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Short communication

Immobilization of *Pseudomonas putida* A (ATCC 12633) cells: A promising tool for effective degradation of quaternary ammonium compounds in industrial effluents

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ABSTRACT

Pseudomonas putida A (ATCC 12633) were encapsulated in Ca-alginate and their ability to degrade the quaternary ammonium compounds (QACs) tetradecylbenzyldimethylammonium chloride (C_{14}BDMA), hexadecylbenzyldimethylammonium chloride (C_{16}BDMA) and benzalkonium chloride (BAC) (a mixture 1:1 of C_{14}BDMA and C_{16}BDMA), was tested and compared with the degradation of the same compounds by freely suspended cells. Degradation was carried out in pH 7.4 buffered medium, at 30 °C with agitation at 100 rpm. After 24 h of incubation immobilized cells degraded 90% of 35–315 mg l^{-1} of C_{14}BDMA, C_{16}BDMA and BAC, whereas free cells were unable to degrade concentrations above 35 mg l^{-1}. Immobilized cells of *P. putida* removed 80% QACs of an industrial effluent in 48 h under both sterile and non-sterile conditions. Degradation of QACs carried out using the non-sterilized effluent showed that the beads could be reused up to four cycles without changing the degradation efficiency. The reduction in the QACs concentration after treatment with immobilized cells can be correlated with the reduction in the phytotoxicity to *Lactuta sativa* seed germination and root elongation. We conclude that immobilized QACs-degrading bacteria represent a promising tool for bioremediation of QACs in industrial wastewater systems.

Introduction

Quaternary ammonium compounds (QACs) are organic molecules extensively used in domestic, agricultural, healthcare, and industrial applications as surfactants, emulsifiers, fabric softeners, disinfectants, biocides, germicides, deodorisers, and cosmetic products (Cross, 1994; Patrauchan and Oriel, 2003). The QACs are routinely deposited on land and into water systems, as either part of an intended process or as industrial and household waste. The discharge of QACs can disturb the purifying activities of natural aquatic systems or public wastewater treatment plants because of their toxicity to microbial life (Laopaiboon et al., 2002; Oh et al., 2013). For this reason, the effective degradation of QACs is necessary to protect the environment. Several methods have been used to control QACs pollution. As one of the most economical alternatives, biological treatment is found to be an effective way to remove QACs. Although QACs are considered biologically degradable under aerobic conditions, it is known that the biodegradability of QACs generally decreases not only with the number of alkyl groups but also by the substitution of a methyl group with a benzyl group (Ying, 2006).

Pseudomonas is frequently reported as the main bacterial genus isolated from QACs contaminated environments and several strains within this genus showed ability to degrade QACs (van Ginkel et al., 1992; Patrauchan and Oriel, 2003; Takenaka et al., 2007). However, the use of free cells to degrade various toxic chemical compounds presents many disadvantages, such as low density of the cell population, low bacterial survival rates, and reduced activity and biodegradation efficiency added to the difficulty in biomass effluent separation (Wang et al., 2007). Immobilization techniques have been developed to cope with such disadvantages. Cross-linked Ca-alginate is one of the most commonly used immobilization polymers due to its highly porous structure that facilitate diffusion of solutes and dissolved gases (Arica et al., 2003). Cross-linking is simple, relatively mild, and does not have any toxic effects on the cells (Sergio and Bustos, 2009). An earlier study performed by our

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research group showed that cells of *Pseudomonas putida* A (ATCC 12633) immobilized in Ca-alginate were able to effectively degrade the QAC tetradecyltrimethylammonium bromide (TTAB) (Bergero and Lucchesi, 2013). Other predominant QACs found in engineered and natural systems is the benzalkonium chloride (BAC) (Kreuzinger et al., 2007; Hajaya and Pavlostathis, 2012). Unlike TTAB, BAC contains a quaternary N bound to two methyl groups, a benzyl group, and a straight-chained alkyl group that varies in length (typically, C12, C14, and C16). Despite being one of the most common QACs used, relatively limited information is available with respect to BAC degradation (Patrauchan and Oriel, 2003; Tezel et al., 2012). This work was focused in testing the ability of free and immobilized cells of *P. putida* A (ATCC 12633) to degrade BAC under aerobic conditions. Degradation assays were performed using two components of BAC of different alkyl chain lengths, tetradecylbenzyldimethylammonium chloride (C_{14}BDMA) and hexadecylbenzyldimethylammonium chloride (C_{16}BDMA) and a 1:1 mixture of C_{14}BDMA and C_{16}BDMA (BAC). The advantages of using *P. putida* A (ATCC 12633) immobilized cells for the development of a clean technology to achieve QACs removal from industrial effluents are also presented.

