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Application of the polymerase chain reaction (PCR) and reverse transcriptase/PCR for determining the fate of phenol-degrading *Pseudomonas putida* ATCC 11172 in a bioaugmented sequencing batch reactor

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Abstract The impact of bioaugmentation on the efficacy of an existing microbial population to detoxify phenol in a laboratory-scale sequencing batch reactor was evaluated. Phenol degradation and the persistence and expression of the catabolic *dmpN* gene were studied for 44 days using a combination of conventional monitoring approaches and molecular techniques. Following addition of the phenol-degrading bacterium, *Pseudomonas putida* ATCC 11172, which converts phenol to catechol by the aerobic *meta*-cleavage pathway, phenol removal in the bioaugmented reactor increased and was maintained at 95%–100%. In the unaugmented control reactor, decreased phenol removal was observed. Correspondingly, *dmpN* DNA, characteristic of *P. putida* ATCC 11172, and its expression were detected in activated sludge biomass from the bioaugmented reactor for over 41 days. The results of this study show that (i) bioaugmentation provides stability in phenol degradation, and (ii) monitoring with molecular techniques such as the polymerase chain reaction (PCR) and reverse transcriptase/PCR can successfully assess the state of a bacterium used in bioaugmentation.

Introduction

A current trend in bioremediation and wastewater treatment practices is the use of bioaugmented systems for the treatment of organic pollutants (Atlas 1991). In these biotreatment processes, an organic contaminant is present in a reactor containing significant concentrations of viable microorganisms, yet the target concentrations for the contaminant are not achieved during the course of the treatment process for a variety of reasons. These reasons include solubility problems, mass-transfer limitations, the presence of inhibitory and/or toxic concentrations of the substrate and the inability of the microbial consortia to achieve sufficient densities to detoxify the contaminant effectively. Bioaugmentation, the addition of specially adapted microorganisms to a bioreactor in which high concentrations of other microorganisms are present along with the target contaminant, attempts to overcome biodegradation efficiency problems by significantly increasing the titer of degradative organisms within a microbial consortium.

Analysis of microorganisms within bioaugmented reactors is essential in understanding the fate of introduced bacteria and their behavior during engineered treatment processes. The availability of DNA probes for a number of degradative genes and the advent of the polymerase chain reaction (PCR) provide new tools with which to explore composition and activity of microbial communities within reactors. Amplification of nucleic acids by PCR and reverse transcriptase (RT)/PCR have now been successfully applied in detecting specific genes and their activities in samples containing nucleic acids from diverse communities (Brauns et al. 1991; Bruce et al. 1992; Herrick et al. 1993; Selvaratnam et al. 1995; Steffan and Atlas 1991; Tsai and Olson 1992a, b).

Few quantitative studies on bioaugmentation strategies have been published (Aggarawal et al. 1991; Atlas 1991; Kaufman and Kreuger 1993). These studies have attempted to link the benefits of bioaugmentation by demonstrating shifts in kinetic parameters and percentage removal of target compounds using traditional methods. However, our study is the first to demonstrate the use of molecular techniques such as PCR and RT/PCR for the analysis of biodegradation performance.

In this study, phenol degradation and the persistence and expression of the *dmpN* gene were studied for 44 days using a combination of biochemical and molecular

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methods. Phenol is a toxic chemical present in most refinery and some chemical plant wastewaters. The specific objectives were (1) to evaluate critically the impact of bioaugmenting a laboratory-scale reactor with the phenol-degrading *Pseudomonas putida* ATCC 11172 on the efficiency of an existing microbial population to detoxify phenol, and (2) to apply PCR and RT/PCR techniques to determine whether the catabolic *dmpN* gene of *P. putida* ATCC 11172 was maintained and expressed in the bioreactor. This would provide direct evidence of the organism's viability. *dmpN* codes for a phenol hydroxylase enzyme that is involved in the conversion of phenol to catechol (Nordlund et al. 1990) by an aerobic pathway.

Materials and methods

Microorganisms

The organism used for bioaugmenting SBR 1 was *Pseudomonas putida* ATCC 11172, which degrades phenol by the aerobic *meta*-cleavage pathway (Molin and Nilsson 1985). The strain, obtained from the American Type Culture Collection (Rockville, Md.), was chosen for the study because of the presence of the *dmpN* gene, which was not found in the municipal sludge used to start up the laboratory-scale reactors (see below). The cells were grown to mid-exponential phase at room temperature with constant shaking (200 rpm) in nutrient broth containing 0.05% phenol before addition to sequencing batch reactor 1. In order to confirm the initial number of cells in the culture, dilutions were plated onto nutrient agar plates containing 0.05% phenol and incubated at room temperature for 48–72 h.

