Biodegradation (2013) 24:353–364
DOI 10.1007/s10532-012-9592-3

ORIGINAL PAPER

Degradation of cationic surfactants using *Pseudomonas putida* A ATCC 12633 immobilized in calcium alginate beads

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Received: 18 June 2012 / Accepted: 10 September 2012 / Published online: 28 September 2012

Abstract In this study, the degradation of tetradecyltrimethylammonium bromide (TTAB) by freely suspended and alginate-entrapped cells from the bacteria *Pseudomonas putida* (*P. putida*) A ATCC 12633 was investigated in batch cultures. The optimal conditions to prepare beads for achieving a higher TTAB degradation rate were investigated by changing the concentration of sodium alginate, pH, temperature, agitation rate and initial concentration of TTAB. The results show that the optimal embedding conditions of calcium alginate beads are 4 % w/v of sodium alginate content and 2×10^8 cfu ml^{-1} of *P. putida* A ATCC 12633 cells that had been previously grown in rich medium. The optimal degradation process was carried out in pH 7.4 buffered medium at 30 °C on a rotary shaker at 100 rpm. After 48 h of incubation, the free cells degraded 26 mg l^{-1} of TTAB from an initial concentration of 50 mg l^{-1} TTAB. When the initial TTAB concentration was increased to 100 mg l^{-1}, the free cells lost their degrading activity and were no longer viable. In contrast, when the cells were immobilized on alginate, they degraded 75 % of the TTAB after 24 h of incubation from an initial concentration of 330 mg l^{-1} of TTAB. The immobilized cells can be stored at 4 °C for 25 days without loss of viability and can be reused without losing degrading capacity for three cycles.

Keywords Biodegradation · Tetradecyltrimethylammonium · *Pseudomonas putida* · Alginate

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Introduction

Quaternary ammonium-based surfactants (QACs) are commercial chemicals that are widely used as disinfectants in textile finishes, antiseptics, pharmaceutical and cosmetics products as well as in the food processing industry (Gilbert and Moore 2005; Zhao and Sun 2007). In quantitative terms, this utilization mainly translates into fabric softeners (66 %), coated clays (16 %) and biocides (8 %) (Cross 1994). Among the QACs, the generic term Cetrimide relates to mixtures of *N*-alkyltrimethyl ammonium bromides, where the *N*-alkyl group is between 8 and 18 carbons long, whereas Cetrimide USP is tetradecyltrimethylammonium bromide (TTAB). Additionally, benzalkonium chlorides are a mixture of *N*-alkyldimethylbenzylammonium chlorides, where the *N*-alkyl groups can be of variable length within a specified range. The QACs are routinely deposited on land and into water systems, as either part of an intended process or as industrial and household waste. Upon release into the environment,

the biodegradability of QACs is limited by their antimicrobial activity (van Ginkel 1991; Nishiyama et al. 1995). While there is little serious risk to the environment from commonly used anionic surfactants, cationic surfactants are known to be much more toxic. For this reason, the effective degradation of cationic surfactants is necessary to protect the environment. Compared to physico-chemical methods, biodegradation is regarded as an appealing alternative method to detoxify or remove pollutant chemicals from the environment because of lower costs and the possibility of complete mineralization. For this reason, the biodegradation of cationic surfactant has attracted increasing attention, and a number of QAC-degrading microorganisms have been isolated and studied (van Ginkel et al. 1995; Nishihara et al. 2000; Kroon and van Ginkel 2001; Kaech et al. 2005; Takenaka et al. 2007). In particular, *Pseudomonas putida* (*P. putida*) A ATCC 12633 is able to completely mineralize TTAB in the presence of Al^{3+} and offers promising opportunities for the efficient biological removal of this or similar QACs (Liffourrena et al. 2008; Lucchesi et al. 2010). The TTAB degradation by *P. putida* A ATCC 12633 is initiated by *N*-dealkylation catalyzed by a TTAB monooxygenase activity resulting in the formation of tetradecylalkanal and trimethylamine (TMA) (Liffourrena et al. 2008, 2009). Tetradecylalkanal is oxidized to tetradecanoic acid and metabolized via β-oxidation while the TMA, is, in part, accumulated inside the cell decreasing the bacterial growth, an effect counteracted by the addition of $AlCl_3$ (Liffourrena et al. 2008) or metabolized to NH_3 through oxidation and demethylation. In the latter case, TMA is oxidized to TMA *N*-oxide through TMA monooxygenase activity which is then demethylated to dimethylamine and methanal by a TMA *N*-oxide demethylase (Liffourrena et al. 2010). However, the use of free microorganisms for the bioremediation of contaminated sites can fail because the inoculants must be able to overcome biotic and abiotic stresses in the environment in which they are introduced. In addition, the free organisms might cause other problems such as secondary pollution due to difficulties in handling and cell separation (Gentili et al. 2006). Immobilized microorganisms offer several advantages over free cells, including protecting cells from the toxic effects of hazardous compounds and increasing their survival and metabolic activity in bioremediation systems, and therefore, it has been receiving increasing attention (Moslemy et al. 2002; Wang et al. 2008; Zhang et al. 2008; Liu et al. 2009). Calcium-alginate cross-linking is one of the most commonly used immobilization methods because the procedure is simple, relatively mild and does not have any toxic effects on the cells (Gouda 2007; Sergio and Bustos 2009; Ha et al. 2009).

In the present study, we determined the optimal entrapping conditions of calcium alginate beads and compared the degradation of TTAB by *P. putida* A ATCC 12633 in cells immobilized to calcium alginate to free cells in batch cultures. The results revealed that the immobilized cell systems can more efficiently degrade TTAB than free cells.