Materials and methods

Organisms and culture conditions

P. putida A (ATCC 12633) were grown aerobically at 30 °C, in a rich Luria–Bertani (LB) medium with shaking. Growth was measured as absorbance at 660 nm (OD_{660}, Beckman DU 640 spectrophotometer). Survival was assessed by viable cell count (cfu ml^{-1}), on LB plates (Duque et al., 2004).

Batch degradation assays with free cells and by Ca-alginate immobilized cells

P. putida A (ATCC 12633) cells were grown in LB medium for 12 h at 30 °C. The cells were harvested by centrifugation at 8000 × *g* for 8 min at 4 °C, washed twice with sterile 0.9% NaCl and resuspended in buffered medium to an OD_{660} of 1.9. After that, 0.75 ml of this suspension were used to inoculate 50 ml of buffered medium (20 mM Tris–HCl pH 7.4, 44 mM KCl, 85 mM NaCl and 0.8 mM $MgSO_4$, Bergero and Lucchesi, 2013) in 500-ml flasks supplemented with BAC, C_{14}BDMA or C_{16}BDMA at either 35 mg l^{-1} or 50 mg l^{-1}. Incubation was performed at 30 °C with shaking (100 rpm). Finally, samples were taken at different times and centrifuged at 8000 × *g* for 10 min at 4 °C; supernatants were used for the analysis of residual surfactants by a colorimetric method (Cross, 1970).

For degradation assays with immobilized cells, *P. putida* A (ATCC 12633) cells were encapsulated in calcium alginate (Ca-alginate) according to Bergero and Lucchesi (2013). To prepare the beads, we used alginic acid sodium salt from brown algae (Sigma Chemical Co., SL, USA). A final Ca-alginate concentration of 2.7% w/v was used and the size of the trapped population was constant (1×10^8 cfu ml^{-1} beads). Degradation was evaluated in batch cultures, performed in 250-ml flasks with 50 ml of buffered medium added with 35–315 mg l^{-1} BAC, C_{14}BDMA or C_{16}BDMA and 1.2 g of alginate beads containing *P. putida* cells (1×10^8 cfu ml^{-1} of beads). Incubation conditions and analysis of residual surfactants were the as explained in the previous paragraph. In order to verify abiotic losses, assays without inoculum addition or with beads without microorganisms were performed under the same experimental conditions.

For the count of viable immobilized cells, at different intervals of time, 10 alginate beads were withdrawn under sterile conditions, rinsed twice with 0.9% NaCl sterile solution, suspended in 250 μl of a sterile solution of 0.16 M phosphate buffer, pH 7.4 and vortexed to achieve a complete dissolution of the alginate and releasing the cells into the medium (Sossa-Urrego et al., 2008). The number of viable cells (cfu ml^{-1}) was determined by plating serially diluted cell suspensions on LB plates (Bergero and Lucchesi, 2013).

Obtaining and processing of effluent samples. Physico-chemical characterization

Effluent samples were obtained from the treatment plant of a poultry industry of Río Cuarto, Córdoba, Argentina. Effluent samples of 5 l were collected in duplicate from the discharge channel, before discharge to the river. All samples were immediately transported and stored refrigerated at 4 °C until use. Effluent samples were filtered to remove large particles and their physicochemical properties were quantified in triplicates using the standard techniques described by (APHA, 2005). The values of parameters analyzed were: pH 6.5; Biochemical oxygen demand: 20 mg l^{-1} (5-Day BOD Test, APHA 5210-B); Chemical oxygen demand: 70 mg l^{-1} (Closed Reflux, Colorimetric Method, APHA 5220-D); oil and grease: 12 mg l^{-1} (Liquid–Liquid, Partition-Gravimetric Method, APHA 5220-B) and suspended solids: 35 mg l^{-1} (Total Suspended Solids Dried at 103–105 °C, APHA 2540-D). Samples used for the isolation of indigenous bacteria were placed in Erlenmeyer flasks at 100 rpm and 30 °C for 24 h. An aliquot of 0.1 ml of serially diluted samples was spread into nutrient agar (LB) plates and solid minimal medium (HPi-BSM) (Lucchesi et al., 1989) with the QAC plus 20 mM glucose and 18.7 mM NH_4Cl as the carbon and nitrogen source. The plates were incubated at 30 °C for 24–48 h and the microorganisms able to grow in both types of plates are selected. Colonies were subcultured to test purity and were then transferred to LB plates and finally stored at 4 °C. Preliminary identification of bacterial isolates was obtained studying morpho-physiological traits that included cells shape, response to Gram stain, colony morphology on solid nutrient media and API profiles using API 20 NE (API®, BioMerieux).