Reactor operation

The inoculum used to start the two 1-1 sequencing batch reactors (SBR 1 and SBR 2) consisted of mixed liquor from a secondary aeration tank from the South Bend Municipal Wastewater Treatment Plant (South Bend, Ind.). The reactors, which are suspended-growth, activated sludge systems, have been effectively utilized for the degradation of organic pollutants (Irvine and Ketchum 1989). This inoculum was chosen because of the absence of indigenous organisms containing the target *dmpN* sequences (data not shown). The reactors were run on two cycles per day, each cycle comprising a 12-h period. A cycle consisted of the following phases: a 1-h fill, a 9-h reaction time, a 1-h settling, a 30-min draw, and a 30-min idle period. On day 4, following the establishment of a steady state, 7×10^{11} cfu P. putida ATCC 11172 (approximately 20%, on a number basis, of the total biomass) resuspended in sterile feed (see below) were added to SBR 1 during the idle phase to facilitate the settling of added bacteria. SBR 2 served as the unaugmented but otherwise identical control reactor. Steady state was determined to be less than 10% change in effluent phenol concentrations measured during three consecutive hydraulic residence times. The hydraulic residence time is the average amount of time spent by a fluid element in the reactor and was 1 day for our experiments. Mixed-liquor-suspended solids in the reactors were maintained at 2000 mg/l by wasting of excess sludge. The sterile feed was composed of phenol (200 mg/l), methanol (6.7 mg/l), sodium acetate (100 mg/l), sodium succinate (66.6 mg/l), glucose (400 mg/l), sodium propionate (26.7 mg/l) sodium phosphate (dibasic; 20 mg/l), potassium phosphate (monobasic; 20 mg/l), and ammonium chloride (78.6 mg/l). During the fill phase of a cycle, 500 ml sterile feed was added to the reactors. The pH of the reactors was maintained between 7.2 and 7.5, and the temperature of the reactors was between 23 °C and 25 °C.

Processing of samples from reactors

Samples of 3 ml (representing 6 mg biomass) mixed liquor (biomass in suspending liquid) were removed from the reactors on various days during the aeration phase of a cycle and frozen immediately at −80 °C until analyzed. Sampling was carried out during the aeration phase because previous results (Selvaratnam et al. 1995) indicated that the *dmpN* gene was expressed during this phase. The biomass was separated by centrifugation and the pellets were resuspended in 1 ml 100 mM sodium phosphate buffer, pH 8 (Sambrook et al. 1989) by shaking at 150 rpm for 15 min. at room temperature. Following a second centrifugation at 4000 rpm for 10 min, the pellets were resuspended in 100 µl diethylpyrocarbonate(DEPC)-treated sterile water (Sambrook et al. 1989) and subjected to freeze/thaw lysis as described below.

Isolation of total nucleic acids from activated sludge

Lysis of bacterial cells from activated sludge biomass was by freeze/thaw (Bej et al. 1991) with certain modifications. Samples were frozen in an ethanol/solid CO_2 bath for 30 s and then thawed at 50 °C for 1 min. Following six freeze/thaw cycles, samples were heated at 85 °C for 5 min to inactivate inhibitors of PCR. Nucleic acids were separated from cellular debris and sludge particles by DEPC-equilibrated Sephadex G-200 spun columns (T sai and Olson 1992b) before treatment with DNase. For RT/PCR, DNA was eliminated from the extracts by the addition of 3 U DNase (Promega Corp., Madison, wis.) and incubating the samples at 37 °C for 15 min.

Amplification of DNA by PCR

For the detection of introduced *P. putida* ATCC 11172, a 199-bp region located within the *dmpN* gene was amplified using two primers (synthesized by the BioCore Facility, University of Notre Dame). DMPN1 (5′-ATC ACC GAC TGG GAC AAG TGG GAA GAC C -3′) was a 28-mer, while DMPN2 (5′- TGG TAT TCC AGC GGT GAA ACG GCG G -3′) was a 25-mer. This gene was chosen because it is commonly found in phenol-degrading bacteria (Nordlund et al. 1990). Sequences for the gene were obtained from GenBank.

A total volume 50 µl PCR mixture containing 2 mM $MgCl_2$, 10 × reaction buffer (New England Biolabs, Beverly, Mass.), 1 mM each dNTP (NEB), 0.15 µM each primer and 1 U (1 µl) Vent DNA polymerase (NEB) was added to each tube. Sterile mineral oil (100 µl) was added to the top of each sample to prevent evaporation of reaction mixture during PCR. Target DNA was amplified in a Thermolyne DNA thermal cycler (Fisher Scientific, Itasca, Ill.), with initial denaturation of the target DNA at 95 °C for 2 min and then 40 cycles of three-step PCR amplifications consisting of denaturation at 95 °C for 1 min, primer annealing at 50 °C, and primer extension at 72 °C for 1 min. Samples were incubated at 72 °C for 5 min at the end of amplification cycles to complete the extension reaction.

