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Short communication

Degradation of trimethylamine by immobilized cells of *Pseudomonas putida* A (ATCC 12633)

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ABSTRACT

Pseudomonas putida A (ATCC 12633) capable of degrading trimethylamine (TMA) was immobilized in calcium alginate. The TMA-degrading capacity of immobilized cells was compared to free cells in batch culture by changing temperature, pH, nutrient supplementation, and initial TMA concentration. Although immobilized cells showed good removal efficiencies in wider ranges of temperature (15 °C–35 °C) and pH (6.5–8.5) than free cells, the optimal conditions for TMA removal for both free and immobilized cells were 30 °C, in buffered medium (pH 7.5) without addition of nutrients. Immobilized cells degraded up 885 mg l^{-1} of TMA completely whereas the free cells degraded only 295 mg l^{-1}. For all the TMA concentrations evaluated with immobilized cells (295–885 mg l^{-1}), after 24 h, the degradation rate started to decline. This effect was attributed to the accumulation of intracellular TMA into the cells (8.3 –15.3 mg l^{-1}) which is sufficient to inhibit the first enzyme of the aerobic degradation of TMA by *P. putida*, the TMA dehydrogenase. The fact that the immobilized cells showed a high stability as regards their viability and degradability of high TMA concentrations revealed a good potential of this system for treating of TMA-contaminated site.

1. Introduction

Trimethylamine (TMA) is a toxic, nitrogen-containing, flammable organic gas with a low odor threshold of 0.0002 ppm (Yaws, 2001), derived from degradation of foodstuffs (Chang et al., 2004; Park et al., 2012), particularly in the manufacturing processes of fish-meal (Rappert and Muller, 2005). It can be emitted from fish-meal manufacturing plants (Kim et al., 2001) and swine waste storage pits (Ho et al., 2008). The source of TMA is not fully established, but there is evidence that it is produced by the activity of microorganisms on choline, betaine or trimethylamine N-oxide (López-Caballero et al., 2001). Besides its malodorous property and chronic harmful effects on humans, it is known that TMA inhibits the synthesis of macromolecules such as DNA, RNA and proteins and has teratogenic effects on mouse embryos (Guest and Varma, 1992). Therefore, TMA removal from gaseous waste is important in the field of environmental engineering. Because of the high cost of employing a physicochemical treatment process, with the occurrence of potential adverse effects resulting from residually persistent unknown by-products in a stream, biological systems have been preferentially adopted (Burgess et al., 2001; Kim et al., 2001; Elias et al., 2002; Park et al., 2005).

Pseudomonas putida A (ATCC 12633) utilize TMA as a sole carbon, nitrogen and energy source (Liffourrena et al., 2010). The aerobic metabolism of TMA by *P. putida* A (ATCC 12633) involves different enzymatic activities to remove the N-trimethylated depending on the conditions where the microorganism is grown: TMA monooxygenase activity or TMA dehydrogenase activity when grown with the cationic surfactant tetradecyltrimethylammonium bromide or TMA, respectively (Lucchesi et al., 2010). This property, together with the broad metabolic versatility of *P. putida* A (ATCC 12633), involves a selective advantage over other strains for the removal of TMA from contaminated environments.

The immobilization of microorganisms, both entrapped and/or adsorbed has many advantages for use in effluent treatment by biofiltration or in bioreactors. The main advantages of using immobilized cells of microorganisms are their higher operational stability, their increased rate of degradation, their high cell density and that they can be stored for long periods without losing their

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degradatives abilities (Quek et al., 2006). It has also been reported that immobilization provides a protective environment against predation and competition by populations of microorganisms that naturally reside in wastewater (Covarrubias et al., 2012).

In the present study, the performance of TMA degradation by Ca^{2+}-alginate-immobilized cells of *P. putida* A (ATCC 12633) was compared with that of its free form counterpart. The efficiency of TMA degradation was optimized by adjusting various parameters, such as the pH value, temperature, initial concentration of TMA, and the supplementation with carbon and nitrogen source. We show that the immobilized cell systems have the potential for the efficient degradation of TMA.

