J Ind Microbiol Biotechnol (2009) 36:275–284
DOI 10.1007/s10295-008-0496-5

ORIGINAL PAPER

Cyanuric acid biodegradation by a mixed bacterial culture of *Agrobacterium tumefaciens* and *Acinetobacter* sp. in a packed bed biofilm reactor

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Received: 22 May 2008 / Accepted: 21 October 2008 / Published online: 11 November 2008

Abstract Cyanuric acid (1,3,5-triazine-2,4,6-triol [OOOT]) is a common biodegradation byproduct of triazinic herbicides, frequently accumulated in soils or water when supplementary carbon sources are absent. A binary bacterial culture able to degrade OOOT was selected through a continuous selection process accomplished in a chemostat fed with a mineral salt (MS) medium containing cyanuric acid as the sole carbon and nitrogen source. By sequence comparison of their 16S rDNA amplicons, bacterial strains were identified as *Agrobacterium tumefaciens*, and *Acinetobacter* sp. When the binary culture immobilized in a packed bed reactor (PBR) was fed with MS medium containing OOOT (50 mg L^{-1}), its removal efficiencies were about 95%; when it was fed with OOOT plus glucose (120 mg L^{-1}) as a supplementary carbon source, its removal efficiencies were closer to 100%. From sessile cells, attached to PBR porous support, or free cells present in the outflowing medium, DNA was extracted and used for Random Amplification of Polymorphic DNA analysis. Electrophoretic patterns obtained were compared to those of pure bacterial strains, a clear predominance of *A. tumefaciens* in PBR was observed. Although in continuous suspended cell culture, a stable binary community could be maintained, the attachment capability of *A. tumefaciens* represented a selective advantage over *Acinetobacter* sp. in the biofilm reactor, favoring its predominance in the porous stone support.

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Introduction

Chlorotriazine herbicides are anthropogenic chemicals frequently found in water bodies, particularly following floods and periods of heavy rain and runoff from agricultural lands. At least atrazine, cyanazine and simazine are harmful to aquatic ecosystems [1], and are listed by the US Environmental Protection Agency (USEPA) as possible carcinogens [2]. Frequently, biodegradation of these pesticides leads to cyanuric acid accumulation. Even though this chemical is not considered harmful to human beings, it has shown chemical toxicity on aquatic organisms such as zooplankton [3] and carps [4]. In larval lampreys and fishes, it causes behavioral perturbations [5]. In addition, this chemical is a suspected gastrointestinal or liver toxicant in humans, and its long-term effects on ecosystems are unknown [6], thus, cyanuric acid, among other s-triazinic compounds, is included in the EC environmental priority pollutants list [7].

Cyanuric acid is a key intermediate in the metabolic pathway of chlorotriazine herbicides [8], or a common byproduct of their chemical [9] or photochemical degradation [10, 11]. For the complete removal of triazine compounds, efficient technologies are required. Direct photo-degradation under sunlight or natural attenuation has little effect on most of these pollutants, and their complete mineralization could not be attained by ozonation, sonolysis, photolysis or photocatalysis. Because of the stability of the N-heterocyclic ring of cyanuric acid, chemical or photochemical methods have shown inadequate to degrade

it [11–13], and expensive advanced oxidation processes (AOPs) are required to obtain high degradation efficiencies of this stable pollutant byproduct [14–17]. To avoid cyanuric acid accumulation, some authors have highlighted the positive effect of coupling photocatalysis and biological process for the treatment of recalcitrant triazines [10].

Although not always possible, chlorotriazine compounds can be completely degraded by microbial action in anaerobic [18] or aerobic conditions. There are reports about their aerobic biodegradation by strains of algae [19], bacteria [20, 21], yeasts [22], fungi [23], or by mixed microbial communities [24]. In addition, some bioprocesses to decontaminate water polluted with cyanuric acid have been reported [25–28]. Besides the high stability of the N-heterocyclic ring, the cyanuric acid biodegradation is problematic because the low nitrogen to carbon ratio in the molecule (C:N = 0.857), lesser than C:N of chlorotriazine herbicides, which varies from 1.20 for simazine to 1.89 for chlorazine. Although carbon to nitrogen ratios from 2.0 to 3.0 have been considered convenient for bacterial growth and secretion of extracellular polysaccharides [29], an average C:N ratio for culture media used for bacterial balanced growth, estimated from data of Atkinson and Mavituna [30] is about 5.4; thus, to improve the removal efficiency and biodegradation of s-triazinic compounds, the addition of assimilable carbon sources to compensate the C:N unbalance is often necessary.