Amplification of RNA by RT/PCR

Reverse transcription and subsequent amplification of target mRNA were performed with a Gene Amp RNA PCR kit (Perkin Elmer-Roche, Norwalk, Conn.) with the following modifications. Briefly, 10 µl RT mixture (made up according to the manufacturer's instructions) containing 5 mM MgCl2, 1 × PCR buffer II, 1 mM each of dGTP, dATP, dTTP, and dCTP, 2.5 µM random hexamers, 1 U RNase inhibitor and 2.5 U reverse transcriptase was added to 10 µl DNase-treated samples. Reverse transcription was carried out in a Thermolyne DNA thermal cycler (Fisher), as follows: 25 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, and 4 °C for 5 min.

The finished RT mixture was combined with the PCR mastermix containing DMPN1 and DMPN2 primers (0.3 µM), and 2.5 U

AmpliTaq DNA polymerase and amplified as described below. A second 10-µl aliquot of each DNase-treated sample (which served as the non-RT control) was combined with 90 µl PCR master mix made up as follows: 2 mM $MgCl_2$, 1X PCR buffer II, 1 mM each of dGTP, dATP, dTTP, and dCTP, 0.15 µM each of the appropriate primers, and 2.5 U AmpliTaq DNA polymerase. Target DNA (or cDNA) was amplified with initial denaturation of the DNA at 95 °C for 2 min and then 40 cycles of three-step PCR amplifications, consisting of denaturation at 95 °C for 1 min, primer annealing at 50° C and primer extension at 72 °C for 1 min. Samples were incubated at 72 °C for 5 min at the end of the amplification cycles to complete the extension reaction.

Analysis of amplification products

PCR and RT/PCR products were visualized by gel electrophoresis using a 2% horizontal agarose gel (Sigma Chemical Co., St. Louis, Mo.) with TAE buffer (Sambrook et al. 1989). Gels were stained in a solution of ethidium bromide, visualized with a UVP UV transilluminator (UVP Inc., San Gabriel, Calif.) and photographed. Amplified *dmpN* products were verified by slot-blot DNA hybridization with a digoxigenin-labeled (Boehringer Mannheim Corporation, Indianapolis, Ind.) internal probe (DMP-INT 5′ TAC CAG GCG GAG AAA GAG AAG AAG-3′) as described by the manufacturer. Binding of probe was detected by autoradiography with Fuji RX film (Fisher) at room temperature for 10–20 min.

Determination of phenol concentrations

Phenol concentrations were determined by high-pressure liquid chromatography (HPLC). Samples were withdrawn from the reactors on various days, in triplicate, and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatants were filtered through 0.45-µM-pore-size Whatman WCN-type cellulose nitrate filters (Fisher) prior to analysis. A Beckman Ultrasphere octyldecylsilane 5-mm column (4.6 mm × 15 cm) was used with a Waters M45 pump and Waters 484 detector set at 254 nm. A Hewlett Packard HP3394 integrator determined the peak areas. The mobile phase consisted of 1:1 methanol and 1% acetic acid. In addition to phenol, a metabolite of the aerobic *meta*-cleavage pathway, namely catechol, was determined by this technique.

Results

The impact of bioaugmentation on phenol and chemical oxygen demand removals in the reactors

Bioaugmentation of SBR 1 with *P. putida* ATCC 11172 resulted in decreased phenol concentrations, as might be expected (Fig. 1). Phenol removal in SBR 1 increased after addition of the organism on day 4 and was maintained at 95%–100% removal through day 44. In the unaugmented SBR 2, phenol removal was initially 100% but decreased to 40% by day 44. Initial phenol removal in both bioreactors was probably due to the activity of bacteria degrading phenol by pathways other than the one probed in these experiments. However, this activity was not maintained in the unaugmented SBR 2, as observed by decreased phenol removal after day 15. In the bioaugmented SBR 1, sustained phenol removal was observed through day 44 presumably because of added *P. putida* ATCC 11172 and a subsequent shift in the degradation process to the aerobic *meta*-cleavage pathway.