Toxicity test

The phytotoxic effect of the effluent was evaluated on *Lactuca sativa* pre and post removal of QACs. In this assay we evaluated seed germination as well as radicle and hypocotyl elongation of the plant. Seeds were kept in a dry environment at 4 °C. A seed was considered germinated when visible appearance of the radicle was detected. Quality controls with a germination percentage over 90 and a coefficient of variation for root elongation below 30% in control treatment (deionized water) were used (Young et al., 2012). In this assay, the buffered medium was replaced by deionized water because the buffered medium caused inhibition of germination and of root elongation. Tests were carried out in 100-mm diameter Petri dishes. Sterile disks of filter paper were placed at the base of each plate, containing either 5 ml of BAC solutions (10, 25 and 50 mg l^{-1}) or 5 ml of effluent samples pre and post removal of QACs, 20 pre-hydrated seeds were placed above each disk. Closed capsules were incubated for 120 h in darkness at 25 °C. After incubation, the number of germinated seeds as well as length of the radicle and hypocotyl of the seedlings were recorded.

Root elongation data was used to calculate the relative growth index (RGI) according to Young et al. (2012). The calculation of phytotoxicity indexes was made by using the equation: RGI = RLS/RLC, were RLS is the radicle length of the sample and RLC is the radicle length of the control. The RGI values were differentiated into categories according to the toxicity effects observed: No significant effects: $0.8 \leq RGI \leq 1.2$; Inhibition of the root elongation: $0 < RGI < 0.8$; Stimulation of the root elongation: $1.2 < RGI$.

Analytical methods

C_{14}BDMA, C_{16}BDMA and BAC were analyzed in the supernatants of the batch cultures using a colorimetric method based on the reaction of QACs with bromothymol blue (Cross, 1970). According to this method, an anionic dye-QAC ion pair is formed, which is then solvent extracted, and the color intensity is measured spectrophotometrically at 420 nm. Analyses were carried out in tubes by addition of 545 μl of 0.2 M phosphate buffer (pH 7.4), 55 μl of 5 mM bromothymol blue and 500 μl of the sample. After addition of 2 ml of chloroform the tubes were mixed vigorously in vortex. After 5 min, the resulting lower phase was removed and collected in 0.5 ml of boric acid solution (2% boric acid in ethanol 92% (v/v)) to stabilize the color complex, the concentration of which was measured at 420 nm in a spectrophotometer Beckman DU 640. The concentration of QAC was calculated by calibration graphs constructed previously. The detection limit, calculated as three times the standard deviation of the blank divided by the absolute value of the slope, was 0.05 mg l^{-1} and the limit of quantification, calculated as ten times the standard deviation of the blank divided by the absolute value of the slope, was found to be 0.17 mg l^{-1}. In this method the interference of amines (nonquaternary species) with the quantification of QACs is insignificant because the extraction of amine-bromothymol blue complexes with chloroform is reduced at pH 7.4 (Gupta and Herman, 1973).

Protein concentrations were measured according to Bradford (1976), with bovine serum albumin (Sigma Chemical Co., SL, USA) as a standard.