The bulk of the published results related to this topic, describes the biodegradation of chlorotriazine herbicides by several microbial species, pure or mixed. However, specific reports about cyanuric acid (2,4,6-trihydroxy-s-triazine [OOOT]) removal processes are not abundant. Most of them are batch or continuous suspended cell cultures [25, 26, 28], even using high concentrations of extra carbon sources to achieve the cometabolic degradation of cyanuric acid [26]. To demonstrate that the rate of OOOT biodegradation is affected by the transport rate of the compound through cell membranes and by the activity of cyanuric acid hydrolase inside the cells, other authors used suspended cell cultures or continuous tubular reactors packed with model soil containing mixed bacterial cultures [53].

Since the volumetric removal rate of a compound R_V depends on the amount and specific removal rate R_X of the reactive biomass retained in the bioreactor, an immobilized cell culture could present kinetic advantages over suspended cell cultures, especially when the compound concentration is low.

For these reasons, in this work, a biological process for the biodegradation of the stable N-heterocyclic ring of cyanuric acid was kinetically evaluated in two situations: (a) when cyanuric acid was supplied to the culture as the only carbon and nitrogen source and (b) when cyanuric acid was used as nitrogen source and glucose as an extra carbon source. The results show that a mixed bacterial culture of *Agrobacterium tumefaciens* and *Acinetobacter* sp., both producers of cyanuric acid amidohydrolase, use OOOT as the sole carbon and nitrogen source, and that its removal rate could be increased by adding small amounts of glucose to the SM medium to compensate the C:N unbalance.

Methods

Chemicals

All components used in culture media were obtained from Merck (Darmstadt, Germany). Cyanuric cid was acquired from Sigma Aldrich, USA. The solvents used for HPLC were purchased from J T Baker (USA).

Microorganisms

By using a successive transfer method on mineral salt medium containing cyanuric acid (50 mg L^{-1}), a binary bacterial culture able to degrade the N-heterocyclic compound was selected from soil samples obtained from corn cultivated lands situated on Teotihuacan Valley, México.

This technique is an intermittent alternative to the use of a chemostat as continuous selector. The successive transfer method is based on the prevalence of a microbial population able to proliferate under a selective pressure exerted by particular environmental variables such as temperature, pH, type and concentration of nutrients or toxic compounds, over a microbial population inoculated in sequential batch cultures. In this case, microorganisms able to grow on cyanuric acid outgrew others unable to use it as nutrient source. By successive transfer of aliquots taken from exhausted culture to a new one, the incompetent microorganisms were diluted. Finally, after ten successive aliquot transfers to the new MSM medium, containing OOOT as the sole carbon and nitrogen source, the culture was plated in MSM-agar medium, isolating the microorganisms selected.

Culture media

Mineral salts (MS) medium

The media composition (in g L^{-1}) was: K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4{\cdot}7H_2O$, 0.20; NaCl 0.1; $CaCl_2$, 0.02. Five milliliter of a microelemental solution was added to obtain a final concentration (in mg L^{-1}) of: $FeSO_4{\cdot}7H_2O$, 2.75; $ZnSO_4{\cdot}7H_2O$, 1.15; $MnSO_4{\cdot}7H_2O$, 0.33; $CoCl_2{\cdot}6H_2O$, 0.24; $CuSO_4{\cdot}5H_2O$, 0.24; $Na_2MoO_4{\cdot}2H_2O$, 0.17. As carbon

and nitrogen source, 33.3 mL L^{-1} of aqueous OOOT were added to obtain a final concentration of 50 mg L^{-1}. Microorganisms were maintained in agar slants with MS medium supplemented with OOOT (50 mg L^{-1}). For viable cell counting (CFU mL^{-1}), Plate Count Agar (Difco) was used.

Bacterial strain identification

Bacterial colonies showing morphologic differences in SM-OOOT agar plates were isolated. DNA from pure strains was extracted, and by PCR amplification, sequencing, and comparison of their 16S rDNA amplicons with the NCBI GenBank, the isolated strains were identified. For 16S rDNA amplification, the primers 8FPL (5′-GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG-3′) and 1,492 RPL (5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′) were used [31].

Bacterial strain differentiation by PCR-TGGE

16S rDNA fragments of about 450 bp were obtained by PCR amplification of extracted bacterial DNA, by using U-968-GC (5′-[GC clamp]-AAC GCG AAG AAC CTT AC-3′) and L-1401 (5′-CGG TGT GTA CAA GAC CC-3′) primers [32]. Amplicons were analyzed by thermal gradient gel electrophoresis (TGGE System Controller, Biometra, Germany).

Random amplification of the polymorphic DNA (RAPD)-PCR

To differentiate the selective detachment of bacterial cells from the porous support, suspended cells flowing outward the packed bed reactor were collected by centrifugation at 13,000 rpm (Refrigerated centrifuge HARMLE Z-252 MK, Germany). DNA from the cell package was extracted (Cells and tissue DNA isolation kit, Genomic Prep, Amersham Pharmacia Biotech Inc, USA) and used as template for a random amplification by using the primer 1,283 (5′-GCGATCCCCA-3′) [33]. Once finished the PBR operation, bacterial cells were detached from volcanic stone samples with the aid of a Vortex agitator. Stone fragments were washed and rinsed until a clear extract was obtained. From the cell package obtained by centrifugation at 13,000 rpm, DNA was extracted and the biofilm microbial diversity was evaluated by RAPD–PCR of the extracted DNA.