Materials and methods

Organisms and culture conditions

Pseudomonas putida A ATCC 12633 were grown aerobically at 30 °C, with shaking in a rich Luria–Bertani (LB) medium or in buffered medium with 50 mg l^{-1} (0.15 mM) tetradecyltrimethylammonium bromide (TTAB) as the carbon and nitrogen source. The buffered medium used contained: 20 mM Tris–HCl pH 7.4, 44 mM KCl, 85 mM NaCl and 0.8 mM $MgSO_4$. To prevent loss of TTAB by adsorption on glass surfaces, all glassware was conditioned by overnight treatment with an aqueous 5 mg l^{-1} TTAB solution (Fernández et al. 1996). Growth was measured as absorbance at 660 nm (D_{660}) with a spectrophotometer (Beckman DU 640). To determine survival, the number of viable cells (cfu ml^{-1}) was determined by plating serially diluted cell suspensions on LB plates (Duque et al. 2004).

Cell immobilization methods

Pseudomonas putida A ATCC 12633 were immobilized in calcium alginate in manner similar to that described in Ravichandra et al. (2008). Briefly, bacteria were grown in LB medium for 12 h at 30 °C. The cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C, washed twice with sterile 0.9 % NaCl and resuspended in buffered medium to a D_{660} of 1.9. Next, 10 ml of this suspension (cell content in terms of wet weight was equivalent to 0.6 g of cells) was added to 20 ml of a 4 % w/v sterilized Na-alginate solution (final concentration of alginate

being 2.7 % w/v). The solutions were subsequently stirred for 10 min, and the resulting alginate/cell mixtures were dripped into ice cold sterile 0.3 M $CaCl_2$ in 25 mM Tris HCl pH 7.4, 50 mM NaCl, which generated gel beads of approximately 3 mm in diameter. The beads were then hardened in fresh $CaCl_2$ solution with gentle agitation for 60 min. Finally, the beads were washed several times with 0.9 % NaCl to remove excess calcium ions and untrapped cells. Following this procedure 1,4 beads were obtained (equivalent to 16.8 g wet weight). The final cell content in the beads reached about 0.036 g g^{-1} beads or 1.0×10^8 cfu ml^{-1} beads.

Degradation by free cells

Pseudomonas putida A ATCC 12633 were grown in LB medium for 12 h at 30 °C. The cells were harvested by centrifugation at 8,000×*g* for 10 min at 4 °C, washed twice with sterile 0.9 % NaCl and resuspended in buffered medium to a D_{660} of 1.9. Next, 0.75 ml of this suspension (cell content in terms of wet weight was equivalent to about 0.045 g of cells) was used to inoculate 50 ml of buffered medium in 500-ml flasks supplemented with 50 mg l^{-1} TTAB. All incubations were done in an orbital shaker at 30 °C and 100 rpm. At different times samples were taken and centrifuged at 8,000×*g* for 10 min at 4 °C, and the supernatant was used for analysis of residual TTAB by a colorimetric method based on the reaction of TTAB with bromothymol blue (Cross 1970).

Degradation by Ca-alginate-entrapped cells

The experiments involving the biodegradation of TTAB were carried out with optimized immobilized cells in batch systems. Batch cultures were performed using 250-ml flasks with 50 ml of buffered medium with different TTAB concentrations (0–330 mg l^{-1}) and 1.2 g (wet weight) alginate beads (equivalent 100 beads) containing immobilized *P. putida* A ATCC 12633 cells. The initial cell content in terms of wet weight in the beads was about 36.2 mg g^{-1} beads (the total inoculum added was equivalent of 0.043 g in 50 ml medium). The degradation process was carried out at 30 °C on a rotary shaker at 100 rpm for the desired incubation period. At different intervals, samples were withdrawn under sterile conditions and used for the analysis of residual substrate TTAB. To analyze the reusability of the immobilized cells, the same beads were used consecutively in batches for seven consecutive cycles.

For the enumeration of viable immobilized cells, 10 alginate beads were rinsed twice with a sterile 0.9 % NaCl solution and suspended in 250 µl of a sterile solution of 0.16 M phosphate buffer, pH 7.4 (Sossa Urrego et al. 2008). The suspension was vortexed to achieve a complete dissolution of the alginate, and the number of viable cells (cfu ml^{-1}) was determined by plating serially diluted cell suspensions on LB plates. The colonies were counted on agar plates after incubation for 24 h at 30 °C. Sterile beads (without microorganisms) were used to monitor the abiotic loss of TTAB. All determinations were made in triplicate.

Preparation of crude extracts

Ten alginate beads containing *P. putida* A ATCC 12633 cells were previously rinsed twice with a sterile 0.9 % NaCl solution and suspended in 450 µl of 0.1 M phosphate buffer, pH 7.4. The suspension was vortexed to achieve a complete dissolution of the alginate. The cells were disrupted by sonication in an ice bath at 20,000 Hz using a Vibra cell ultrasonic processor 10 times for 10 s each time. Whole cells and cell debris were removed by centrifugation at 14,000×*g* for 15 min, and the supernatant of the cell extracts was used for trimethylamine (TMA) and TTAB monooxygenase determination.

Scanning electron microscopy analysis of beads

For each observation with scanning electron microscopy (SEM), beads were fixed using the method described by Covarrubias et al. (2011), with some modifications. Briefly, 10 beads were fixed for 5 h in 5 % (v/v) formaldehyde solution in 0.1 M Tris–HCl buffer pH 7.4), washed 3 times in the same buffer and then dehydrated with ethanol at the following concentrations: 25 % for 10 min, 50 % for 30 min, 70 % for 10 h and 100 % for 60 min. The whole procedure was carried out at 4 °C. The samples were dried under a CO_2 atmosphere in a critical point dryer (CPD 030, BAL-TEC AG.). The dried samples were mounted on a stub, covered with gold, and examined under a LEO 1450VP scanning electron microscope at 15 kV.

