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RESEARCH ARTICLE

Assessment of *Arthrobacter viscosus* as reactive medium for forming permeable reactive biobarrier applied to PAHs remediation

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are significant environmental contaminants as they are present naturally as well as anthropogenically in soil, air and water. In spite of their low solubility, PAHs are spread to the environment, and they are present in surface water, industrial effluent or groundwater. Amongst all remediation technologies for treating groundwater contaminated with PAHs, the use of a permeable reactive biobarrier (PRBB) appears to be the most cost-effective, energy efficient, and environmentally sound approach. In this technology, the microorganisms are used as reactive medium to degrade or stabilize the contaminants. The main limits of this approach are that the microorganisms or consortium used for forming the PRBB should show adequate characteristics. They must be retained in the barrier-forming biofilm, and they should also have degradative ability for the target pollutants. The aim of the present work is to evaluate the viability of *Arthrobacter viscosus* as bioreactive medium for forming PRBB. Initially, the ability of *A. viscosus* to remove PAHs, benzo[a]anthracene 100 μM and phenanthrene 100 μM was evaluated operating in a batch bench-scale bioreactor. In both cases, total benzo[a]anthracene and phenanthrene removals were obtained after 7 and 3 days, respectively. Furthermore, the viability of the microorganisms was evaluated in the presence of chromium in a continuous mode. As a final point, the adhesion of *A. viscosus* to sepiolite forming a bioreactive material to build PRBB was demonstrated. In view of the attained results, it can be concluded that *A. viscosus* could be a suitable microorganism to form a bioreactive medium for PAHs remediation.

Keywords *A. viscosus* · Benzo[a]anthracene · Bioreactive medium · PAHs · Phenanthrene · PRBB

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are significant environmental contaminants as they are present naturally as well as anthropogenically. Their presence in surface water and groundwater is due to the atmospheric sedimentation, rainwater, industrial effluent (He and Balasubramanian 2010) and soil leaching (Manoli and Samara 1999). PAHs degradation is interesting because of their biological and mutagenic effects, toxicity and elevated carcinogenic grade (Haritash and Kaushik 2009). Nowadays, the water decontamination process is limited by an excessive usage of chemicals, expensive plant requirements and high operational costs (Quintelas et al. 2010). Amongst all remediation technologies for treating contaminated groundwater, the use of permeable reactive barriers (PRBs) appears to be the most cost-effective, energy efficient and environmentally sound approach (Thiruvenkatachari et al. 2008).

In this approach, groundwater flows through a highly permeable reactive zone of the system, under the effect of

the natural hydraulic gradient. Therefore, the dissolved contaminants in the plume can be captured by sorption in the barrier filling material, removed by chemical reactions or biodegraded by the microorganisms attached to the barrier. PRBs are considered passive systems, that is to say that only limited maintenance is necessary and no ongoing energy input is required (Saponaro et al. 2009).

This technology can increase its efficiency by the coupling of another remediation technique such as bioremediation. The main principle of bioremediation technology is to remove pollutants from the natural environment and/or transform the pollutants to less dangerous products using microbiological activity. Several researches have determined the biodegradative capacity of PAHs of both bacteria and fungi in contaminated land and waters (Bamforth and Singleton 2005). It involves the breakdown of organic compounds through mineralization into inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic). Thus, PRBs can be coupled with bioremediation forming the named permeable reactive biobarrier technology (PRBB). In this technology, the microorganisms retained in a porous support are used as reactive medium to degrade or stabilize the contaminants (Tiehm et al. 2008). The main limits of this approach are that the microorganisms or consortium used for forming the PRBB should show adequate characteristics such as the barrier-forming biofilm with degradative ability for the target pollutants.