RAPD–PCR was performed in a volume of 20 μL containing 2 μL of 10× Taq DNA polymerase buffer, 2 μL of 2.25 mM $MgCl_2$, 2 μL of 0.2 mM of dNTP mix, 1 μL of a solution containing 15 pM of each primer, 2 μL containing 0.4 ng genomic DNA, and 1 unit of Taq DNA polymerase (Platinum, Invitrogen USA). DNA amplification was performed in a GeneAmp PCR System 2400 (Applied Biosystems USA). After initial incubation for 3 min at 95°C, the samples for enzymatic amplification were subjected to 40 cycles of the following thermal cycle: 30 s at 94°C, 1 min at 36°C and 1 min at 72°C. The final extension at 72°C for 10 min ended the program. The reaction products were fractionated by electrophoresis in 1.5% agarose gels; the gels were stained with ethidium bromide and photographed under UV light using a Gel documentation system (Kodak, USA). A 100 bp DNA ladder (Invitrogen, USA) was used as the molecular standard in all gel electrophoresis.

Packed bed biofilm reactor (PBR)

The lab-scale fixed-bed reactor (Fig. 1) used in this study was composed by three flanged glass modules joined by metallic clamps and sealed with neoprene gaskets. A sintered glass plate constituted the basis of the lower (1) and central (2) modules. The third is the reactor's cover, which has entries for acid or alkali addition and for air venting.

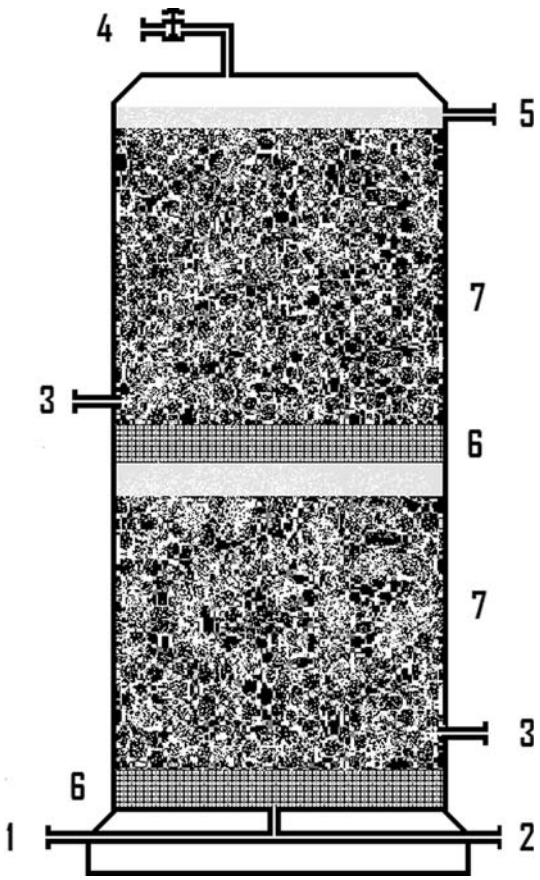

Fig. 1 Lab scale packed bed biofilm reactor used for cyanuric acid biodegradation. Liquid input (1), air input (2), sample ports (3), air exhaust (4), liquid output (5), sintered glass plates (6), packed bed support in lower and upper PBR's stages (7)

Table 1 Characteristics of porous volcanic material and packed bed stages

Characteristics of porous volcanic material		
Intra-particle porosity of volcanic stone [ε_p]	0.1043	
Inter-particle porosity of volcanic stone bed [ε_e]	0.4148	
Total bed porosity [$\varepsilon_{tot} = \varepsilon_e + \varepsilon_p$]	0.519	
Dry material bulk density [ρ_{TZ}]	1.857 (g cm^{-3})	
Characteristics of packed bed, stages L and C	Stage 1 (cm^3)	Stage 2 (cm^3)
Packed bed volume [V_{PB}]	530	550
Packed bed void volume [$V_{void} = V_{PB}$ ($\varepsilon_e + \varepsilon_p$)]	275.6	285.6
Packed bed circulating liquid volume [V_{PB} (ε_e)]	220.2	228.2
Packed bed stagnant liquid volume [V_{PB} (ε_p)]	55.4	57.4

Table 2 Operative conditions and overall behavior of the two-stage packed bed reactor