Analytical methods

TTAB concentrations were analyzed in the supernatants of the batch cultures using a colorimetric method based on the reaction of TTAB with bromothymol blue (Cross 1970). According to this method, an anionic dye-TTAB 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 achieved the stabilization of the solution and subsequently, was measured at 420 nm in a 1 cm cuvette using a spectrophotometer Beckman DU 640.The concentration of TTAB was calculated by calibration graphs constructed previously. The detection limit, calculated as 3 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 10 times the standard deviation of the blank divided by the absolute value of the slope, was found to be 0.17 mg l^{-1}.

The production of TMA was analyzed in the crude extracts using the fluorochrome 2′,3,4′,5,7-pentahydroxyflavone (morin reagent) with the addition of $AlCl_3$, as described by Liffourrena et al. (2008). The fluorescence of the Al^{3+}-morin complex was measured with a Fluoromax-3 spectrophotometer (Jobin–Yvon Inc, Edison, NY, USA) using an excitation wavelength of 440 nm and an emission wavelength of 494 nm. The concentration of TMA was calculated using the calibration graphs constructed by plotting the fluorescence intensity of Al^{3+}-morin complex versus the TMA concentration.

TTAB monooxygenase activity was assayed at 30 °C in a 1 ml reaction mixture containing 0.5 mM TTAB plus 0.5 mM NAD(P)H, 14 mM phosphate buffer (pH 7.4), and 0.3 mg ml^{-1} protein. After 30 min of incubation at 30 °C, the reaction was stopped by the addition of 0.5 ml of 37.5 % TCA. After centrifugation at 12,000×*g* for 10 min, aliquots of 1 and 100 µl of the supernatant were used to detect the product TMA by GC–MS (Hewlett Packard model 5890 with a mass detector HP 5972) or fluorescence, respectively, as described by Liffourrena et al. (2009).

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

Results and discussion

Effect of the concentration of the sodium alginate solution on the beads

Different concentrations of sodium alginate solutions (3, 4 and 5 % w/v) were used to produce Ca-alginate gel beads and test the shape and diameters of the beads and the viability of entrapped cells (Table 1; Fig. 1). For all of the alginate concentrations used, the bead diameter size ranged between 2.7 and 3.1 mm with an average of 3.0 mm, and the trapped population size was constant (approximately 10^8 cfu ml^{-1}). SEM images show that at a low concentration of alginate (3 %), the shape of the beads was more irregular (Fig. 1a), while regular spherical beads were formed when the concentration of alginate was 4 and 5 % w/v. As shown in Fig. 1b, c, in beads with 4 and 5 % w/v of alginate, the microorganisms are uniformly distributed inside the beads, showing that the microorganisms and materials in the mixture were properly mixed prior to the polymerization and that *P. putida* A ATCC 12633 was successfully embedded inside of the beads. However, the alginate beads prepared with 5 % of alginate showed a more compact surface structure with decreased surface roughness and porosity (Fig. 1c). These characteristics are known to increase the mass transfer resistance (Ravichandra et al. 2008; Lu et al. 2012). The mechanical stability, estimated by the

Table 1 Physical properties and cell viability of *P. putida* A ATCC 12633 beads prepared with different concentration of sodium alginate solution

Na-alginate (% w/v)	3 %	4 %	5 %
Diameter of the beads (mm)	3.0–3.1	2.9–3.0	2.7–2.8
Stability	Not stable	Stable	Stable and rigid
Viability (cfu ml^{-1})			
Time (h)			
0	3.00×10^8	2.60×10^8	2.60×10^8
24	1.35×10^8	2.15×10^8	3.20×10^8
72	2.00×10^6	2.30×10^8	2.20×10^8