Arthrobacter species are of particular interest because of their high potential for bioremediation. These bacteria can detoxify metal wastewater by reduction or accumulation inside the cells and/or adsorption on their surface (Blázquez et al. 2009). Furthermore, several strains of this genus have been reported as degrading of aromatic compounds which include 4-fluorophenol, 4-chlorobenzoate, phenol, *p*-hydroxybenzoic acid and mono- and dichlorinated biphenyls (Ferreira et al. 2008; Furukawa and Chakrabarty 1982; Karigar et al. 2006; Zaitsev et al. 1991). Amongst these strains, the species *Arthrobacter viscosus* shows high potential as bioreactive medium for the development of PRBB. This microorganism is well known for the removal of several organic compounds such as diethylketone, phenol, chlorophenol, *o*-cresol (Costa et al. 2012; Quintelas et al. 2010) as well as metals (Lameiras et al. 2008; Pazos et al. 2010). On the other hand, *A. viscosus* is a non-pathogenic soil bacterium that produces a high amount of viscous extracellular polysaccharides (López et al. 2003). This characteristic permits prediction of good adhesion to different support structures favouring its use as bioreactive medium. Nevertheless, to the best of our knowledge, there is no report in the literature on the use of PAHs as carbon source and energy by *A. viscosus*.

Based on these previous results, the aim of the present work is to assess the viability of *A. viscosus* as bioreactive medium for forming PRBB to PAHs removal. Accordingly, the ability of *A. viscosus* to PAHs removal was assayed in a bench-scale bioreactor, in batch and continuous mode. Finally, the growth of *A. viscosus* over a low-cost support was evaluated.

Materials and methods

Microorganism and growth

A. viscosus (CECT 908) was obtained from the Spanish Type Culture Collection of the University of Valencia (Spain). The bacterium was maintained at 4 °C on solid agar petri plates that contained per litre: 10 g glucose, 5 g peptone, 3 g malt extract, 3 g yeast extract and 15 g agar

The bioreactor inoculum was obtained by submerged bacterium cultures prepared in 250-mL Erlenmeyer flasks with 50 mL of a culture medium (CM). The CM contained per litre: 10 g glucose, 5 g peptone, 3 g malt extract and 3 g yeast extract. The medium pH was initially adjusted to 7 with HCl 0.5 M. The CM was autoclaved at 121 °C for 20 min. The flasks were inoculated, plugged with cellulose stoppers for passive aeration and incubated in an orbital shaker at 28 °C and 180 rpm.

Support

Naturally occurring sepiolite, provided by Tolsa S.A., was assayed as a low-cost support for the retention of *A. viscosus* bacterium. This support has high porosity and allows the exchange of nutrients and/or the flow of the medium through it. The clay mineral has a particle size between 0.5 and 0.125 mm, and its composition is detailed in Table 1.

Polluted media

The polluted medium (PM) was composed of malt extract 3 g/L, yeast extract 3 g/L, Tween 80 1 %, acetone 2 % and 100 μM PAHs (Moscoso et al. 2012a, b; Rosales et al. 2012).

Table 1 Sepiolite mineralogical analysis by X-ray fluorescence (FRX)

Compound	Percentage
SiO_2	63.45
MgO	22
Al_2O_3	6.81
CaO	3.48
K_2O	1.9
Fe_2O_3	1.63
Na_2O	0.3
TiO_2	0.246
SO_3	0.11
MnO	0.04
P_2O_5	0.04

In the experiment where the presence of metals was evaluated, this medium also contained 100 mg/L of Cr(VI). The pH of the PM was initially adjusted to 7 with HCl 0.5 M, and after that, the medium was autoclaved at 121 °C for 20 min. Pollutant concentration was analysed after the sterilization process to assure that the pollutant was not destroy/volatilized during this process.

Bench-scale bioreactor experimental set-up

A Biostat B airlift bioreactor (B. Braun, Germany) with a working volume of 1.5 L was employed (Fig. 1). The bioreactor can operate in batch and continuous mode. The temperature was maintained at 28 °C by circulation of thermostatted water. The bioreactor was inoculated with actively growing cells from flask cultures (6 % *v*/*v*). Humidified air was continuously supplied at flow rate of 0.33 vvm, and samples were regularly taken during the experimental period. The continuous experiments were developed with similar operational conditions than the bench-scale bioreactor connected with peristaltic pumps.