Run	Overall D (day^{-1})	HRT (day)	[C:N]$_{in}$	$B_{V\text{-}OOOT}$ (mg L^{-1} day^{-1})	$B_{V\text{-}GLC}$ (mg L^{-1} day^{-1})	$B_{V\text{-}N}$ (mg L^{-1} day^{-1})	$R_{V\text{-}OOOT}$ (mg L^{-1} day^{-1})	$R_{V\text{-}GLC}$ (mg L^{-1} day^{-1})	$R_{V\text{-}N}$ (mg L^{-1} day^{-1})	$R_{X\text{-}OOOT}$ [mg (mg protein)$^{-1}$ day^{-1}]	η_{OOOT} (%)	η_N (%)
1	0.142	7.04	0.86	7.13	–	2.32	6.77	–	2.04	57.6	95.4	88.0
2	0.278	3.60	3.80	13.92	33.42	4.53	13.87	33.42	4.53	117.0	99.9	99.9
3	0.465	2.15	3.80	23.25	55.8	7.57	23.11	55.8	7.56	195.0	99.4	99.7
4	0.621	1.61	3.80	31.18	74.52	10.11	30.92	74.52	9.91	261.6	99.16	98.0

An air entrance is located in a void space beneath the porous glass-plate of the L module. Modules 1 and 2 have lateral ports for sampling or liquid input. Both modules were packed with porous volcanic stone as cell support material. Characteristics of porous volcanic material and packed bed stages are shown in Table 1.

The reactor was upstream operated, with air and liquid concurrently fed. The gas flow rate, measured with a gas rotameter, was maintained at 400 ± 20 cm^3 min^{-1}. Initially, the PBR was fed with MS medium containing cyanuric acid (50 mg L^{-1}). In the next three runs, the feeding C:N ratio was increased by adding glucose (120 mg L^{-1}) to the MS medium and the volumetric loading rate was also increased by augmenting the flow rate (Table 2).

Packed bed biofilm support

A vesicular basaltic-andesitic-scoria accumulated in the Trans-Mexican Volcanic Belt by volcanic eruptions, and named tezontle in Mexico, was used as packed bed biofilm support. The rough particles have a porous microstructure; consequently, the fluid in their interior is considered stagnant and intraparticular mass transfer occurs by diffusion. Porous fragments were considered ellipsoid bodies with three characteristic radii: a, b and c. Using the equation $V_P = \frac{4\pi}{3}abc$, the particle volume V_P was calculated and used to obtain the equivalent diameter d_P of spherical particles from the equation $d_P = \left[\frac{6V_P}{\pi}\right]^{\frac{1}{3}}$.

Porosity of volcanic stone was determined according to the method described by Hodge and Devinny [34]. The equivalent diameter d_p obtained from a sample of 50 stone fragments was 8.18 ± 0.88 mm.

Operation of the packed bed reactor

The PBR was initially operated at a dilution rate $D = 0.142$ day^{-1} in abiotic conditions (abiotic blank), feeding it with MS medium containing cyanuric acid (50 mg L^{-1}). The feeding was maintained until the output's concentration reached the input's OOOT concentration, meaning that support was saturated. Then, the MS medium was partially drained, and a cell suspension of the binary culture previously grown in cyanuric acid was injected into the lower reactor's module. Once completed the reactor volume with fresh medium; the reactor was batch operated for 72 h; then, the feeding was restarted, maintaining the same dilution rate. Along the continuous process, the cyanuric acid concentration was measured by liquid chromatography. When glucose was added to the feeding medium, it was enzymatically quantified (Glucose oxidase-peroxidase kit; Sigma Aldrich, USA). Cell detachment from porous support was periodically evaluated by viable cell counting of the liquid stream flowing from the PBR. To evaluate the composition of detached bacterial cells, the random amplification of the polymorphic DNA extracted from suspended cells leaving the reactor was done.

Analytical methods

Bacterial cells suspended or immobilized in porous support

Along PBR's continuous operation, protein and cell concentration were determined in cell free extracts of centrifuged liquid samples, by the Lowry method [35], and viable cell counting (CFU mL^{-1}), respectively. Once that continuous culture runs were finished, volcanic stone fragments were sampled for attached viable-biomass determination (CFU [g porous dry support]$^{-1}$). Cells were water-extracted from volcanic stone samples with the aid of a Vortex agitator. Stone fragments were washed and rinsed until a clear extract was obtained; then, appropriated dilutions of the collected cell suspension were used for plate counting. Another aliquot was used for cell protein determination.

Cyanuric acid amidohydrolase activity

When both bacterial strains were individually batch cultivated on OOOT plus glucose, both showed cyanuric acid amidohydrolase activity. Enzyme activity was determined in cell free extracts obtained by mechanical rupture using Ballotini glass beads (45 μm), with the aid of a Vortex agitator. By measuring OOOT disappearance at 212 nm in a Beckman DU spectrophotometer, the enzyme activity was determined [36]. To estimate changes in their specific activity, the protein concentration in cell free extracts was determined by the Lowry method.

Cyanuric acid determination

From sample filtrates, OOOT was determined by column liquid chromatography using a Beckman HPLC System equipped with an Altech Inertsil column and a diode array detector (UV 280 nm). An isocratic mobile phase of 5.0 mM sodium octane sulfonate on 0.05% H_3PO_4 was injected at a flow rate of 1.0 mL min^{-1} [37].