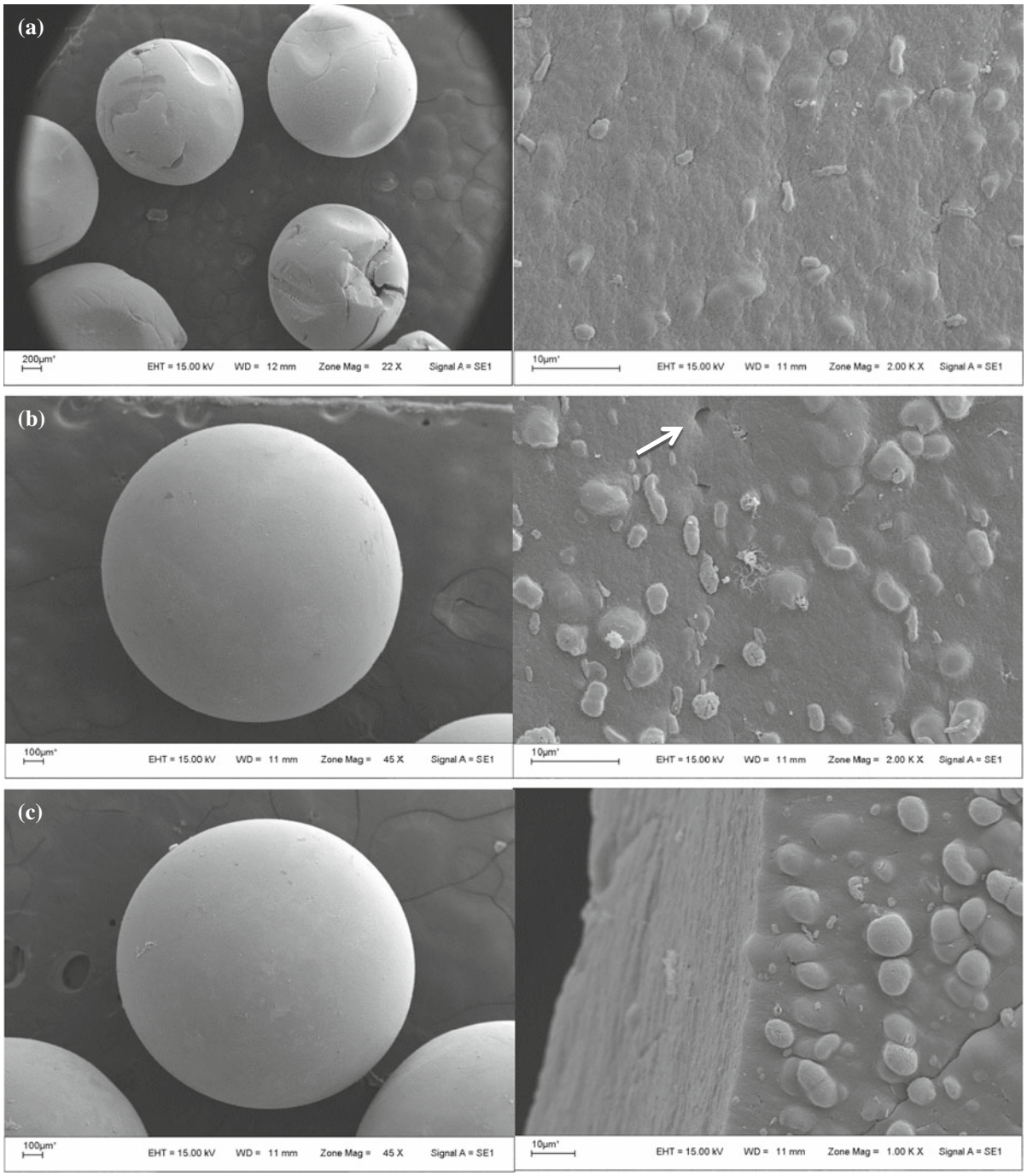

Fig. 1 Scanning electron micrographs of the whole beads and the surface of the beads. **a** Alginate 3 % **b** Alginate 4 % **c** Alginate 5 %. *Arrow head* show the pore

disintegration of beads when they were tested in batch culture, increased with increasing alginate concentrations. The beads made with 3 % alginate did not maintain their stability over time and broke after 72 h of the culture, with a corresponding loss of cells into the medium. As shown in Table 1, the initial number of entrapped bacteria was similar to the number of cells contained within the beads after 72 h of incubation for

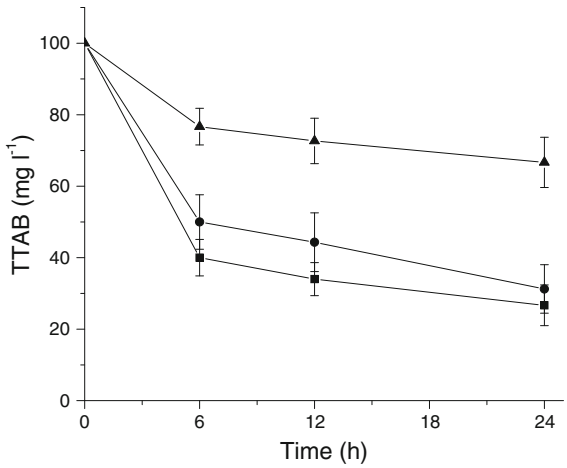

Fig. 2 Degradation of TTAB with beads of different Na-alginate concentrations: 3 % (*filled square*), 4 % (*filled circle*) and 5 % (*filled up pointing triangle*). The initial TTAB concentration was 100 mg l^{-1} and the *P. putida* A ATCC 12633 cell content in the beads was 0.045 g (w/v). Values are the mean ± SD ($n = 3$)

beads containing either 4 or 5 % alginate (approximately 10^8 cfu ml^{-1}). Although beads with both 4 and 5 % alginate had good mechanical stability and did not display any structural changes or damage after incubation, the beads obtained with 4 % of alginate showed a uniform and crinkled surface structure with good distributions of pores on the surface (Fig. 1b), which helps to reduce mass-transfer resistance. The Fig. 2 shows the data of degradation of 100 mg l^{-1} TTAB with beads of different alginate concentrations. Beads prepared with 3 and 4 % w/v of alginate, degraded approximately 70 % of the TTAB after 24 h of incubation whereas the removal efficiency of TTAB was reduced to 30 % with beads of 5 % w/v of alginate. Similar results were obtained for 200 mg l^{-1} of TTAB (not shown). Considering the good mechanical stability of the beads and the porosity and maintenance of cell viability (Table 1; Fig. 1), together with the increased use of TTAB the medium (Fig. 2), 4 % Na-alginate was selected for subsequent experiments.

Effect of storage on the viability of immobilized cells of *P. putida* A ATCC 12633

Immobilized cells were stored at 4 °C in tubes containing 1.2 g (wet weight) of alginate beads. The cfu ml^{-1} counts in fresh-made beads and after 7, 15, 25 and 40 days of storage at 4 °C show that the immobilized cells can be stored at 4 °C for at least 25 days with no appreciable loss of viability. After 40 days of storage at 4 °C, the cell viability decreased from 4.5×10^8 to 2.0×10^6 cfu ml^{-1}.