Immobilization onto sepiolite experimental set-up

Immobilization of *A. viscosus* onto sepiolite was carried out via submerged aerobic culture prepared in a 250-mL Erlenmeyer flask with 50 mL of PM and 9.5 g of sepiolite clay. The flask containing the sepiolite and PM was sterilized at 121 °C for 20 min. After that, it was inoculated and incubated for 7 days at 28 °C and 180 rpm with a passive aeration permitted by cellulose stoppers.

Sample treatment and analysis

PAHs analysis

PAHs concentration in the supernatant was determined by HPLC (Agilent 1100) equipped with an XDB-C8 reverse-phase column (150×4.6 mm i.d., 5 μm). Prior to injection, the samples were centrifuged (10,000 rpm, 5 min) and filtered through a 0.45-μm Teflon filter. The injection volume was set at 5 μL, and the isocratic eluent (60:40 acetonitrile/water) was pumped at a rate of 1 mL/min for 10 min. Detection was performed with a diode array detector from 200 to 400 nm, and the column temperature was maintained at 20 °C (Alcántara et al. 2009).

Extracellular polysaccharide measure

The samples were centrifuged (8,000 rpm, 4 °C and 30 min), and the polysaccharides were estimated in the supernatant using ethanol precipitation (2:1, *v*/*v*) in the presence of 1 % KCl. After 24 h at 4 °C, the precipitated biopolymer was separated by centrifugation (8,000 rpm for 30 min at 4 °C) and quantified by dry-weight determination (López et al. 2003).

pH

The pH in liquid samples was measured directly with an IQ Scientific Instruments pH metre (model Stainless Steel ISFET pH Probes).

Biomass determination

Cell growth was determined by spectrophotometer Helios Beta (Thermo Electron Corporation) at 620 nm. Previously, the samples were centrifuged (10,000 rpm, 5 min), the pellet was resuspended in distilled water, and the obtained values were converted to gram cell dry wt per litre using a calibration curve (López et al. 2003).

Scanning electron microscopy analyses

A series of environmental scanning electron microscopy (ESEM) images was taken to provide a visual characterization of the *A. viscosus* grown over sepiolite. Images were collected on a FEI-Quanta 200 environmental scanning electron microscope using an accelerating voltage of 15 kV (Electron Microscopy Service, C.A.C.T.I., University of Vigo).

Results and discussion

The reactivity of the materials used to form a barrier is crucial in the design of PRBB. Therefore, the objective in this work was to evaluate the viability of *A. viscosus* as bioreactive medium for PAHs removal. For this purpose, several experimental tests were designed in order to determine the two main factors: degradation and adhesion ability. Initially, the ability of *A. viscosus* to PAHs removal was assayed in a bench-scale bioreactor in batch mode. Two different PAHs were tested, and the influence of chromium in the aqueous environment was also evaluated. After that, the removal capacity of the microorganism along the time was analysed in a continuous assay. Finally, the bacterial growth over a low-cost support, the sepiolite clay, was studied.

PAHs removal by *A. viscosus*: batch assays

The capability of the bacterium *A. viscosus* to degrade PAHs was evaluated using a bench-scale bioreactor in batch mode. Two different PAHs, phenanthrene (PHE), a three-ring aromatic compound, and benzo[a]anthracene (BAA), a four-ring aromatic compound, were selected. These substances are widely used as indicators of PAHs pollution and have been appointed as EPA priority pollutants (Alcántara et al. 2009;