2. Materials and methods

2.1. Organisms and culture conditions

P. putida A (ATCC 12633) was grown aerobically at 30 °C, with shaking in a rich Luria–Bertani (LB) medium or in buffered medium with 148, 295, 590 and 885 mg l^{-1} (2.5, 5, 10 and 15 mM) of TMA as the carbon and nitrogen source. When necessary, 20 mM glucose and 0.1% NH_4Cl were added. The buffered medium contained: 20 mM Tris–HCl pH 7.4, 44 mM KCl, 85 mM NaCl and 0.8 mM $MgSO_4$. Growth was measured as absorbance at 660 nm (DO_{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).

2.2. Cell immobilization methods

P. putida A (ATCC 12633) was immobilized in calcium alginate (Ca^{2+}-alginate) in a manner similar to that described in Bergero and Lucchesi (2013). Alginate final concentration was 4% w/v, the bead diameter size ranged an average of 3.0 mm, and the trapped population size was constant (approximately $1–5.10^6$ cfu ml^{-1} beads). For the observation with scanning electron microscopy (SEM), beads were treated and examined under a LEO 1450VP scanning electron microscope at 15 kV as described Bergero and Lucchesi (2013).

2.3. Degradation of TMA by free cells and by Ca^{2+}-alginate immobilized cells

P. putida A (ATCC 12633) 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 a OD_{660} of 1.9. Next, 0.75 ml of this suspension was used to inoculate 50 ml of buffered medium in 500-ml flasks supplemented with 295, 590 and 885 mg l^{-1} TMA. All incubations were done in an orbital shaker at 30 °C and 100 rpm. At different times, samples were taken and centrifuged at 8000 × *g* for 10 min at 4 °C, and the supernatant was used for analysis of residual TMA by fluorescence, as described previously Liffourrena et al. (2009).

For immobilized cells, the batch incubation was performed using 250-ml flasks with 50 ml of buffered medium with different TMA concentrations and 100 alginate beads (equivalent to 2.5 ml of beads) containing *P. putida* A (ATCC 12633) cells (6.4×10^6 cfu ml of $beads^{-1}$). The degradation process was carried out at 30 °C on a rotary shaker at 100 rpm for the desired incubation period. At different intervals of time, samples were withdrawn under sterile conditions and used for the analysis of residual substrate TMA.

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 TMA. All determinations were made in triplicate.

The cellular fraction volume was determined by the product of the number of cells obtained after 10 alginate beads had been treated with phosphate buffer and the volume of one cell ($0.8\ m^3$ (8×10^{-10} μl)), that was calculated as described by Roy and Packard (1998).

To obtain cell-free extracts, 100 alginate beads were treated with phosphate buffer as described above, where the cells were removed by centrifugation at 10,000 × *g* for 10 min at 4 °C, washed and suspended with 20 mM Tris–HCl pH 7.4. Next, cells were disrupted by sonication at 20,000 Hz using a Vibra cell ultrasonic processor for 10 cycles, with 10 s for each cycle, and after centrifugation at 16,000 × *g* for 10 min at 4 °C, the soluble fraction was collected and used for intracellular TMA determination and for TMA dehydrogenase activity assay.

2.4. Analytical methods

The consumption of TMA was analyzed in the supernatants of the batch cultures using the fluorochrome 2′,3,4′,5,7-pentahydroxyflavone (morin reagent) with the addition of $AlCl_3$, as described Liffourrena et al. (2009). 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.