Results and discussion

Degradation of C_{14}BDMA and C_{16}BDMA using free-cells of P. putida A (ATCC 12633)

To evaluate consumption of C_{14}BDMA and C_{16}BDMA by free-cells we used 35 mg l^{-1} as initial concentration to supplement media since we have previously determined that this value corresponds to the lowest concentration of C_{14}BDMA and C_{16}BDMA that does not affect cell viability. When *P. putida* A (ATCC 12633) was cultivated with BAC (a mixture 1:1 of C_{14}BDMA and C_{16}BDMA) at an initial concentration of 35 mg l^{-1}, the bacterial survival was less than 10%, indicating increased susceptibility to the surfactant mixture. For this reason, the consumption of BAC by free-cells was not evaluated.

P. putida A (ATCC 12633) was able to use C_{16}BDMA as the only source of carbon and nitrogen. Cells presented a doubling time of 13 h and after 48 h they entered in stationary phase (Fig. 1). Growth, expressed as the increase in protein production by the cultures through time, was clearly correlated with the removal of C_{16}BDMA from cultures. Consequently, the net increase in cell protein content when the culture reached stationary phase was of 5.80 mg l^{-1}, and C_{16}BDMA consumption was of 24 mg l^{-1} (70% of initial concentration). Considering that the average N content of proteins is about 15%, the N incorporated into the cell biomass was about 0.87 mg l^{-1}. Although this represented about 90% of the initial N–C_{16}BDMA incorporated into the cell biomass, 0.06 mg l^{-1} of N produced was not incorporated in protein synthesis, indicating that the C_{16}BDMA was not completely metabolized. Similarly to results obtained for C_{16}BDMA, *P. putida* A (ATCC 12633) was able to use C_{14}BDMA as source of carbon and nitrogen but the degradation efficiency was lower (48% of initial concentration). C_{14}BDMA and C_{16}BDMA degradation ceased after 48 h of incubation (Fig. 1) and this may due to the catabolic pathway used by *P. putida* A (ATCC 12633) during their degradation under aerobic conditions and the biochemical properties of the enzymes involved. Several

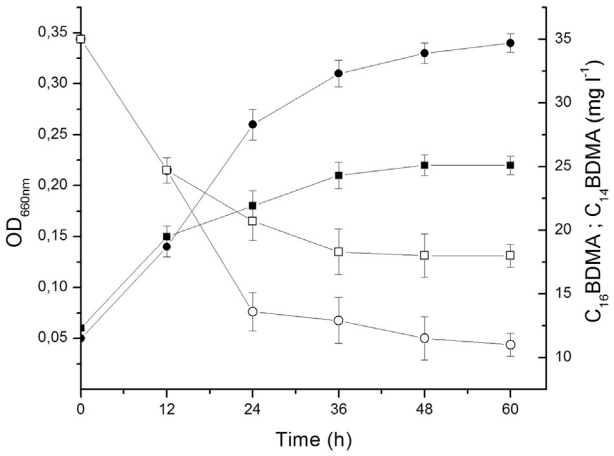

Fig. 1. Growth (filled symbols) and consumption (empty symbols) of C_{16}BDMA and C_{14}BDMA by free cells of *P. putida* A (ATCC 12633). Cells were incubated in buffered medium pH 7.5, at 30 °C with shaking in presence of 35 mg l^{-1} of C_{16}BDMA (circle) and C_{14}BDMA (square). Values are means ± SD ($n = 3$).

biotransformation pathways of QACs by microorganisms have been postulated. An initial cleavage of the C_{alkyl}-N bond was proposed as a general strategy of microorganisms to gain access to the alkyl chains of QACs (van Ginkel, 1996; Patrauchan and Oriel, 2003; Takenaka et al., 2007; Lucchesi et al., 2010; Tezel et al., 2012). The aldehyde formed from cleavage of the C_{alkyl}-N bond will be oxidized and metabolized via β oxidation as has been previously reported in other QAC-utilizing bacteria (Nishihara et al., 2000; Patrauchan and Oriel, 2003; Liffourrena et al., 2008). Nitrogen for growth may be released from benzyldimethylamine produced after the dealkylation. Benzyldimethylamine can then be metabolized through demethylation and debenzylation (Patrauchan and Oriel, 2003; Tezel et al., 2012). Although we have not examined the aerobic metabolism of C_{14}BDMA or C_{16}BDMA by *P. putida* A (ATCC 12633), this interpretation is supported by the fact that *P. putida* was able to grow on 5 mM benzylamine as the sole carbon and nitrogen source, confirming that this strain was able to remove the benzyl-N of this compound. Probably the benzyl-N group results in a lower biotransformation rate as compared to the oxidation of aldehyde formed from cleavage of the C_{alkyl}-N bond. Thus, the catalytic activity of the enzymes involved in removal of the N from benzylamine are not sufficient to completely metabolize the QAC incorporated in the cell and in consequence, benzyl-containing amines might accumulate in sufficient quantity to inhibit the QAC utilization, as reported for the degradation of C_{12}–C_{14}BDMA by a pure culture of *Aeromonas hydrophila* K (Patrauchan and Oriel, 2003).