Effects of incubation conditions on TTAB degradations

It is widely known that the pH value of the degradation medium can affect the activity of enzymes, microbial growth and the structure of the beads. To obtain the optimum pH for the degradation of TTAB by immobilized *P. putida* cells, pH values of 6.5, 7.4 (original pH for the growth medium of free-cells) and 8.5 were studied under identical conditions: 30 °C, agitation at 100 rpm and an initial TTAB concentration of 100 mg l^{-1}. After 48 h of incubation time, the immobilized cells degraded almost 75 % of the TTAB present in the buffered medium at pH 7.4, whereas the removal efficiency of TTAB was reduced to 58 % at both pH 8.5 and 6.5. This result is in agreement with the optimum pH value of cell growth and the degradation of TTAB by free cells (Lucchesi et al. 2010). Hence, the optimum pH for the degradation of TTAB by immobilized *P. putida* cells is pH 7.4.

In order to evaluate the effect of temperature on the process of degradation by immobilized cells, temperatures of 30 and 16 °C were tested. For an initial TTAB concentration of 100 mg l^{-1} at pH 7.4 and 30 °C without agitation, approximately 60 % of the cationic surfactant disappeared from the medium after 48 h of incubation, while at 16–18 °C, this percentage dropped to 40 %. As expected, at a lower temperature the bacterial metabolism is slower, which is reflected in less degradation of the detergent. When the same study was carried out with agitation, an increased degradation of TTAB was detected (approximately 63 and 75 % of TTAB disappeared after 48 h at 16 and 30 °C, respectively). Diffusional problems have been studied in immobilized systems and may result in a decreased enzymatic activity for the system due to the diffusional barrier generated by the polymer and reduced availability of oxygen to immobilized cells (Kanasawud et al. 1989; Mamo and Gessesse 1997). Because the agitation promotes the exchange of oxygen between the beads and the environment and because the degradation of TTAB is an aerobic process that involves the oxidation of this substrate (Liffourrena et al. 2008), the results obtained on the

degradation of TTAB affirm the importance of using the immobilized system under controlled conditions of aeration to avoid the negative effect of low oxygen availability.

Degradation of different concentrations of TTAB by both immobilized and free *P. putida* A ATCC 12633 cells

Degradation of different concentrations of TTAB was studied with free cells and immobilized cells in batch systems. Figure 3 shows the relation between TTAB removal versus time for all of the TTAB concentrations tested. Because the bead population size was kept constant for all of the conditions (cell content in terms of wet weight was about 0.045 g) the TTAB degradation would be mediated by the same total number of cells, resulting in similar degradation efficiencies. It can be seen that the rate degradation was the fastest in the first 2 h and slows down after this period, which can likely be attributed to the availability of the biomass and its need to consume a carbon and nitrogen source (Fig. 3a). The maximum rates of degradation of TTAB for immobilized system under batch conditions were found to be 7.6 ± 1.6, 36.3 ± 3.5, 55.3 ± 4.3 and 77.9 ± 4.9 mg l^{-1} h^{-1} ($n = 3$) at concentrations of 100, 200, 260 and 330 mg l^{-1}, respectively. However, as seen in Fig. 3b, the immobilized cells degraded approximately 75 % of the TTAB after 24 h of incubation from an initial 330 mg l^{-1} of TTAB. Similar degradation efficiencies were obtained when the initial concentrations were 100, 200 and 260 mg l^{-1} of TTAB (70, 76 and 77 %, respectively) indicating that the efficiency of TTAB degradation by immobilized cells is similar as the concentration increases. This results clearly suggests that immobilized *P. putida* had high tolerance to changes in TTAB concentration. Using an initial TTAB concentration of 50 mg l^{-1}, it took both immobilized and free cells less than 24 h to degrade 50 % of the TTAB (Fig. 3b). However, no bacterial growth and degradation occurred at an initial TTAB concentration of 100 mg l^{-1} or greater with free cells. This same behavior which implies no growth or degradation of TTAB at 100 mg l^{-1} was reported for free cells of *P. putida* A ATCC 12633 cultivated in a saline medium with high concentrations of phosphates (Liffourrena et al. 2008). For comparative purposes, in the present study, the phosphate was replaced by