TMA dehydrogenase activity was assayed at 30 °C in a 1 ml reaction mixture containing 0.5 mM TMA, 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 addition of 0.5 ml of 37.5% TCA. After centrifugation at 12,000 × *g* for 10 min, aliquots of 100 μl of the supernatant were used to monitor TMA by fluorescence (Liffourrena et al., 2009). TMA dehydrogenase activity was expressed as nmol of TMA min^{-1} mg $prot^{-1}$. Protein concentrations were measured by the Bradford (1976) method with bovine serum albumin (BSA, Sigma Chemical Co., SL, USA) used as a standard.

3. Results and discussion

3.1. Effect of pH and temperature on TMA degradation by free and immobilized cells of P. putida A (ATCC 12633)

To determine the optimum pH and temperature for TMA removal by free and immobilized *P. putida* A (ATCC 12633) cells, pH values ranging from 6.5 to 9.0 and temperature ranging from 15 °C to 40 °C were studied under identical conditions: agitation at 100 rpm and an initial TMA concentration of 295 mg l^{-1} (5 mM). Since the Ca^{2+}-alginate is sensitive to acid pH (Yang et al., 2013), we have avoided using pH under than 6.0. Besides, when the initial pH was 5, the surface of the beads showed deterioration (Fig. 1). The effect of pH on the removal of TMA by immobilized cells showed that variation of initial pH between 7.5 and 8.5 had no effect on degradation (Table 1). When the initial pH value was decreased from 7.5 to 6.5, the TMA removal efficiency decreased from 99.5% to 71.4%, with only 32% at pH 9. The lower TMA degradation efficiency detected at pH 6.5 can be attributed to a decrease of the diffusion of TMA into the alginate beads due to the high degree of protonation

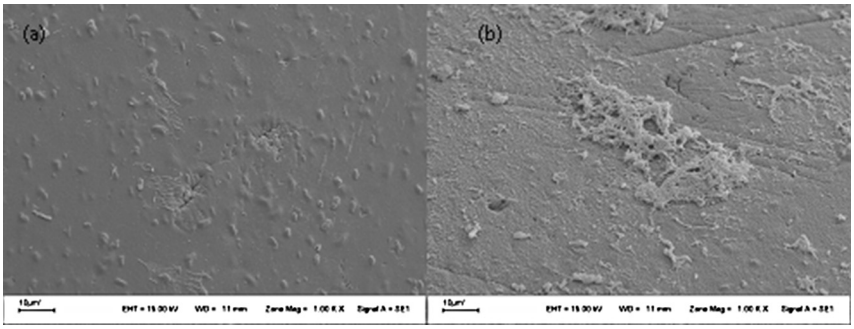

Fig. 1. Scanning electron micrographs (SEM) of the surface of Ca-alginate beads containing cells of *P. putida* incubated at different pH values. The beads were incubated 48 h at 30 °C with stirring in presence of TMA 10 mM, Tris–HCl 20 mM, pH 7 (a) and CH_3CO_2H/CH_3CO_2Na 20 mM, pH 5 (b).

of TMA at this pH (pKa 9.76) which increases its interaction with polyanionic alginate (pKa 3.38–3.65) (Azarnia et al., 2008; Chen et al., 2012). In contrast, the free cells showed good performance only at pH 7.5 (Table 1), which is in agreement with the values of pH most favorable for the enzyme activities involved in TMA oxidation (Chang et al., 2004).

Similarly to what occurs with the pH, immobilized cells are active in a temperature range greater than free cells, probably due to the protection provided by alginate (Table 1). The optimum temperature of TMA degradation for both free and immobilized cells was 30 °C (97.6% and 97.8% of 295 mg l^{-1} TMA degradation, respectively, after 48 h of incubation). Immobilized cells also showed good activity at 20 °C while at 35 °C and 40 °C the degradation efficiency was lower (Table 1). These results are consistent with the mesophyllic characteristic of *P. putida* A (ATCC 12633) and the fact that the exposure to high temperatures may have negative effects on the bacterial enzymes responsible for TMA oxidation.

