to be immobilized. On the other hand, calcium alginate is usually costlier than the supports used for biofilm formation. Hence a compromise relationship between time, costs and efficiency must be taken when deciding the more suitable system for PAH removal from a liquid phase.

5. Conclusion

In the present study, alternative culture media formulated with industrial by-products were successfully used for the production of biomass with PAH removal capability. This biomass was immobilized in different supports either by biofilm formation or by cell entrapment, generating different PAH remotion systems. In general, the systems with immobilized cells were more efficient for PAH removal than the systems that consisted of the supports alone. The combined PAH removal of the bacterial cells and the supports plus the low-cost biomass production make this technology a very promising one that deserves further research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2017.01.038.

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