Previous studies performed by our research group have demonstrated that *P. putida* A (ATCC 12633) is capable of degrading, in 48 h, 75% of 50 mg l^{-1} of QAC Br-tetradecyltrimethylammonium (TTAB) (Liffourrena et al., 2008). Neither growth nor degradation was observed for free cells of *P. putida* A (ATCC 12633) when using an initial concentration of C_{14}BDMA or C_{16}BDMA of 50 mg l^{-1} or greater. These results confirm the importance of the molecular structure of the compound to be biodegraded, as stated by Garcia et al. (2001) when analyzing the degradation of two families of surfactants, wherein substitution of a methyl group by a benzyl, caused a marked decrease in their biodegradability. It has also been reported that the degradation rate of Cl-TTAB by a microbial community of *Pseudomonas* spp was 5 times higher than for the Cl–C_{14}BDMA, indicating that the presence of the benzyl group resulted in a slower rate of degradation (Tezel et al., 2012).

C_{14}–C_{16}BDMA and BAC degradation using immobilized cells of P. putida A (ATCC 12633)

Degradation of C_{14}BDMA and C_{16}BDMA in concentrations that ranged from 35 to 315 mg l^{-1} was studied using immobilized cells in batch systems. After 24 h, 90% of initial 105, 210 and 315 mg l^{-1} of C_{14}BDMA or C_{16}BDMA were removed by immobilized cells of *P. putida*. When the same process was evaluated using empty beads without microorganisms, no degradation was obtained (not shown). The fastest degradation rate when using 35 mg l^{-1} of C_{14}BDMA or C_{16}BDMA was obtained using immobilized cells while values registered for free cells were lower (Table 1). This result may be attributed to the accelerated reactions caused by high local cell density in the immobilized matrix and also to the ability of the polymer to protect the cells from the surrounding environment and improve the tolerance against toxicity of high concentration of pollutants (Moslemy et al., 2002; Chen et al., 2007; Parameswarappa et al., 2008; Tallur et al., 2009). As shown in Fig. 2 and in Table 1, the immobilized cells could degrade 35–315 mg l^{-1} of BAC at a similar degradation rate to the one obtained for each compound when used separately. When using free cells neither growth nor degradation were achieved under 35 mg l^{-1} of BAC or under initial concentration of C_{14}BDMA or C_{16}BDMA of 50 mg l^{-1}. These results clearly point out the immobilized cells as the preferable system since Ca-alginate beads protected bacteria from surfactant toxicity and maintained cell viability. Beads with immobilized cells were reused in four consecutive degradation experiments, and BAC was almost 90% degraded in each cycle (data not shown). Thus, results from this experiment revealed that the immobilized cells could be reused without reduction in their BAC degradation ability.

Overall, these results reveal an advantage of the immobilized system over free cells showing high tolerance to elevated concentrations of substrate and high biodegradation efficiency.

QACs degradation from an industrial effluent by free and immobilized P. putida A (ATCC 12633) cells

Characterization of the effluent revealed a pH of 6.5, chemical oxygen demand value of 70 mg l^{-1}, QACs content of 28 ± 3 mg l^{-1} and high turbidity due to the amount of total suspended solids. The analyzed samples contained microorganisms further isolated and identified as members of the species: *Serratia marcescens, Hafnia alvei, Salmonella* and *Pseudomonas luteola.* In order to analyze the influence of the microbial community of the effluent on the degradation efficiency of *P. putida* A (ATCC 12633) we performed experiments using sterilized and non-sterilized effluent samples.

Table 1
Maximal degradation rate of C_{14}BDMA, C_{16}BDMA and BAC by free and immobilized cells of *P. putida* A (ATCC 12633).