Nitrogen determination

From sample filtrates, total nitrogen was determined by the HACH Test N Tube Persulfate Method No 10071 [38].

Protein determination

To determine the protein content in the cellular suspension obtained from porous support material samples, four milliliters of cell suspension were mixed with 1.5 mL of 20% NaOH, heated in a boiling water bath for 10 min, and cooled. This suspension was used for protein determination by the Lowry method [35]. This method combines the biuret reaction (reaction of peptide bonds with copper ions in an alkaline medium producing a purple protein–copper complex) with reduction of the Folin–Ciocalteau phenol reagent (phosphomolybdic–phosphotungstic acid) by tyrosine and tryptophan residues. The reduction is promoted by the copper–protein complex, giving a blue color whose intensity is proportional to the protein concentration, and depends on its content of tyrosine and tryptophan.

Microscopic methods

Fragments of volcanic stone containing the microbial biofilm sampled at various points of the packed bed were broken into small pieces. After fixing with 2% glutaraldehyde, washing twice with phosphate buffer at pH 7, post-fixing with 1% osmium, dehydrating with ethanol, drying, and finally covering with gold, micrographs of samples were obtained in an electronic scanning microscope JEOL, JSM-5800 LV (Japan).

Results and discussion

Bacterial strains identification

After the enrichment procedure, a binary culture was obtained. By PCR amplification, sequencing of bacterial 16S rDNA amplicons, and comparison with known sequences of 16S rDNA from the NCBI GenBank, the isolated strains were identified as: *A. tumefaciens* with accession number: AY850392 [98% homology] and *Acinetobacter* sp. with accession number: AF467306 [96% homology]. Reported species with the best homology matches were regarded as the isolated species. The primers 8FPL and 1492 RPL [31] were used for PCR amplification. The purity of both strains was verified by the TGGE technique. Figure 2 shows the TGGE of 16S rDNA fragments of about 450 bp obtained by PCR amplification of bacterial DNA extracted from each bacterial strain.

Cyanuric acid amidohydrolase activity in bacterial strains

When growing on cyanuric acid, both strains showed cyanuric acid amidohydrolase activity. When enzyme activity was determined on actively growing cells, the specific enzyme activity reached by *Acinetobacter* sp. was higher than that of *A. tumefaciens* (Fig. 3). In slow-growing cells differences in enzyme activity between them was not observed. Although both strains showed cyanuric acid amidohydrolase activity (3.5.2.15), it was presumed that the gene codifying for this enzymatic activity is different from that codified by *AtzD* or *TrzD* genes [39], since

Fig. 2 TGGE of a 450 bp fragment of 16S-rDNA from isolated bacteria. *Lane 1* *Acinetobacter* sp., *lane 2* *Agrobacterium tumefaciens*

specific initiators for the enzyme [AtzD392f, AtzD942r, and TrzD274f, TrzD936r] gave negative results for the amplification of any of these bacterial genes.

Cyanuric acid biodegradation in the PBR

Because nitrogen heterocyclic molecules, such as cyanuric acid, have high nitrogen to carbon ratio, their biodegradation often requires the addition of assimilable carbon sources to compensate the C:N unbalance. Once balanced, their biodegradation rate could be improved [40]. In the first run, the biofilm reactor was fed with cyanuric acid as the sole carbon and nitrogen source (C:N = 0.857), corresponding to a volumetric loading rate $B_{V,OOOT}$ of 7.13 mg $L^{-1}day^{-1}$. In this condition, only a 95% of the cyanuric acid and 94% of the nitrogen could be removed, with a corresponding volumetric degradation rate $R_{V,OOOT}$ of 6.77 mg $L^{-1}day^{-1}$ and a $R_{V,N}$ of 2.04 mg $L^{-1}day^{-1}$ (Fig. 4; Table 2). When the C:N ratio was increased to 3.8, by glucose addition, and $B_{V,OOOT}$ values were raised (runs 2–4) by increasing the dilution rate D, cyanuric acid concentration at the PBR's outflowing liquid diminished noteworthy (Fig. 4). In consequence, the $R_{V,OOOT}$ values were remarkably increased and the removal efficiencies for nitrogen η_N, and cyanuric acid η_{OOOT} reached values

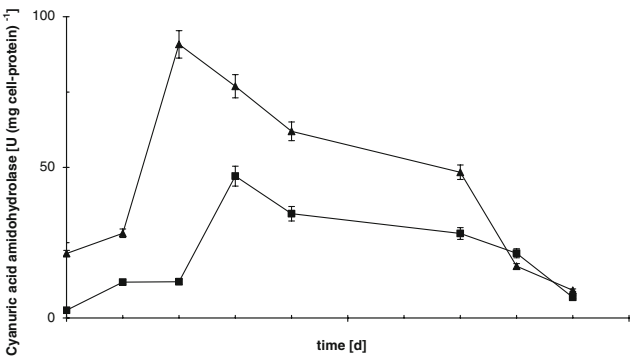

Fig. 3 Cyanuric acid amidohydrolase activity in cell free extracts of bacteria batch cultivated on OOOT. *Filled circle Acinetobacter* sp.; *triangle Agrobacterium tumefaciens*