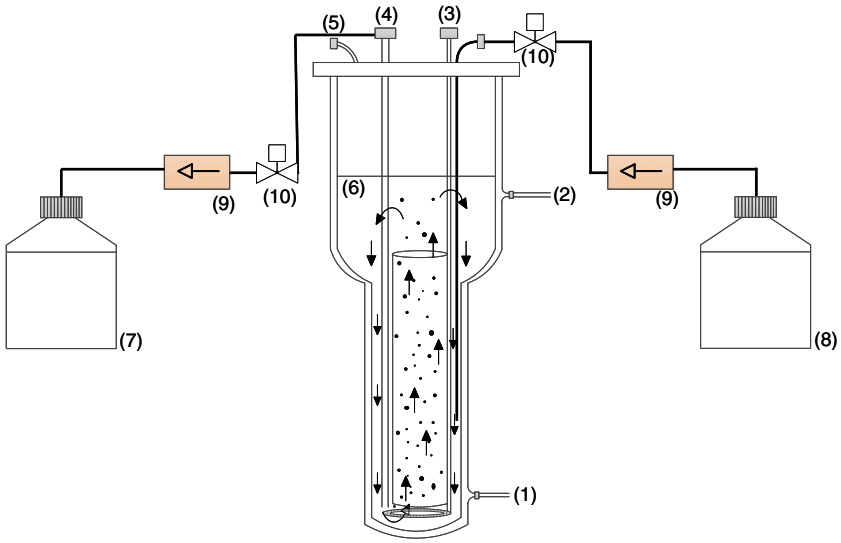

Fig. 1 Bench-scale bioreactor. Batch process *1*, heating water in; *2*, heating water out; *3*, air inlet, *4*, sampling, *5*, air outlet and *6*, microorganism and PM. Continuous process *7*, PM bottle outlet; *8*, PM bottle inlet; *9*, peristaltic pump and *10*, switch for continuous process

Sack et al. 1997). Initially, batch assays using PHE as pollutant were performed in the bioreactor (Fig. 2). In the first batch, the fast removal of PHE from aqueous solution during the first day is attributed to the adsorption of aromatic compound to the bacterium biomass. According to Raghukumar et al. (2006), once the PAHs are adsorbed by the biomass, they are subsequently metabolized by the microorganisms as carbon source. Samanta et al. (1999) determined that 30 % of removal of PHE was achieved by *Arthrobacter sulphurous* after 18 h. In the following batches, the PHE removal rates were decreased, around 40 % lower than obtained in the first batch. This fact could be due to biomass saturation as a result of the adsorption experimented in the first batch. The pH in the reactor was monitored during the treatment, and alkaline environment, pH=8–9, was determined in all batches. These results are in accordance with those reported by Peng et al. (2012). They determined that keeping the water body under a slightly alkaline condition is propitious for PAHs degradation by *Arthrobacter* species.

In a second stage, the removal of the four-ring aromatic compound, BAA, was evaluated (Fig. 3). The rapid adsorption of the pollutant in the first hours of treatment was also observed in this experiment. The removal obtained after the first day was higher than 70 %. Similarly to the previous experiment, the environment was propitious to PAHs removal because alkaline conditions, pH=8–9, were developed in the reactor during the experiments. As it was expected, according to its greater structure, the observed removal rate was lower than the previous one. Total BAA removal was obtained after 7 days. This fact is a promising result because less is known about the bacteria capable of utilizing PAHs containing four or more rings as a carbon and energy source, and it has been reported that only fungi can degrade these types of PAHs (Peng et al. 2008; Rosales et al. 2012).

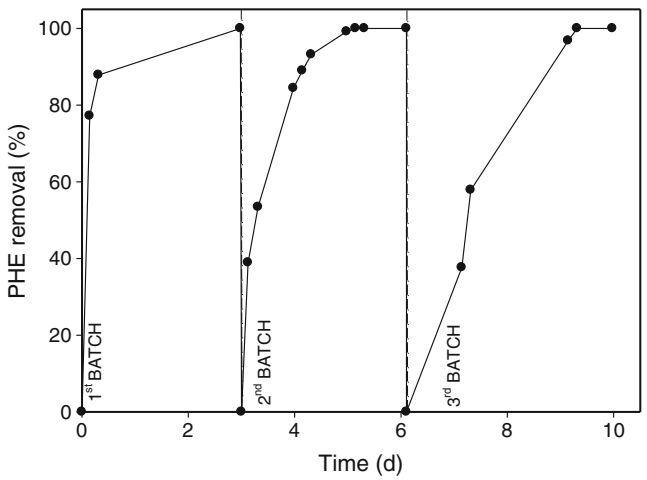

Fig. 2 PHE removal in bench-scale bioreactor by *A. viscosus* during three successive batches