3.2. Effect of initial TMA concentration

In order to evaluate the effect of initial TMA concentration on the process of degradation by free and immobilized cells, identical conditions were used: buffered medium (pH 7.5), 30 °C and agitation at 100 rpm. The period for complete degradation varied according to the initial TMA concentration. For 295 mg l^{-1}, complete removal was reached in 48 h for free cell cultures of *P. putida* A (ATCC 12633). Using an initial TMA concentration of 590 mg l^{-1} free cells degrade 60% of TMA and after 24 h the consumption of TMA stopped and the residual TMA concentration detected was approximately 242 mg l^{-1} (Fig. 2A). In addition, no bacterial growth and degradation occurred at an initial TMA concentration of 885 mg l^{-1} (Figs. 2A and B). Immobilized cells were able to degrade 100% from an initial 295 mg l^{-1} and 590 mg l^{-1} of TMA after 48 h and 84 h of incubation, respectively. For 885 mg l^{-1}, 100% of degradation occurred in 96 h (Fig. 3A). These results reveal an advantage of the immobilized system over free cells showing that the tolerance to concentration of substrate (Fig. 3B) and efficiency of degradation (Fig. 3A) by immobilized cells is higher than free cells.

Table 1
Efficiency of TMA removal by free cells and immobilized cells of *P. putida* A (ATCC 12633).

	TMA removal efficiency (%)	
	Free cells	Immobilized cells
Temperature (°C)		
15	21.2 ± 3.2	52.5 ± 3.2
20	62.6 ± 5.1	92.6 ± 2.6
30	97.6 ± 3.3	97.8 ± 1.9
35	21.2 ± 2.1	47.5 ± 2.7
40	5.2 ± 3.1	36.5 ± 3.1
pH		
6.5	27.4 ± 2.6	71.4 ± 2.3
7.5	97.6 ± 3.6	99.5 ± 2.6
8.5	48.2 ± 2.9	99.1 ± 3.3
9	15.7 ± 1.5	32.8 ± 2.5

The experiments were performed in presence of TMA 295 mg l^{-1}.
Values are the mean ± SD ($n = 3$).

The percent of TMA depletion measured in batch system may resulted from the combination of degradation by immobilized microorganisms and TMA adsorbed by the support. To corroborate if TMA is metabolized by immobilized cells, we determined the TMA dehydrogenase activity in cell-free extracts obtained from the beads. In cells of *P. putida* A (ATCC 12633) growing on TMA, the TMA dehydrogenase is the first enzyme responsible for catalyzing the oxidation of TMA to dimethylamine, which is then oxidized to methanal and methylamine by dimethylamine dehydrogenase (Liffourrena et al., 2010). In cell-free extracts obtained from beads after 24 h of contact with 885 mg l^{-1} of TMA, the specific activity of TMA dehydrogenase was 0.041 nmol min^{-1} mg $prot^{-1}$, similar to those reported for cell-free extracts obtained from free cells grown with 295 mg l^{-1} of TMA (0.031 nmol min^{-1} mg $prot^{-1}$) (Liffourrena et al., 2010). This result clearly showed that the immobilized cells metabolized TMA once it entered the bead. In addition to this, no degradation was obtained when the process was evaluated using empty beads without microorganisms (not shown) indicating that the disappearance of TMA from the culture medium is due to degradation by *P. putida* A (ATCC 12633) and not a nonspecific process of adsorption.

