Community evolution during the acclimation process of activated sludge to p-chlorophenol

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Abstract: The present work describes the evolution of the kinetics curves and the changes in the community conformation of a mixed culture of microorganisms during acclimation to 4-chlorophenol as a model of chlorophenols compounds in a sequencing batch reactor. Activated sludge from a municipal wastewater treatment plant was acclimated to 200 mg/l of 4-chlorophenol. To determine the diversity and dynamics of the microbial community on the activated sludge, PCR, DGGE, cloning and sequencing techniques were used. The results showed a reduction in the degradation time during the acclimation process. DGGE analysis show that the community varies before and after the acclimation process.

Keywords: acclimation, activated sludge, DGGE, community evolution, SBR, chlorophenol

Introduction

Chlorophenols are common environmental pollutants that arise from extensive use of wood preservatives, pesticides, herbicides and fungicides and they are found frequently in pulp and paper effluents and industrial wastewater (Kringstad and Lindström, 1984; Valo *et al.,* 1985). Monochlorophenols can be formed during wastewater chlorination, and as a result of the breakdown of pesticides and chlorinated aromatic compounds (Pritchard *et al*., 1987). Chlorophenols can easily migrate within different aqueous environments and contaminate groundwater (Smith and Novak, 1987), since their solubility in water is relatively high. Due to these environmentally hazardous properties, intensive attention has been paid to the biological chlorophenols degradation. It was observed that the chlorophenols can cause death, respiratory failure, bone marrow atrophy, and skin damage in animals (Kintz *et al.,* 1992) and confers a distasteful taste and odor to food and water (Buikema *et al.,* 1979). Because of its manufacture in large quantities, animal effects, and organoleptic qualities, chlorophenols are an EPA priority pollutant (Wentz, 1989).

The first step to biodegrade chlorophenols in a wastewater treatment plant is the acclimation of the microorganisms to this type of toxic compounds. In a favorable environment, when microorganisms are put in contact with toxic compounds, acclimation to these compounds may occur (Aelion *et al.* 1989). Different mechanisms have been described to explain the acclimation phase. Wiggings *et al.* (1987) suggested that there is a selection and a multiplication of specialized microorganisms during this phase and physiological transformations occur in the metabolic system of the microorganisms, i.e., alterations at the enzymatic level, regulation and production, mutations, etc. In aerobic microbial communities, the acclimation periods range from several hours to several days (Wiggings *et al*. 1987).

Many microorganisms have been reported to degrade chlorophenols as a sole carbon and energy source, including different species of the generous as *Pseudomonas* (Knackmuss and Hellwig, 1978; Radehaus and Schmidt, 1992), *Arthrobacter* (Stanlake and Finn, 1982), *Flavobacterium* strains (Saber and Crawford, 1985), *Commamonas* (Hollender *et al*., 1994), *Streptomyces* (Yee and Wood, 1997) *Phanerochaete* (Perez *et al.,* 1997) and specialized species as *Rhodococcus chlorophenolicus* (Apajalahti *et al.,*1986) *Herbaspirillum chlorophenolicum* (Wan-Taek *et al.,* 2004), *Arthrobacter chlorophenolicus* (Westerberg et al., 2000). Also, high efficiencies of chlorophenol degradation are reported on chlorophenol degradation by mixed cultures (Moreno and

Buitrón, 2004; Quana *et al.,* 2004; Gallego *et al.,* 2003; Kim *et al.,* 2002; Puhakka *et al.,* 1995; Klecka and Maier, 1985). However, not much information is available concerning the activity and the evolution of a microbial consortium during the acclimation to chlorophenols degradation.

The present work describes the evolution of the kinetics curves and the changes in the community conformation of a mixed culture of microorganisms during acclimation to 4-chlorophenol as a model of chlorophenols compounds in a sequencing batch reactor.

Methodology

Pilot reactor

An aerobic automated Sequencing Batch Reactor (SBR) system with a capacity of 7L and an exchange volume of 57% was used (Figure 1). The airflow rate was 1.5 L/min and the temperature was maintained at 20 ºC inside the reactor. The reactor was inoculated with microorganisms coming from a municipal activated sludge treatment plant. A synthetic wastewater containing 4CP was used as a sole source of carbon and energy. Nutrients such as nitrogen, phosphorus, and oligoelements were added following the techniques recommended by AFNOR (1985). The SBR was operated under the following strategy: preaeration time (15 min), filling time (5 min), reaction time (variable depending on the necessary time to reach 99% of removal efficiency of 4-CP), settling time (12 to 30 min) and draw time (1 min).

Figure 1 Automated pilot reactor utilized for 4CP degradation

Substrate concentration was measured taking samples and processing them offline using the colorimetric technique of 4-aminoantipyrine (APHA, 1992). Total and volatile suspended solids (TSS and VSS) analyses were determined according to the APHA (1992).

Acclimation

The reactor was inoculated with activated sludge from a municipal wastewater treatment plant containing 2000 mgVSS/L. The biomass was acclimated using a variable cycle strategy, i.e., the reaction phase duration was variable and stopped when the removal of 4CP was equal or greater than 99%. The concentration of 4CP in the influent was 350 mg/L, giving an initial concentration in the tank of 200 mg/L due to the dilution by the volume exchange each cycle (4L exchanged in a total volume of 7L).