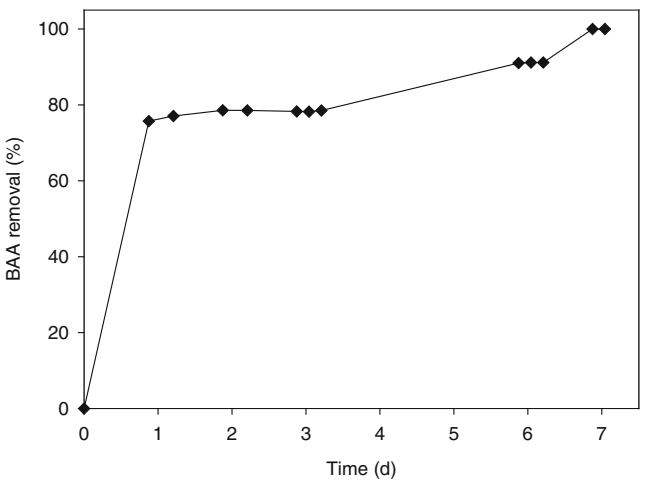

Fig. 3 BAA removal in bench-scale bioreactor by *A. viscosus*

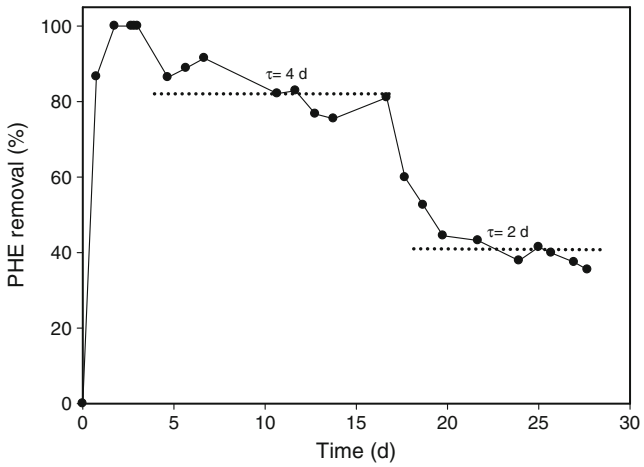

Fig. 4 PHE continuous removal in bench-scale bioreactor by *A. viscosus*

PHE removal by *A. viscosus*: continuous assay

To evaluate the loss of removal property of the microorganism along the time, a continuous experiment was developed in the bench-scale bioreactor. Two different residence times, 4 and 2 days, were tested (Fig. 4). Working at the residence time of 4 days, the reactor reached a stable performance with an average removal degree of 80 %. This removal was lower than what was obtained in batch assays, where after 3 days, total removal was obtained. This fact could be attributed to the decrease of biomass around 30 % inside the reactor. Similar behaviour was detected when the PM flow was increased. Thus, at the residence time of 2 days, the cell wash-out increased, meaning a low removal rate of around 40 %.

PHE and Cr(VI) removal by *A. viscosus*

The existence of metals in the water bodies is frequently found along with organic pollutants in the environment. Their presence can have significantly detrimental effects on the flora, fauna and human health (Akpor and Muchie 2010; Nadal et al. 2011). The common metals that have been identified in polluted water include arsenic, copper, cadmium, lead, chromium, nickel, mercury and zinc (Akpor and Muchie 2010). Therefore, the influence of PAHs remediation by *A. viscosus* in the presence of soluble metal, such as Cr(VI), was evaluated. For this purpose, a new experiment in batch mode was designed in which PHE and Cr(VI) were in the medium. In Fig. 5, the removal degrees during the time of both pollutants in the bench-scale bioreactor are shown. Following the behaviour observed in the previous studies, the PHE removal rate was higher during the first days, reaching 80 % of removal after 2 days. This fact is also related with the increase of biomass in the system of around 2 g/L. In this experiment, the presence of Cr(VI) reduced the PHE removal rate. The PHE total removal was reached after 8 days. It is postulated that the microorganisms need more time to adapt their metabolic activity to both pollutants. Nevertheless, around 72 % of Cr(VI) was also eliminated from the medium. The ability of Cr(VI) anions to overcome the permeable membrane of the bacterium can be attributed to the chemical similarity between CrO_4^{2-} and SO_4^{2-} ions (Mabbett and Macaskie 2001). Once the Cr(VI) penetrates into the cell, it can be reduced to Cr(III) that is known to be the main detoxification mechanism of *A. viscosus* due to its reductase activity (Çetin et al. 2008; Srinath et al. 2002). After treatment, total chromium was measured in the liquid medium in order to determine the chromium absorption by the biomass. Around 42 % of initial Cr(VI) was liberated to the medium as Cr(III), meaning that the biomass absorption of chromium was around 30 %. These results are promising because in our knowledge, the removal of Cr(VI) and PHE via *A. viscosus* bacterium was not reported previously.