It is a well-known fact that most degradation obeys the first order kinetics, where the rate of reaction is a function to its initial concentration. For all of the TMA concentrations evaluated with immobilized cells (295–885 mg l^{-1}), after 24 h, the degradation rate started to decline (Fig 3A). For the first 24 h, the rates of degradation of TMA for immobilized system under batch conditions were found to be 10.3 ± 1.2, 12.6 ± 0.9 and 15.2 ± 1.1 mg l^{-1} h^{-1} ($n = 3$) at concentrations of 295, 590, and 885 mg l^{-1}, respectively. For the next 24 h, the rates of degradation of these TMA concentrations were 1.9 ± 0.4, 6.9 ± 0.9 and 9.1 ± 0.6 mg l^{-1} h^{-1} ($n = 3$), respectively. The enhanced degradation in the first time was probably due to the accelerated reaction rates caused by high local cell density in the immobilized matrix. Apparently, the decline of TMA degradation is not related to the loss of cells into the medium because we found that the bead population size was kept constant for this conditions ($1–5 \times 10^6$ cfu ml^{-1}) (Fig. 3B). We explained the reduction in the degradation rates considering the catabolic pathway used by *P. putida* A (ATCC 12633)

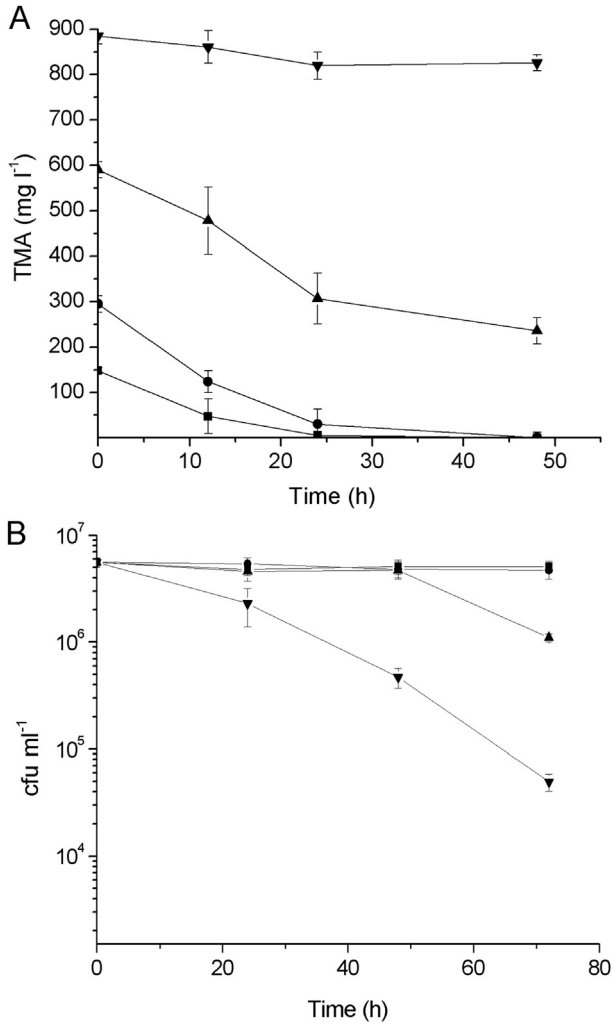

Fig. 2. Degradation of TMA by free cells of *P. putida* A (ATCC 12633) (A) and cell viability (B). Cells were incubated in buffered medium pH 7.5, at 30 °C with stirring in presence of 148 (square), 295 (diamond), 590 (up pointing triangle) and 885 (down pointing triangle) mg l^{-1} of TMA. Values are the mean ± SD ($n = 3$).