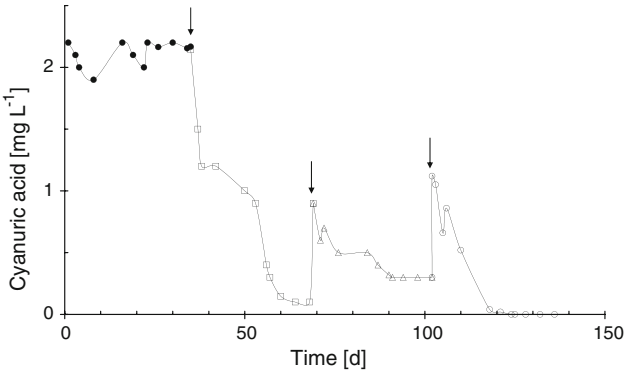

Fig. 4 Remaining OOOT in the PBR's outflowing liquid. *Filled circle* Run 1, OOOT (50 mg L^{-1}) as the sole carbon source; *open square* Run 2; *triangle* Run 3; and *open circle* Run 4, mixed carbon sources OOOT (50 mg L^{-1}) and glucose (120 mg L^{-1}). Operative conditions for each run as described in Table 2. *Arrows* indicate changes in overall dilution rate, once reached the system steady state

higher than 98% (Table 2). Glucose was never detected in the exhausted liquid leaving the PBR.

Once finished the fourth run, the bioreactor was disassembled and attached cells were extracted from samples of porous support obtained from each reactor's stage. Afterwards, cell plate count and cell protein were determined. Both determinations showed a noteworthy difference in cell mass attached at each stage. Total cell protein content in first and second reactor's stages was respectively, 121.3 and 7.2 mg cell protein per stage. The total cell counts were 6.32×10^{11} and 8.49×10^{10} CFU per stage, respectively. Because the second stage is fed with the exhausted medium outflowing from the first one, the difference in cell growth can be attributed to differences in substrate availability along the packed bed reactor.

DNA extracted from cells attached to porous support and from suspended cells sampled from the liquid output was analyzed by RAPD–PCR. Although several minor bands from amplicons could be observed by gel electrophoresis (Fig. 5), the DNA amplified from *A. tumefaciens* showed a major band of about 1,400 bp and *Acinetobacter* sp., one of about 400 bp. In Fig. 5, a clear predominance of *A. tumefaciens* was observed in PBR's suspended (lanes 1–3) or attached cells (lanes 4–5). This fact could be explained because *Agrobacterium* surface carbohydrates (capsular polysaccharides, lipopolysaccharides, oligosaccharides and cyclic glucanes), fulfill an essential function in bacterial adhesion to plant surface. In particular, β-1,2-glucanes have been isolated in all the studied species of *Agrobacterium* [41, 42].

The nonappearance of *Acinetobacter* in the PBR hardly can be attributed to antagonism with *Agrobacterium* because in suspended batch cultures carried out for selecting microorganisms able to degrade OOOT, by the

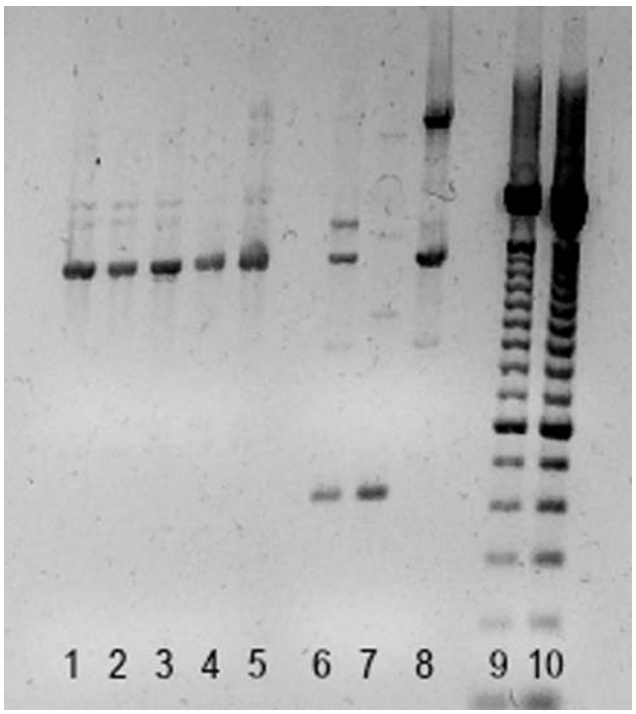

Fig. 5 Random Amplification of the Polymorphic DNA RAPD–PCR, using the 1,283 primer. *Lanes 1–3* cells from PBR's effluent, *lanes 4–5* attached cells, *lane 6* mixed bacteria; *lane 7 Acinetobacter* sp., *lane 8 Agrobacterium tumefaciens*; *lanes 9–10* 100 bp DNA ladder