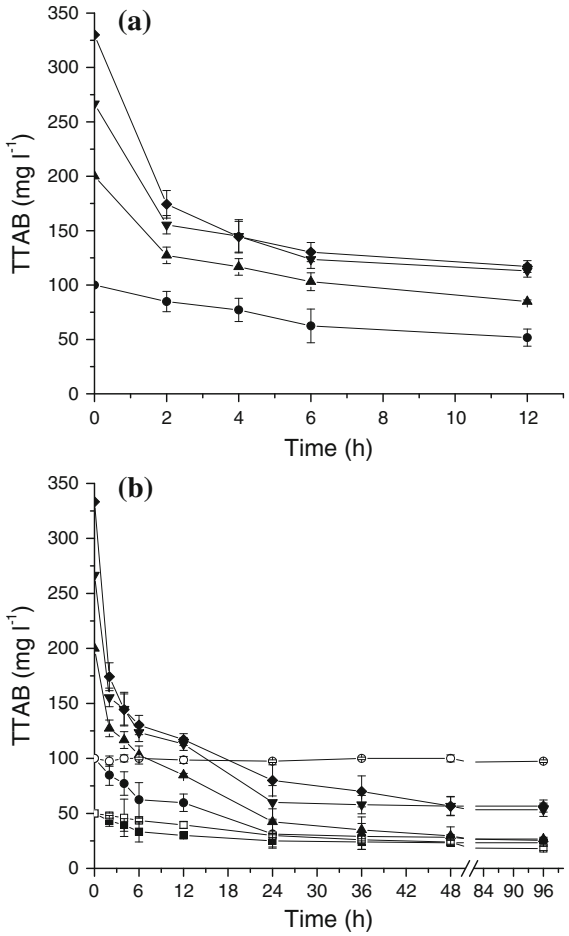

Fig. 3 Degradation for short time (**a**) and long time (**b**) of different initial concentrations of TTAB by 4 % Ca-alginate beads containing *P. putida* A ATCC 12633 cells. The cell content in terms of wet weight in the beads was 0.043 g. TTAB initial concentrations: 50 mg l^{-1} (*filled square*), 100 mg l^{-1} (*filled circle*), 200 mg l^{-1} (*filled up pointing triangle*), 260 mg l^{-1} (*filled down pointing triangle*), 330 mg l^{-1} (*diamond*). For *P. putida* isogenic mutant strain (*open circle*) and free-cells (*open square*), the initial concentration of TTAB was 100 mg l^{-1} and 50 mg l^{-1}, respectively. Values are the mean ± SD ($n = 3$)

Tris–HCl as a buffering agent because the phosphate caused extensive disruption of the Ca-alginate beads, releasing the cells into the medium. Hence, consistent with the immobilized system capacity to degrade higher concentrations of TTAB than free-cells, the beads made with Ca-alginate were able to protect the bacteria from the toxicity of TTAB. It is know that the toxicity of cationic surfactants is centered upon the physical disruption and partial solubilization of the cell wall and membrane, resulting in the death of

the bacterial cell (Macdonnell and Russell 1999). The tolerance of the immobilized cells to higher concentrations of substrate, which reveals a further advantage of the immobilized system over free cells, can be attributed to the ability of the polymer to protect the cells from the surrounding environment (Kewellow et al. 1989; Moslemy et al. 2002; Quek et al. 2006; Chen et al. 2007; Tallur et al. 2009). In addition, the immobilization provides a type of membrane stabilization, which is assumed to be responsible for cell protection and better degradation rates in immobilized cells compared to free cells (Cassidy et al. 1996; Hall and Rao 1989; Manohar and Karegoudar 1998).

The metabolic activity during the biodegradation of a pollutant may involve induction or inhibition, depending on the substrates and microbial species present. Typically, enzymes are responsible for the degradation of hazardous organic compounds (Chan and Lai 2010). To corroborate whether TTAB is metabolized by the immobilized cells, we determined the TTAB-monooxygenase activity in cell-free extracts obtained from the beads. TTAB-monooxygenase is the first enzyme of *P. putida* A ATCC 12633 that is responsible for catalyzing the breakdown of the C–N bond of TTAB (Liffourrena et al. 2008). In the immobilized system, the specific activity of TTAB-monooxygenase increased after the first 30 min of contact of the beads with 100 mg l^{-1} of the detergent (from 0.083 ± 0.03 ($n = 3$) nmol TMA min^{-1} mg $protein^{-1}$ to 1.31 ± 0.13 ($n = 3$) nmol TMA min^{-1} mg $protein^{-1}$), which was kept constant during the degradation process. This result suggests that the immobilized cells metabolized TTAB once it entered the bead. As shown in Fig. 3b, no degradation occurred at an initial TTAB concentration of 100 mg l^{-1} when beads were loaded with 2×10^8 cfu ml^{-1} of *P. putida* ASL1 cells, a knockout-mutant in which the cells lack TTAB monooxygenase activity and are unable to utilize TTAB as a growth substrate (Liffourrena 2010). In addition, no degradation was obtained when the process was evaluated using empty beads without microorganisms (not shown). Together, these data suggest that the disappearance of TTAB from the culture medium is due to degradation by *P. putida* A ATCC 12633 and not a nonspecific process of adsorption.

For all of the TTAB concentrations evaluated with immobilized cells (50–330 mg l^{-1}), after 24 or 48 h, the consumption of TTAB stopped, and a residual TTAB concentration was detected, which was approximately 20 mg l^{-1} for lower initial concentrations of TTAB (50–200 mg l^{-1}) (Fig. 3b). The addition of new beads to the media did not alter the performance of the degradation process, keeping the residual concentration at approximately 20 mg l^{-1} of TTAB. These data, coupled with the absence of degradation of an initial concentration of 20 mg l^{-1} TTAB (not shown) suggest that there is a necessary minimum concentration of detergent to achieve a gradient to facilitate its entry into the beads.

For higher initial concentrations of TTAB (260 and 330 mg l^{-1}), the residual detergent concentration was higher, reaching values close to 50 mg l^{-1}. This phenomenon can be explained by the catabolic pathway used by *P. putida* A ATCC 12633 during the TTAB degradation process. Previous studies in our laboratory have demonstrated that TTAB degradation is initiated by *N*-dealkylation catalyzed by a TTAB monooxygenase, resulting in the formation of tetradecylalkanal and TMA. The TMA product is used by *P. putida* A ATCC 12633 as a nitrogen source through the activity of trimethylamine monooxygenase and also accumulates inside the cell affecting both the bacterial growth and TTAB consumption. We have shown that the low catalytic activities of trimethylamine monooxygenase were not sufficient to completely metabolize the TMA produced in the cell by TTAB monooxygenase and, therefore, intracellular TMA is accumulated in the cell in sufficient quantities (8.9×10^{-2} mg TMA mg $protein^{-1}$) to inhibit TTAB monooxygenase (Liffourrena et al. 2010, Lucchesi et al. 2010). Thus, as it occurs with free cells, the halt in degradation capacity detected with immobilized cells may be the result of a high concentration of intracellular TMA. Figure 4a, b shows the production of intracellular TMA and the consumption of TTAB from initial TTAB concentrations of 100 and 260 mg l^{-1} using immobilized cells. When the initial concentration of TTAB in the medium was 100 mg l^{-1}, the consumption of TTAB, after a period of 24 h, was accompanied by an increase in the intracellular concentration of free TMA (9.2×10^{-3} mg TMA mg $protein^{-1}$) (Fig. 4a). At 48 h of incubation, the consumption of TTAB stopped, and the intracellular content of TMA decreased slightly to 8.1×10^{-3} mg TMA mg $protein^{-1}$ due to its metabolism by the