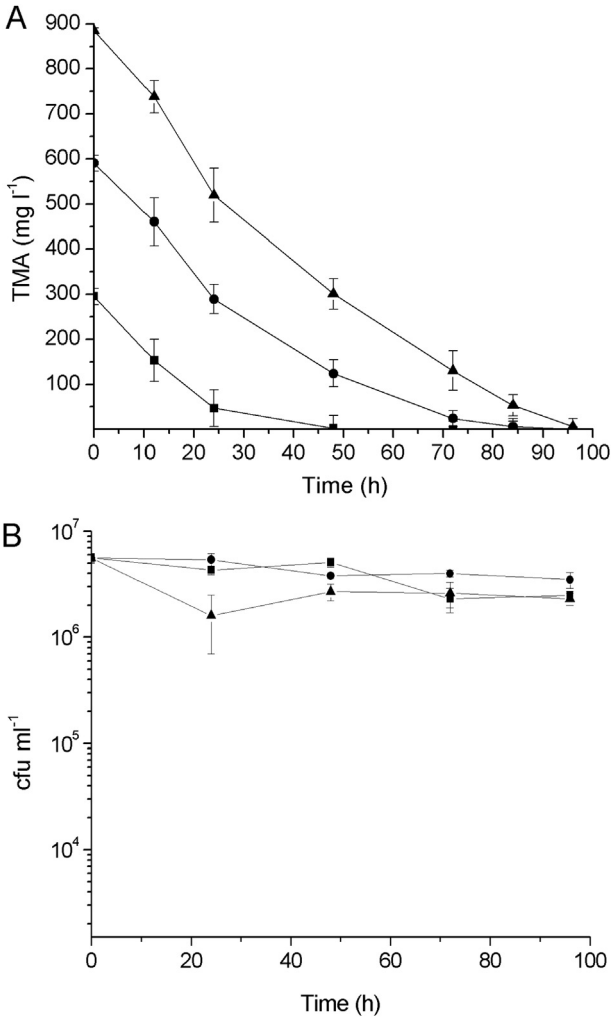

Fig. 3. Degradation of TMA by immobilized cells of *P. putida* A (ATCC 12633) (A) and cell viability (B) in the presence of different concentrations of TMA. Ca^{2+}-alginate beads containing cells of *P. putida* were incubated in buffered medium pH 7.5, at 30 °C with stirring in presence of 295 (square), 590 (diamond) and 885 (up pointing triangle) mg l^{-1} of TMA. The initial *P. putida* A (ATCC 12633) cells content in the beads was 6.4×10^6 cfu ml of beads^{-1}. Values are the mean ± SD ($n = 3$).

during TMA utilization under aerobic conditions and the biochemical properties of the enzymes involved. Previous studies in our laboratory have shown that the TMA dehydrogenase has a lower catalytic activity and was inhibited by TMA from 1 mM (Liffourrena et al., 2010). The TMA incorporated into the beads might not be to completely metabolized due to low catalytic activity of this enzyme. In consequence, TMA might have accumulated into the beads in quantity sufficient to inhibit the TMA dehydrogenase. In order to verify so, we determined the intracellular content of TMA utilizing cell-free extracts obtained from the beads. The intracellular TMA values found after 24 h of incubation with 295, 590 and 885 mg l^{-1} of substrate were 8.3 ± 0.6, 12.4 ± 0.9 and 15.3 ± 0.8 mg l^{-1} ($n = 3$), respectively (equivalent to 1.4, 2.1 and 2.6 mM of TMA, based on cell volume) which are sufficient to inhibit the TMA dehydrogenase activity. Thus, as it occurs with free cells (Liffourrena et al., 2010), the downturn in degradation capacity detected with immobilized cells may be the result of a high concentration of intracellular TMA. However, the fact that the cells remain viable over time suggests that these concentrations of TMA are not toxic to cells and that the alginate-entrapped cells of *P. putida* A (ATCC 12633) although slower, can degrade high TMA concentration which is an advantage of the immobilized system in relation to free cells.