Changes in the community by the acclimation

Samples of the reactor, for the acclimation period, were taken each 24 h during 5 days, i.e., samples were collected at 24, 48, 72, 96 and 120 h, in order to determine the diversity and dynamics of the microbial community on the activated sludge. PCR, DGGE, cloning and sequencing techniques were used. Total DNA was extracted from 2 mL samples of activated sludge using an Ultra clean™ Soil DNA isolation kit (MoBio). PCR amplifications were conformed with a BioRad temperature cycle, with the following program: initial activation 95°C (15 min), 35 cycles of: denaturation 94°C (1 min), annealing 53°C (1 min) and elongation 72°C (1 min) and a final extension of 72°C (1 min). The universal primers GC-338F and 518R were used for amplification (Muyzer *et al.,* 1995). The PCR products were separated by DGGE using a D-code universal mutation detection system (BioRad, Hercules, CA) as described by Muyzer *et al.* (1995). The central portions of selected bands were excised and extracted amplicons were used as template for the re-amplification. These PCR products were cloned into pCR2.1-TOPO plasmid (Invitrogen, USA). The positive clones were sequenced with the primer M13 reverse (5´-CAGGAAACAGCTATGAC) in an ABI Prism 3100 DNA sequencer. The identification of the clones is being processed at this moment. A search of GenBank database will be conducted using the BLAST software (National Center for Biotechnology Information).

Results and Discussion

Degradation kinetics

The degradation kinetics for the acclimation process was followed. For the initial concentrations of 4CP used, acclimation was obtained in 8 degradation cycles. During the acclimation, 4CP was degraded with efficiencies higher than 99% as 4CP. During the acclimation of the activated sludge, the relationship between residual 4CP concentration and incubation time gradually changed, and then stabilized, in this case the degradation times were reduced from 48 h to 2.5 h, i.e., after 128 h of total operation (Cycle 1 to 8).

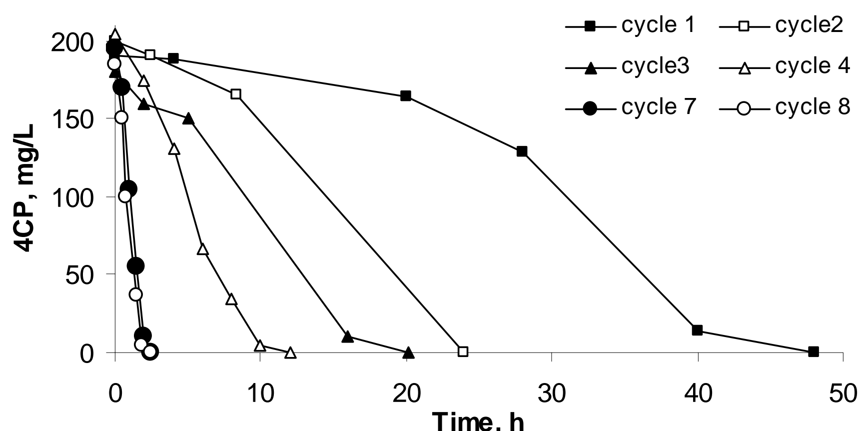

Figure 2 Degradation kinetics during the acclimation process to 200 mg/L of 4CP

DGGE analysis of the bacterial community in the consortium and bacterial identification

The V3 hypervariable region of the 16S rRNA gene was obtained by PCR amplification using the DNA obtained from the Easy-DNA kit method as template. The PCR amplification products of template DNA; 200-bp fragments were obtained as expected. The number of individual bands obtained in a DGGE analyses is related to the number of bacterial species in the tested sample. Figure 3 shows the DGGE community fingerprint of the consortium. This analysis revealed a moderately diverse bacterial community, eleven DGGE band were visible, these suggesting that minimum eleven different bacteria were present. Six of the detected bands were excised from the gel and sequenced. The most closely related sequences in the GenBank nucleotide database were

determined (The results are processing at this moment). The most relevant results are show in figure 3. It is possible to observe that at the beginning of the acclimation the species corresponding to the bands 1, 3, 4, 5 and 6 prevailed during all the acclimation period. The band 2 at the beginning of acclimation was not present. Nevertheless, at second day this band appears and it was conserved until the end of acclimation, indicating the reproduction of this specialized organism. In the non acclimated activated sludge it is possible to observe a diffuse band (band **7**) which disappears once the acclimation occurs. It was not possible to cut this band it due to the diffuse presented.

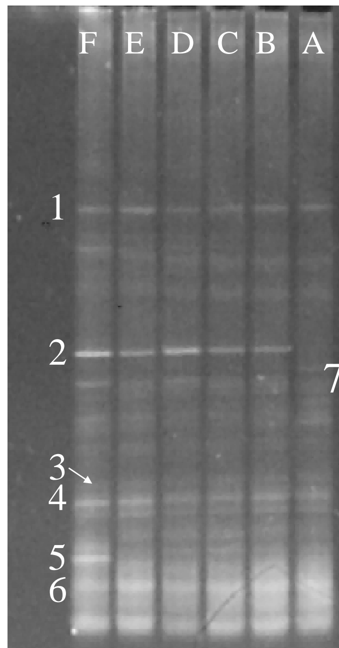

Figure 3 DGGE analysis of the bacterial community in the consortium during the acclimation. A) Initial condition, B) 24h, C) 48h, D) 72h, E) 96h, F) 120h

Conclusions

The results showed a reduction in the degradation time during the acclimation process. DGGE analysis show that the community varies before and after the acclimation process, mainly in for the bands 2 and 7. The specie of band 2 appeared during acclimation and the band 7 disappeared. The species will be identified to determine their importance in the degradation of the toxic compound.

Aknowledgements

Thanks to DGAPA (IN102005) and CONACYT (46093) support. This paper includes results of the EOLI project that is supported by the INCO program of the European Community (Contract number ICA4-CT-2002-10012). The authors thank Gustavo Navarrete for his technical support.

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