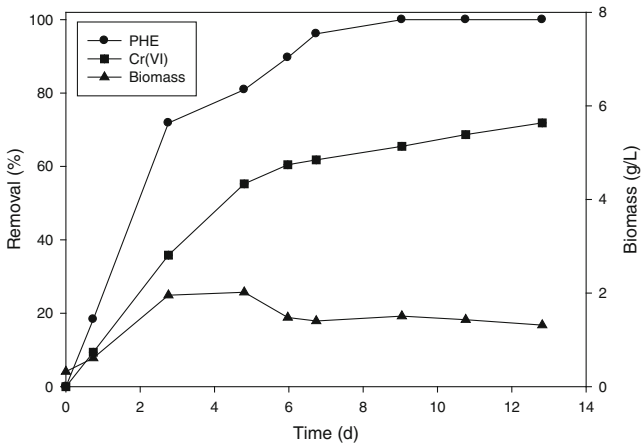

Fig. 5 Biomass concentration, PHE and Cr(VI) removal profiles in bench-scale bioreactor

A. viscosus immobilization onto sepiolite

Once the capacity of pollutant removal using the *A. viscosus* bacterium was demonstrated, the following issue was to evaluate microorganism adhesion onto an adequate support. General requirements such as porous structure, low cost, hydraulic conductivity, eco-friendliness and mechanical stability should be taken into consideration for the selection of appropriate support to PRBBs. Among the different available supports, the clay mineral sepiolite seems to be an appropriate candidate. Sepiolite exhibits microfibrous morphology with a high specific surface area (around 340 m^2/g) and a large micropore volume (around 0.44 cm^3/g) due to the existence of intracrystalline cavities ("tunnels") (Rytwo et al. 1998). Furthermore, this clay has powerful sorbent properties and ability to adsorb inorganic and organic species too (Kocaoba 2009; Quintelas et al. 2011).

On the other hand, *A. viscosus* belongs to the soil bacterium species that produces a high amount of viscous extracellular polysaccharides (López et al. 2003) which favour bacterium

