p-Cresol mineralization and bacterial population dynamics in a nitrifying sequential batch reactor

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Abstract
The ability of a nitrifying sludge to oxidize *p*-cresol (0-200 mg C L^{-1}) and its intermediates was evaluated throughout the operation cycles of a sequential batch reactor (SBR). *p*-Cresol was first transformed to *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate, which were later completely mineralized. Kinetic results indicated that the SBR system allowed an increase in the metabolic ability of the sludge to oxidize *p*-cresol and its intermediates throughout the cycles. The ability of the sludge to consume *p*-cresol and its intermediates might be associated with the presence of organotrophic species related to *Variovorax paradoxus* and *Thauera mechernichensis*. When *p*-cresol was added from 25 to 200 mg C L^{-1}, the SBR culture showed high nitrification performance with an ammonium consumption efficiency of 99.0% ± 0.5 and a yield of 0.98 ± 0.07 g NO_3^--N g^{-1} NH_4^+-N consumed. This may be the result of the high stability observed in the ammonia- and nitrite-oxidizing communities. Results indicate that nitrifying SBR may be a good alternative to eliminate simultaneously ammonium and aromatic compounds from wastewaters, maintaining a high stability in the respiratory process and the bacterial community structure.

Keywords
Ecological index; nitrification; *p*-cresol oxidation; population dynamics; sequential batch reactor

INTRODUCTION

The use of sequential batch reactors (SBR) in the biological treatment of wastewaters has been widely extended from lab-scale studies to wastewaters treatment plants. The SBR technology can be used for eliminating ammonium (NH_4^+) from the water through the coupled respiratory processes of nitrification and denitrification (Puig *et al*., 2004). Nitrification is an aerobic respiratory process carried out by two groups of gram negative lithoautotrophic bacteria phylogenetically unrelated where NH_4^+ is oxidized to nitrite (NO_2^-) by the ammonia-oxidizing bacteria (AOB) and subsequently NO_2^- is oxidized to nitrate (NO_3^-) by the nitrite-oxidizing bacteria (NOB). A wide variety of organic compounds could be toxic or provoke inhibitory effects on nitrification (Zepeda *et al*., 2007). However, knowledge on the inhibitory effects of cresols on the nitrification respiratory process is still limited (Silva *et al.*, 2009). Phenolic compounds including cresols are toxic and they can cause persistence and bioaccumulation effects in animal and vegetable organisms. Nevertheless, several studies have reported the ability of nitrifying consortia to oxidize various organic compounds, including phenolic compounds (Beristain-Cardoso *et al*., 2009). Further work is required to kinetically characterize the ability of nitrifying SBR cultures for oxidizing inhibitory compounds such as *p*-cresol throughout the cycles and evaluate the accumulation of intermediates.

Nowadays, attempts are made to relate the physiological response of microbial consortia with their structure and population dynamics. Denaturing gradient gel electrophoresis (DGGE) is a powerful tool to determine the genetic diversity of microbial communities and to identify the phylogenetic affiliation of community members in bioreactors operated under different experimental conditions (Martínez-Hernandez *et al.*, 2009). Therefore, the aim of this study was to evaluate the ability of a nitrifying sludge to consume *p*-cresol and its intermediates as well as to monitor the population dynamics of the bacterial community throughout the operation cycles in a SBR system.

MATERIALS & METHODS

Nitrifying sequential batch reactors

The sludge used for inoculating the SBRs was obtained from a continuous reactor in steady-state nitrification (Silva *et al*., 2009). Two laboratory-scale SBRs were operated with cycles of 12 h. The initial concentration of volatile suspended solids (VSS) was of 1 g L^{-1}. The 2 L SBR_A was fed with culture medium and used as control while the 1.7 L SBR_B was fed with different initial *p*-cresol concentrations (25-200 mg C L^{-1}). At the beginning of each cycle, the initial concentration of NH_4^+-N was 100 mg L^{-1}. A constant aeration and agitation of 225 rpm were maintained into the cultures.

Culture evaluation

The ammonium consumption efficiency (E_{NH4}) was expressed as (g NH_4^+-N consumed g^{-1} NH_4^+-N initial) × 100 and nitrate production yield (Y_{NO3}) as g NO_3^--N g^{-1} NH_4^+-N consumed. To determine the biomass yield (Y_{BM}, mg biomass-C produced mg^{-1} consumed-C), it was considered that 50% of the microbial biomass (VSS) is carbon. The Gompertz model was used to analyze the kinetic data of *p*-cresol consumption (Draper and Smith, 1981).