Initial concentration (mg l^{-1})	Degradation rate (mg l^{-1} h^{-1})		
	C_{14}BDMA	C_{16}BDMA	BAC
Free cells			
35	0.6 ± 0.07	0.89 ± 0.06	ND
Immobilized cells			
35	1.09 ± 0.32	1.14 ± 0.33	1.2 ± 0.21
50	4.13 ± 0.49	4.31 ± 0.51	4.52 ± 0.91
105	12.82 ± 1.04	13.37 ± 1.23	14.03 ± 1.55
210	24.99 ± 1.46	24.02 ± 1.68	25.31 ± 1.48
315	41.37 ± 2.59	35.12 ± 2.35	41.91 ± 2.36

The values of maximal degradation rate were obtained from the slope of the linear portions of the plots of QACs removal vs time for all of the QACs concentrations tested. ND: not determined; Mean ± standard deviation (n = 3).

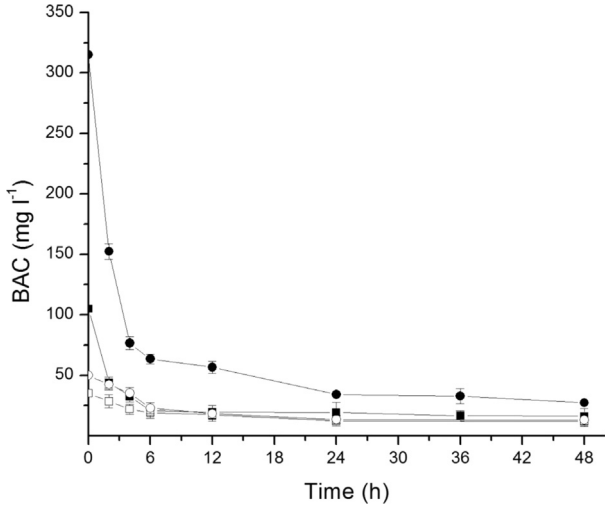

Fig. 2. Degradation of different concentrations of BAC by immobilized cells of *P. putida* A (ATCC 12633). 1.2 g of Ca-alginate beads containing cells of *P. putida* (1×10^8 cfu ml^{-1} of beads) were incubated in buffered medium pH 7.5, at 30 °C with shaking in presence of 35 (□), 50 (○), 105 (■) and 315 (●) mg l^{-1} of BAC. Values are means ± SD ($n = 3$).

Fig. 3 shows that immobilized cells degraded, after 48 h of incubation, approximately 90% of QACs presents in the sterilized or non-sterilized effluent sample. No degradation was obtained when the process was evaluated using empty beads without microorganisms indicating that the disappearance of QACs from the effluent was not due to a process of adsorption. In contrast with results obtained for immobilized cells, free-cells of *P. putida* A (ATCC 12633) degraded only a 50% of QACs present in the non-sterilized sample (Fig. 3) while when using sterilized samples, an increase of cellular mass was registered and was positively correlated with an increase in the consumption of QACs (80% after 48 h of incubation). This difference in the ability of free cells of *P. putida* A (ATCC 12633) to remove QACs when using sterilized or non sterilized samples suggest that competition between this bacteria and indigenous microorganisms of the non-sterilized samples possibly promoted a decrease in *P. putida* biodegradation efficiency.

The results of repeated batch degradation of QACs by Ca-alginate immobilized *P. putida* cells carried out with the non sterilized effluent sample showed that the beads could be reused

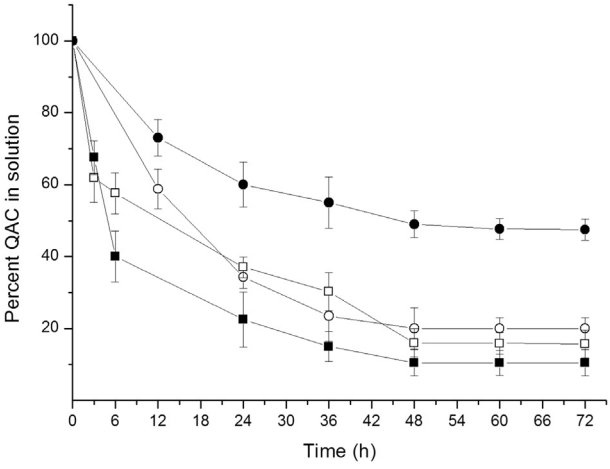

Fig. 3. Reduction in QACs content in a sterilized (empty symbols) and non-sterilized (filled symbols) effluent sample treated with free cells (circle) and immobilized cells (square) of *P. putida* A (ATCC 12633). The initial QACs concentration in the effluent was 28 ± 3 mg l^{-1}. Values are means ± SD ($n = 3$).