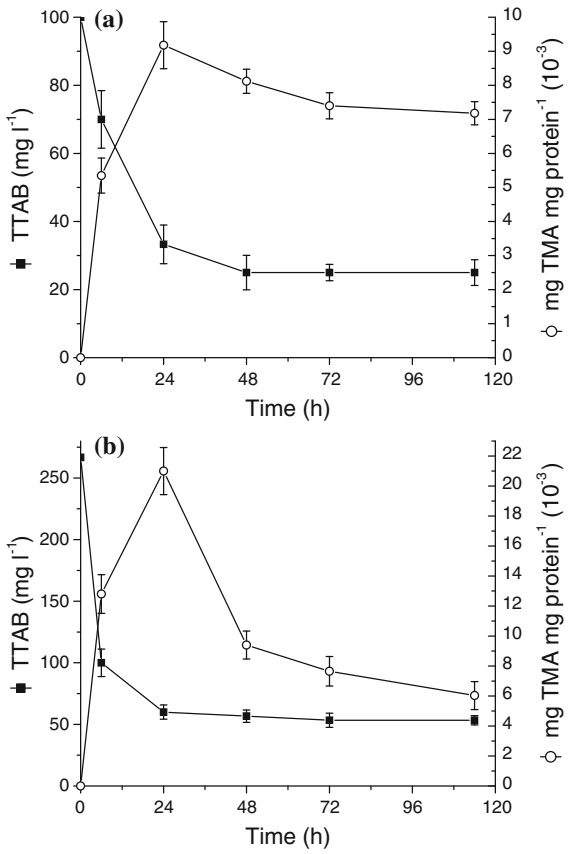

Fig. 4 TTAB consumption and production of intracellular TMA by *P. putida* A ATCC 12633 immobilized in Na-alginate. Beads were incubated in buffered medium (pH 7.4) containing **a** 100 mg l^{-1} and **b** 260 mg l^{-1} of TTAB. At different times, samples of beads were withdrawn, and cellular extracts were obtained as described in the "Materials and methods" section. The TMA concentration (*open circle*) was determined using the morin reagent with the addition of $AlCl_3$. The residual TTAB concentration (*filled square*) was analyzed in the supernatants of the batch cultures by a colorimetric method. Values are the mean ± SD ($n = 3$)

TMA monooxygenase activity (liffourrena et al. 2010). Thus, as seen along of the degradative process, the values of TMA detected were lower than the detected as inhibitory for bacterial growth and TTAB monooxygenase activity with free-cells (8.9×10^{-2} mg TMA mg protein^{-1}) (Liffourrena et al. 2008). Therefore, while the production of TMA is an indicator that the TTAB is being metabolized, the slowing of TTAB degradation by immobilized cells in these conditions is not due to the high accumulation of TMA and can instead be because the residual concentration of TTAB detected (approximately 20 mg l^{-1}) is not appropriate to allow for its entry into the beads, as described above.

When we evaluated the disappearance of higher initial concentrations of TTAB (260 mg l^{-1}), the intracellular TMA value found after 24 h of incubation was 2.1×10^{-2} mg TMA mg protein^{-1}, which is similar to what is inhibitory for cell-free systems (8.9×10^{-2} mg TMA mg protein^{-1}) (Lifourrena et al. 2008). Thus, the concentration of intracellular TMA may explain, in part, the detention of the degradation process in an immobilized system. However, the fact that the cells remain viable over time (approximately 1×10^8 cfu ml^{-1} after 8 days of incubations) suggests that these concentrations of TMA are not toxic to cells, which is another advantage of the immobilized system.

The results of repeated batch TTAB degradation with Ca-alginate immobilized *P. putida* showed that the beads could be reused without changing the degradation efficiency up to three cycles and that the efficiency decreased from 70 to 50 % of degradation in the subsequent cycles (for seven cycles). It has been previously described that the mechanical instability and gradual cell leakage from the beads decreases the degradation rate with increased cycle number (Trevors et al. 1992). In studies of the biodegradation of p-cresol with cells of *Bacillus* sp. PHN1 or in the bioremediation of mercury by *Enterobacter* sp. encapsulated in alginate, the decrease in efficiency of degradability with increased cycle number has been attributed to the loss of viability of cells or to the progressive saturation of the adsorption sites (Tallur et al. 2009; Arvind and Sunil 2011). Although we did not observe any visual disintegration of the alginate beads, during the recycling of immobilized microbial systems, there was leakage of cells from the matrix and changes in the surface of the beads. Figure 5 shows the surface images of alginate beads after one and three cycles of reutilization. The SEM images clearly show that after three cycles, the morphology of the surface of the beads change and that protuberances were formed over the surface of the beads. Some of these bulges are broken with the subsequent release of trapped cells (Fig. 5b). In contrast, after one cycle, the outer morphology of the beads was similar to that observed in fresh alginate beads (Figs. 1b, 5a). Therefore, the reduction in efficiency of degradability during repeated cycles may be due to cell leakage from the beads.