Molecular analysis

DNA extraction and 16S rRNA gene amplification

Duplicate samples were taken from both reactors at the first and the last cycle of operation for each *p*-cresol concentration tested in SBR_B. DNA was extracted using the UltraCleanTM Soil DNA Isolation kit (MO BIO Laboratories, USA). The V6-V8 regions of the 16S rRNA gene were amplified by using the bacterial primers 968-f with clamp and 1401-r. The polymerase chain reaction (PCR) amplification was performed in a thermocycler CG1-96 (Corbett Research, Australia) (García-Saucedo *et al*., 2008).

Denaturing gradient gel electrophoresis

Amplification products were separated by DGGE (García-Saucedo *et al*., 2008) in a universal DCode mutation detection system (Bio-Rad Laboratories, USA) at 60°C. Polyacrylamide gels were used at 6% (w/v) and two denaturant gradients were used: 30-38% and 45-60%. Species richness (S) and evenness (J) of the microbial community were estimated (Martínez-Hernandez *et al*., 2009). The richness index S represents the total number of bands in a lane. The J index reflects an overview of the predominance of species within a microbial community. The bands were excised and reamplified. The PCR products were purified (Wizard ® SV gel and PCR clean-up system, Promega, USA) and sequenced in the Genetic Analyzer ABI Prism 3100 (Applied Biosystems, UK) using ABI Prism Big-Dye Terminator 3.1 ready reaction cycle sequencing. Phylogenetic affiliations of the partial sequences were estimated using the program Basic Local Alignment Search Tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (McGinnis and Madden, 2004).

RESULTS AND DISCUSSION

p-Cresol oxidation by nitrifying SBR culture

p-Cresol was totally consumed from the SBR_B culture (Figure 1). The specific rate of *p*-cresol consumption (q_{pcr}) increased with the number of cycles until 100 mg C L^{-1} (Table 1). When *p*-cresol was added into the SBR_B for the first time at 200 mg C L^{-1} (cycle 296), the q_{pcr} showed a drastic decrease, but 26 cycles later the q_{pcr} increased again. These results showed that the metabolic capacity of the consortium to oxidize *p*-cresol increased throughout the cycles. *p*-Cresol was oxidized to *p*-hydroxybenzaldehyde (pOHBD) and *p*-hydroxybenzoate (pOHBT). The consortium showed also a higher metabolic capacity for oxidizing the intermediates throughout the cycles (data not shown). The total organic carbon concentration was negligible after 10 h in all cases, indicating that there was no accumulation of carbonaceous products. Furthermore, Y_{BM} was only of 0.01 mg biomass-C produced mg^{-1} consumed-C, showing that the process was clearly dissimilative. These results suggested that the main end product from *p*-cresol oxidation was CO_2.

In spite of the *p*-cresol addition, the nitrification performance did not change significantly in the SBR_B. E_{NH4} was of 99.0% ± 0.5 and Y_{NO3} of 0.98 ± 0.07 g NO_3^--N g^{-1} NH_4^+-N consumed. Similar results were obtained in the control SBR_A reactor without *p*-cresol addition.

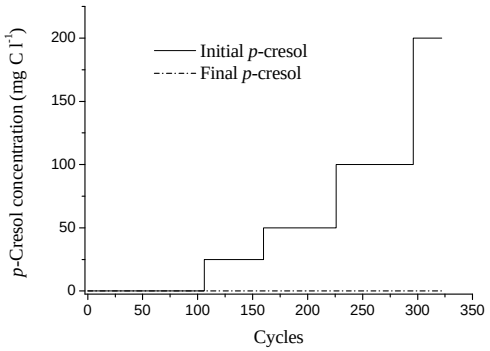

Figure 1. *p*-Cresol consumption in a nitrifying sequential batch reactor.

Table 1. Specific rates of *p*-cresol consumption in a nitrifying SBR culture.

Cycles	*p*-Cresol conc. (mg C L^{-1})	Specific rate (mg C g^{-1} VSS h^{-1})
106	25	30 ± 4
160	50	60 ± 6
226	100	160 ± 10
296	200	10 ± 2
322	200	40 ± 4

DGGE profiles of bacterial communities

DGGE analysis was performed in the SBR_A and SBR_B (Figure 2). Both reactors presented a high similarity in their S values throughout the cycles (SBR_A: 7.5 ± 1.5 and SBR_B: 7.2 ± 1.7). Thus, the addition of *p*-cresol into the SBR_B did not cause a significant change in the richness of the community. In both reactors, up to the cycle 295 (lanes 15 and 16), the J index presented an average value of 0.68 ± 0.03, indicating that the evenness in both SBRs was similar and constant. The bacterial communities of both SBRs showed a high stability independently of the *p*-cresol addition.