successive transfer method, both strains could grow without problems in mixed culture. On the other hand, although several species of the genus *Acinetobacter* are unable to metabolize glucose [43, 44], the *Acinetobacter* strain isolated by us, grew well on this substrate (data not shown). Therefore, its absence from the porous support could not be attributed to its inability to use glucose as a carbon source when a mixed substrate (glucose–OOOT) was fed to the PBR, but to its phenotypical inability to remain attached to the support surface. Surface adherence is a characteristic presented only by some *Acinetobacter* phenotypes; particularly by those presenting pilus structures, mainly observed in pathogenic adhesive species [45]. Microbial associations showing outcompeting of one species over another in biofilm co-culture is not an unusual phenomenon [46], thus, the adhesion capacity of *Agrobacterium* could represent a selective advantage over *Acinetobacter* in the packed bed reactor.

Although kinetic information about cyanuric acid biodegradation is not abundant, the results obtained in this work were compared with those obtained with several published bioprocesses. The terms volumetric removal rate R_V and compound's removal efficiency η, give information about the bioprocess performance, while the term specific removal rate R_X is a cell's metabolic quotient that provides information about the biodegradation capability of particular microbial strains or microbial consortia. In a continuous system, the removal efficiency can be calculated as $\eta = R_V/B_V$. Both terms, η and R_V, depend on the operational variables of the process, but also on the reactive cell mass concentration x and on its specific removal rate R_X. Thus, the bioprocess performance can be augmented, by using microorganisms presenting higher R_X values or by increasing the cellular concentration into the reactor. In Table 3 can be observed that, although the volumetric removal rates obtained in this work are comparatively low, the binary culture selected presents rather high values of R_X; when growing in OOOT as the sole carbon and nitrogen source, or in glucose plus OOOT. Thus, by increasing the attached biomass of the selected culture, the volumetric removal rate could be remarkably increased in a biofilm reactor.

Conclusions

Although important removal efficiencies for nitrogen η_N, and cyanuric acid η_{OOOT} (Table 2) were obtained when cyanuric acid was supplied as the sole carbon and nitrogen source ($[C:N]_{in} = 0.857$); when glucose was added to increase the $[C:N]_{in}$ to 3.8, the OOOT concentration at the PBR's outflowing liquid diminished noteworthy. In consequence, the removal efficiencies for nitrogen and cyanuric acid reached values closer to 100%.

When a binary bacterial community selected by successive transfer method on suspended batch culture was cultivated on a biofilm reactor fed with OOOT or with OOOT plus glucose, by the RAPD–PCR technique, it was evidenced that only one of the species; *A. tumefaciens*, predominates in the system. In spite of its lesser cyanuric acid amidohydrolase activity, compared with that shown by *Acinetobacter* sp., the ability to become attached to the support material could be selective advantage of *A. tumefaciens* over *Acinetobacter*, and this ability could be associated to the medium C:N ratio. It has been demonstrated that the bacterial polysaccharide biofilm production can be manipulated by varying the nutrient carbon-to-nitrogen ratio. Both, total polysaccharide production and ratio of polysaccharide to protein in biofilm cultures are increased with increasing C:N ratios [47]. In addition, differences in the number of attached cells are not necessarily a result of the individual actions of bacterial strains, but a metabolic response to the medium's composition [48]. Finally, by microscopic analysis, a biofilm exhibiting a complex architecture with typical cell surface structures, possibly *A. tumefaciens* microcolonies [49, 50], glued together by a bacterial exopolymeric material was evidenced (Fig. 6).