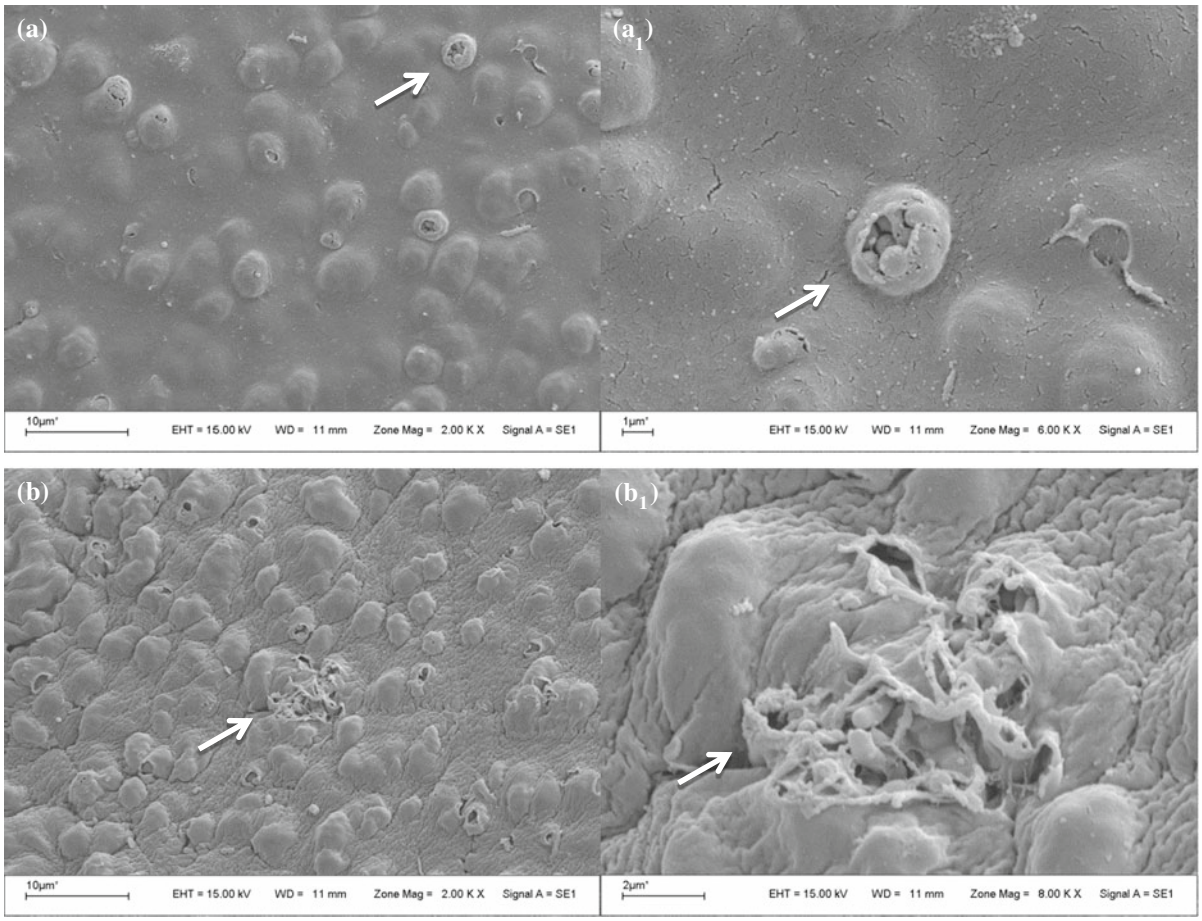

Fig. 5 Scanning electron micrographs of the surface of the beads after of (**a**) one and (**b**) three cycles of reutilization. **a**, **b** Magnification ×2,000; (**a**$_1$) magnification ×6,000; (**b**$_1$) magnification ×8,000

Conclusions

In this study, we immobilized cells of *P. putida* A ATCC 12633 in Ca-alginate matrix and demonstrated the ability of this system to degrade the cationic surfactant TTAB. The concentration of 4 % w/v Na-alginate was optimal for the effective encapsulation of the cells, resulting in spherical beads of regular size (3 mm) and good mechanical stability, which allows for both diffusion and degradation of the substrate without loss of cell viability.

The immobilization of *P. putida* for further TTAB decontamination treatment showed numerous advantages over the use of free-microorganisms: (i) The cells are more resistant to the toxic effect of the detergent. While the immobilized cells tolerated up to 330 mg l^{-1} of the detergent without loss of cell viability, 100 mg l^{-1} of TTAB leads to cellular death for free cells. (ii) The biodegradative process is more efficient. The beads used 330 mg l^{-1} of TTAB, degrading 75 % of that initial concentration after 24 h. Free-cells degraded approximately 50 % of 50 mg l^{-1} TTAB after 24 h. (iii) It is possible to reuse the same beads and continue to efficiently degrade TTAB. The TTAB removal efficiency with the same beads was maintained, with little change, for at least three cycles of reuse.

Overall, this study shows that the use of *P. putida* A ATCC 12633 cells immobilized in Ca-alginate matrix provides a promising alternative to achieve efficient environmental removal of TTAB or other similar cationic detergents.

Acknowledgments GIL is Career Members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). MFB is grateful for fellowships from CONICET-Ministerio de

Ciencia y Tecnología de Córdoba. We thank Andrés Liffourrena and Gisel Esteves (Universidad Nacional Río Cuarto) for their collaboration in conducting experiments. This work was supported by Grants from CONICET, MCyT Córdoba and SECYT–UNRC of Argentina.

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