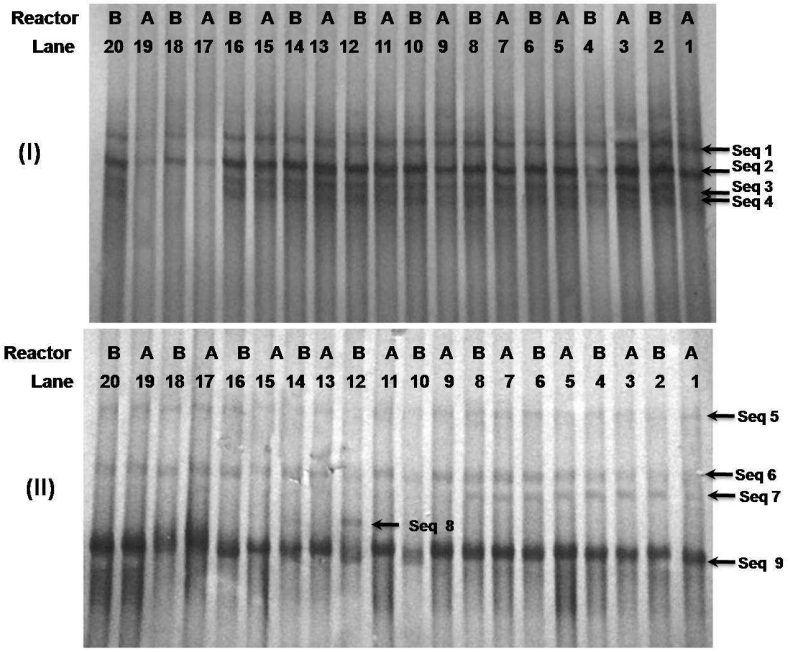

Figure 2. DGGE profiles of 16S rRNA from a nitrifying sludge in SBRs. I: Gradient 30-38%, II: Gradient 45-60%. A: SBR_A without *p*-cresol addition and B: SBR_B with *p*-cresol addition. SBR_B: lanes 2 and 4, without *p*-cresol addition at cycles 1 and 105; lanes 6 and 8, with 25 mg *p*-cresol-C L^{-1} at cycles 106 and 159; lanes 10 and 12, with 50 mg C L^{-1} at cycles 160 and 225; lanes 14 and 16, with 100 mg C L^{-1} at cycles 226 and 295; lanes 18 and 20, with 200 mg C L^{-1} at cycles 296 and 322. SBR_A: lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19, at 0 mg *p*-cresol-C L^{-1} at the following respective cycles: 1, 105, 106, 159, 160, 225, 226, 295, 296 and 322. Identified DGGE bands are labeled to the right.

Sequence analysis of DGGE bands

The bands related to *Nitrosomonas halophila* (Seq. 1), an *uncultured ammonia-oxidizing bacterium* (Seq. 2) and *Nitrosomonas oligotropha* (Seq. 5) were kept constant during the whole operation of both reactors. The band associated with *Nitrosomonas europaea* (Seq. 3) only remained until the cycle 295 in both reactors (lanes 15 and 16) and was again present in the cycle 322 of SBR_B (lane 20). Two bands related to *Nitrospira sp*. (Seq. 7) and *Nitrobacter sp*. (Seq. 9) were the NOB detected in the consortium. In both SBRs, the band related to *Nitrospira sp*. only was present until the cycle 159 (lanes 7 and 8). NO_2^- conversion to NO_3^- was conducted ($Y_{NO3} = 0.98 \pm 0.07$) due to the presence of *Nitrobacter sp*. Our results showed the high stability in the nitrifying community throughout the cycles of both reactors independently of the *p*-cresol addition. In addition, two sequences were associated with organotrophic microorganisms: *Variovorax paradoxus* and *Thauera mechernichensis* (Seq. 4 and 6) that would be involved in the *p*-cresol oxidation. The band related to *Thauera mechernichensis* remained in both cultures during all the operation period while the band associated with *Variovorax paradoxus* was observed in both reactors until the cycle 295 (lanes 15 and 16).

CONCLUSION

The sludge showed high nitrification performance ($E_{NH4} = 99.0\% \pm 0.5$ and $Y_{NO3} = 0.98 \pm 0.07$) up to 200 mg C L^{-1} of *p*-cresol. The sludge was able to totally mineralize *p*-cresol and its intermediates through a dissimilative process. The bacterial communities of both SBRs showed a high stability throughout the cycles independently of the *p*-cresol addition. This may be associated with the high stability of the nitrifying performance obtained in SBR cultures. The *p*-cresol mineralization was probably realized by some bacteria related to *Variovorax paradoxus* and *Thauera mechernichensis*. The increase in the metabolic activity of the sludge to oxidize *p*-cresol and its intermediates throughout the cycles may not clearly be associated with changes in the bacterial community composition, suggesting that the increase was rather due to a higher enzymatic capacity of the species present in the consortium.

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