3.3. Effect of nutrients supplementation on the degradation of TMA

The biodegradation process of some compounds in the presence of nutrients enhance the ability of the bacteria to metabolize the target chemical effectively (Gupta and Bhaskaran, 2004). In order to determine if the availability of nitrogen and carbon might affect the removal of TMA, the degradation of this compound by immobilized cells of *P. putida* A (ATCC 12633), in buffered medium pH 7.5 at 30 °C, with and without nutrient supplementation, was investigated. Bacteria viability during 72 h was not altered in experiments where the nutrients were added or not (Fig. 4B). Also the beads remained intact and cell leakage was slight during the 72 h experiment (not shown). The analysis of Fig 4A showed that in relation to control, the degradation of initial 590 mg l^{-1} of TMA was faster in the first 24 h when the process was evaluated in LB medium (13.7 ± 1.2 mg l^{-1} h^{-1}) or in buffered medium with the addition of glucose (12.1 ± 1.5 mg l^{-1} h^{-1}) or glucose and NH_4^+ (11.4 ± 1.3 mg l^{-1} h^{-1}). After this time, the degradation rate started to decline but the TMA degradation after 84 h was 95–99% for all conditions (Fig. 4A) which indicated that the addition of carbon and nitrogen source or the use of rich media is not important in order to

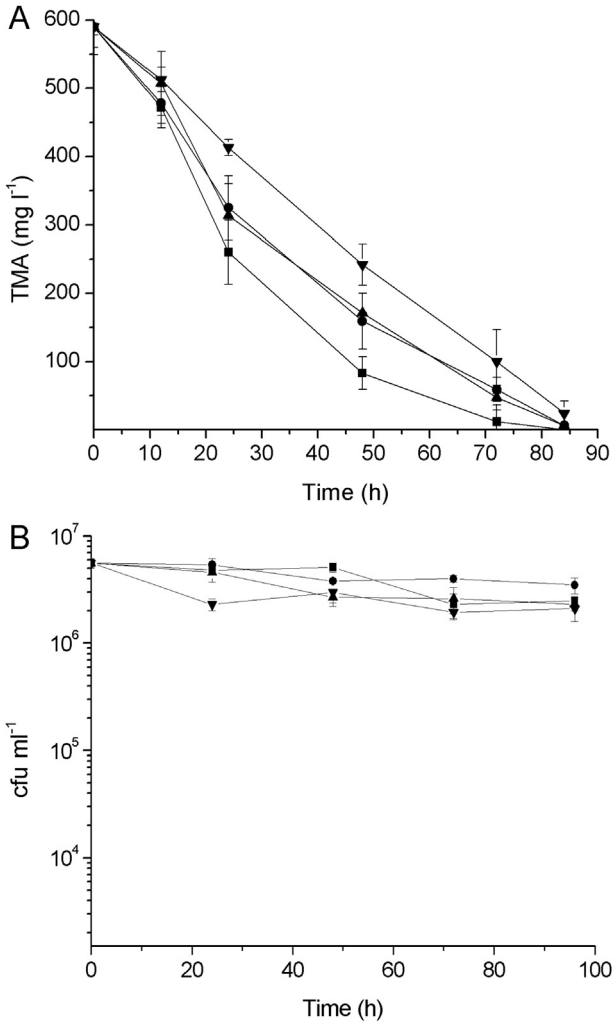

Fig. 4. Degradation of TMA by immobilized cells of *P. putida* A (ATCC 12633) (A) and cell viability (B) in different nutritional conditions: LB medium (square), buffered medium pH 7.5 (down pointing triangle), with 20 mM glucose (up pointing triangle), or with 20 mM glucose and 0.1% NH_4Cl (circle). Ca^{2+}-alginate beads containing cells of *P. putida* were incubated at 30 °C with stirring. TMA initial concentration was 590 mg ml^{-1} and the initial *P. putida* A (ATCC 12633) cells content in the beads was 6.4×10^6 cfu ml of $beads^{-1}$. Values are the mean ± SD ($n = 3$).

obtain the highest possible degree of TMA degradation. Possibly, this is due to the number of cells of *P. putida* A (ATCC 2633) into the beads has remained constant in all conditions and therefore the degradative capacity were the same in all conditions tested.

In summary, in this study we have successfully established key operating parameters (i.e. temperature, pH and TMA concentration) for TMA degradation using immobilized cells of *P. putida* A (ATCC 12633) and provide useful guideline for future application of the alginate beads with this strain for the treatment of industrial wastewater containing TMA and possibly other related N-trimethylated contaminant.

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