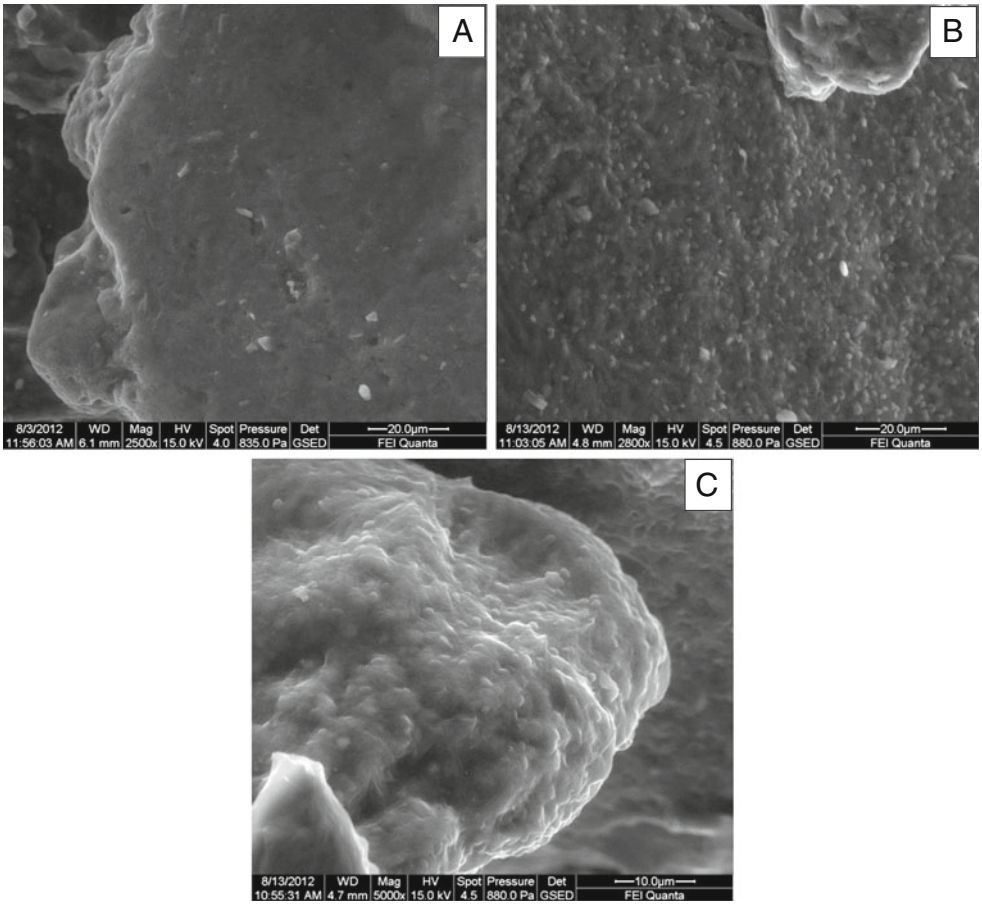

Fig. 6 ESEM images of *A. viscosus* supported on sepiolite in presence of 100 μM PHE and BAA and 100 mg/L Cr (VI). **a** initial, **b** after 7 days and **c** surface detail after 7 days

adhesion to different supports. This fact, along with sepiolite characteristics, make their combination an adequate system to form bioreactive material for PRBB. It is expected that sepiolite porous structure will favour bacterium adhesion and will allow the retention of pollutant, increasing the contact time between the pollutant and the bacterium.

The adhesion of the microorganisms onto sepiolite was studied by ESEM microscopy, and it was accomplished in the presence of 100 μM PHE and BAA and 100 mg/L Cr (VI). In Fig. 6, the ESEM images of sepiolite initially (Fig. 6a) and after 7 days (Fig. 6b and c) demonstrated that the bacterium colonized the sepiolite surface. In these images can be clearly appreciated high biomass density after 7 days. This experiment was performed in the presence of PHE, BAA and Cr (VI) in order to analyse their influence on polysaccharide secretion. After 7 days, the extracellular polysaccharides were determined in the supernatant, and a moderate level (1.89 g/g biomass) of polysaccharides was detected. These results are in agreement to that reported by López et al. (2003) who obtained similar levels of polysaccharide production by *A. viscosus*. Accordingly, it can be concluded that the presence of pollutants did not interfere in the secretion of polysaccharides. These results clearly show that sepiolite is a suitable support for *A. viscosus* to form bioreactive material for the development of PRBB.

Conclusions

In this work, the *A. viscosus* soil bacterium was assessed to form PRBB applied to PAHs removal. In a first step, *A. viscosus* bacterium was evaluated to remove high and low molecular weight PAHs operating in a batch bench-scale bioreactor. For both cases, total BAA and PHE removals were obtained after 7 and 3 days, respectively. Furthermore, the bacterium's ability for BAA and PHE removal was positively evaluated during the time period in the presence of Cr(VI). As a final point, the adhesion of *A. viscosus* to sepiolite forming a bioreactive material to build PRBB was demonstrated. Accordingly, in the assayed conditions, the developed bioreactive material can be an appropriate candidate to be employed in PRBB applied to treat aqueous samples polluted with PAHs. Further studies are necessary, using real samples to assess the efficiency of the technology.

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