without changing the degradation efficiency up to 4 cycles and decreased to 75% after 5 cycles (data not shown). Similar degradation efficiencies were obtained for the sterilized sample demonstrating the advantage of the immobilized cells over free cells for degradation of the target compounds. Therefore, in addition to the ability of the immobilized system to degrade higher concentrations of QACs and the possibility of its reutilization, the beads made with Ca-alginate were able to protect *P. putida* A (ATCC 12633) from native competitors of the effluent. As described by Covarrubias et al. (2011), immobilization in alginate beads provides a physical protection for the trapped microorganism from competitors and adverse conditions of wastewater systems.

When testing toxicity of BAC to *Lactuca sativa* we found that 25 and 50 mg l^{-1} inhibited seed germination and root elongation while no significant adverse effects were observed in seeds exposed to 10 mg l^{-1} of BAC (93% of seed germination, RGI = 0.9; Fig. 4). The assay performed with the non-sterilized effluent samples resulted in a seed germination percentage of 43 and RGI was 0.18, thus showing high inhibition of the root elongation (Fig. 4). However, after 48 h of treatment with immobilized *P. putida* cells the toxicity of the non-sterilized effluent decreased, obtaining 75% of seed germination and value of 0.93 for the RGI. As described above, immobilized cells degraded in 48 h, approximately 90% of QACs present in the non-sterilized effluent sample, reducing their concentration from 28 ± 3 mg l^{-1} to 4 ± 1 mg l^{-1} (Fig. 3).

In summary, results obtained in the present study indicate that an effective degradation of QACs could be achieved by using immobilized cells of *P. putida* A (ATCC 12633). More than 80% of QACs present in the analyzed industrial effluent were degraded within 24 h by *P. putida* A (ATCC 12633) immobilized cells, the beads could be reused without changing the degradation efficiency after four cycles. Thus, the data reported here could be used in order to design an economically viable process using batch or stirred tank reactors to achieve degradation of QACs of industrial effluents.

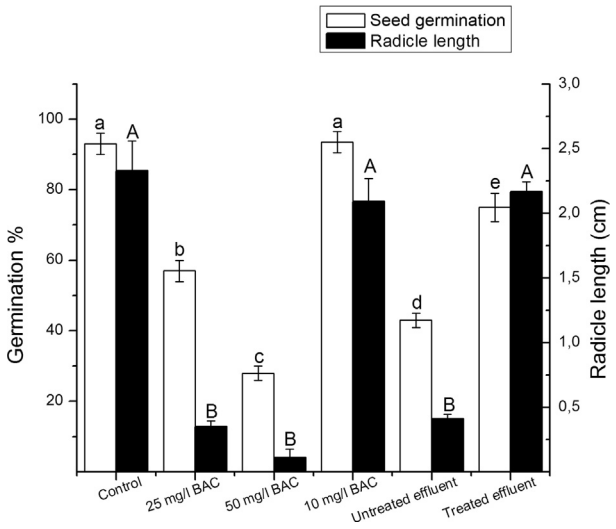

Fig. 4. Effect of QACs on germination and root elongation of *Lactuca sativa* seeds. Seeds were exposed to different concentrations of BAC and to the non-sterilized effluent by 120 h. QACs concentration of the effluent before and after of treating with immobilized cells of *P. putida* A (ATCC 12633) by 48 h, was of 28 ± 3 mg l^{-1} and 4 ± 1 mg l^{-1}, respectively. Deionized water was used as control. The number of germinated seeds and root elongation were determined as described in Materials and methods section. Bars represent means ± standard deviations of results obtained in three independent experiments from 20 seeds per sample analyzed. Bars denoted by a same letter are not significantly ($p \leq 0.05$) different according to the ANOVA test.

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