Table 3 Cyanuric acid biodegradation processes

Process	Culture system and bioreactor	Primary substrate	Microorganisms	η_{OOOT} (%)	Cell growth yield (%)	$R_{V\text{-}OOOT}$ (mg L^{-1} h^{-1})	$R_{X\text{-}OOOT}$ mg (g cell)$^{-1}$ h^{-1}	Reference
Aerobic/batch	Suspended cells culture	Glucose	*Penicillium* and *Hormondendrum*	90–96			0.7–1.2	[51]
Anaerobic/batch	Suspended cells culture	Raw sewage	Mixed culture	100		0.55		[25]
Aerobic/batch	Immobilized in sandy loam soil		*Hendersonula toruloidea* and *Stachybotrys chartarum*				0.08	[52]
Anoxic/ continuous	Suspended cells culture	Raw sewage	Mixed culture	100		1.25	0.3–2.4	[25, 26]
Aerobic/batch	Suspended cells culture	Glucose or saccharose (0.5%)	*Sporothrix schenkii* 6.2 NRRL Y-11307				20–30	[26]
Aerobic/batch	Suspended cells culture	Glycerol 10 mM	*Rhodococcus corallinus NRRL B-15444*	100	150 mg protein (mMol OOOT)$^{-1}$			[28]
Anoxic/batch	Immobilized cells in packed bed column/silica sand support		*Pseudomonas* sp. NRRL B-12227			5.4	4×10^{-14} mmol cell^{-1} h^{-1}	[53]
Aerobic/ continuous	Immobilized cells in packed bed reactor/volcanic stone support		*Acinetobacter* sp. and *Agrobacterium tumefaciens*	95		0.28	2.4 or 7.9×10^{-14} mmol cell^{-1} h^{-1}	This work
Aerobic/ continuous	Immobilized cells in packed bed reactor/volcanic stone support	Glucose 120 mg L^{-1}	*Acinetobacter* sp. and *Agrobacterium tumefaciens*	100		1.29	10.9 or 36.3×10^{-14} mmol cell^{-1} h^{-1}	This work

Comparison based on removal efficiencies η_{OOOT}, volumetric $R_{V,OOOT}$, or specific removal rates $R_{X,OOOT}$

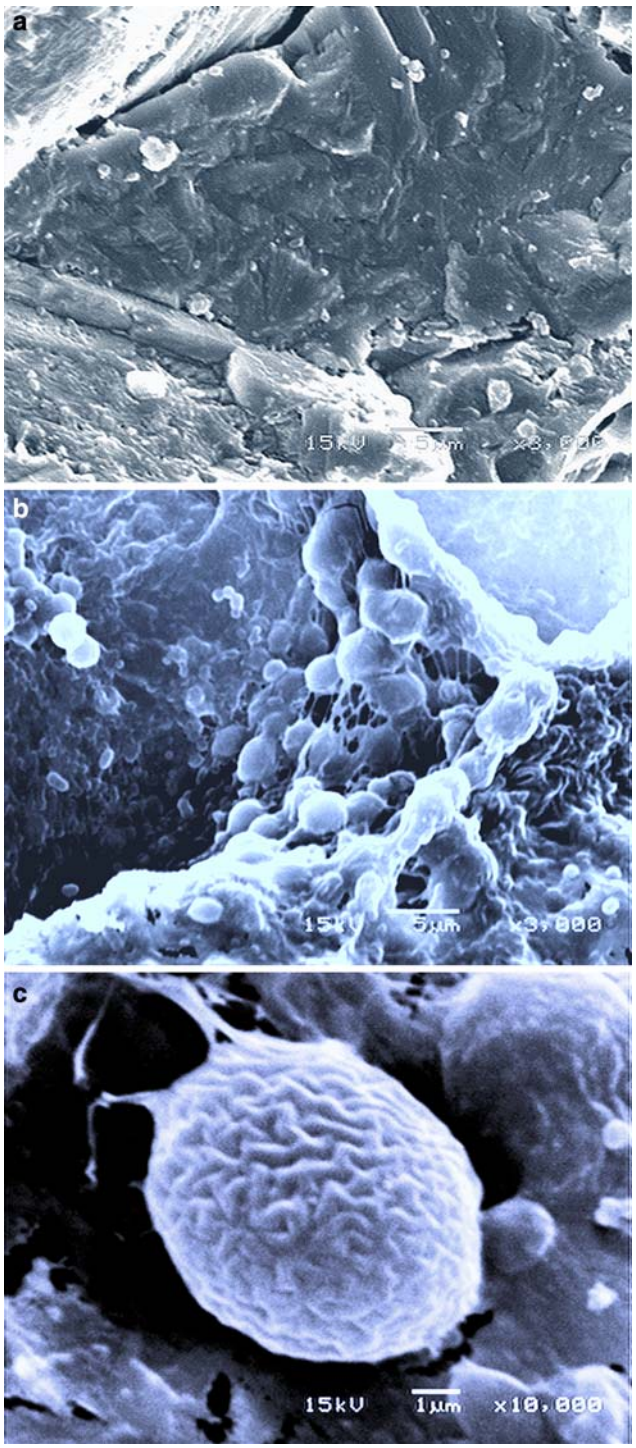

Fig. 6 Micrographs of bacteria attached to volcanic stone. **a** Porous stone structure without biofilm (×3,000); **b** porous stone vesicle showing attached biomass (×3,000); **c** detail of an *Agrobacterium tumefaciens* microcolony united by biopolymeric material (×10,000)

Acknowledgments S.P. Galíndez-Nájera and M.A. Llamas-Martínez, are holders of a research grant from PIFI-IPN. C. Juárez-Ramírez, N. Ruiz-Ordaz, D. Ahuatzi-Chacón, and J. Galíndez-Mayer, are holders of grants from COFAA-IPN, SIP-IPN, and SNI-Conacyt.

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