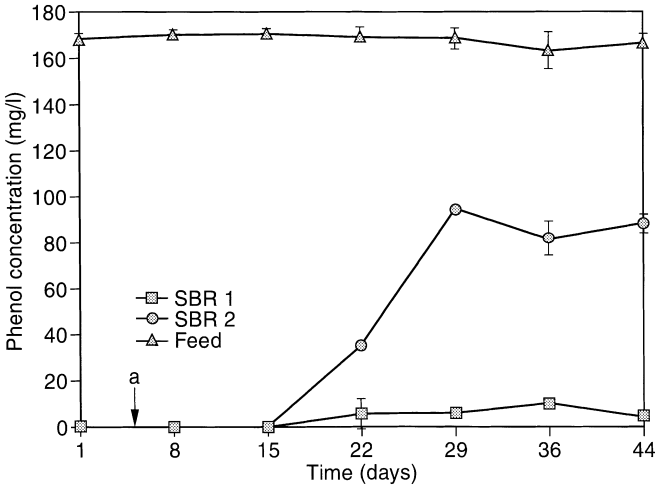

Fig. 1 Comparison of phenol removal in bioaugmented SBR 1 and unaugmented control SBR 2. *a* 7×10^{11} cfu *P. putida* ATCC 11172 were added to SBR 1 on day 4 following establishment of a steady state. Each datum point is an average of three samples

Detection of *dmpN* gene in activated sludge biomass by PCR

The amplified *dmpN* product was detected in activated sludge biomass from SBR 1 for at least 41 days after the addition of *P. putida* ATCC 11172 (Fig. 2). PCR products were observed in lanes D, F, H, and J from samples obtained on days 10, 27, 34, and 44 respectively. No amplified product was observed in activated sludge samples from the unaugmented SBR 2 reactor on identical days (lanes E, G, I, and K respectively), or in those from both reactors prior to the addition of the organism (day 1; lanes B and C representing samples from SBR 1 and SBR 2 respectively). Similar results were observed for samples from other assay times (data not shown). These results indicated the absence of *dmpN* sequences in the indigenous populations of both bioreactors prior

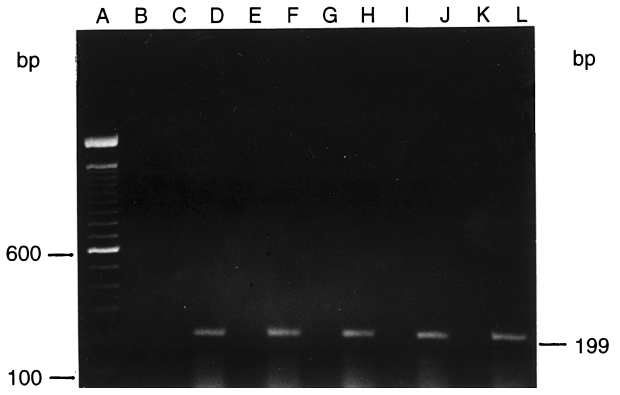

Fig. 2 DNA from 6-mg activated sludge samples was amplified by the polymerase chain reaction (PCR) with primers for the *dmpN* gene. *A* 100-bp DNA ladder size marker; *B* day-1 sample from sequencing batch reactor (SBR) 1; *C* day-1 sample from SBR 2; *D, F, H, J* SBR 1 samples from days 10, 27, 34, and 44 respectively; *E, G, I, K* SBR 2 samples from days 10, 27, 34, and 44 respectively; *L P. putida* ATCC 11172 DNA as a positive control

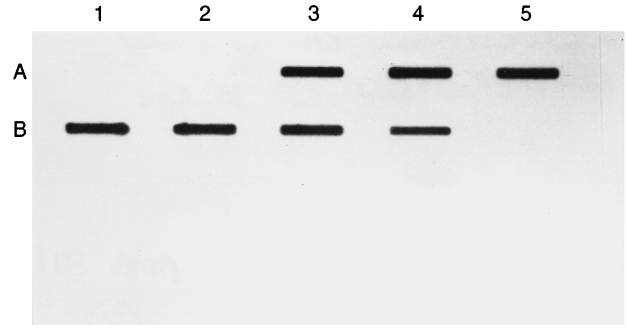

Fig. 3 PCR mixtures containing DNA from activated sludge samples were analyzed by slot-blot hybridization with the digoxigenin-labeled DMP-INT probe. *A1* PCR reaction mixture from SBR 2 obtained on day 20; *A2* PCR reaction mixture from SBR 1 obtained on day 1; *A3–5*, *B1–3* PCR reaction mixtures from SBR 1 obtained on days 10, 20, 27, 34, 38, and 44 respectively; *B4* 1 μg purified DNA from *P. putida* ATCC 11172 as a positive control

to bioaugmentation with SBR 1. d*mpN* product was also observed in lane L, corresponding to purified DNA from the positive control *P. putida* ATCC 11172.

Results obtained by PCR were confirmed by slot-blot hybridization of amplified products from SBR 1 with the digoxigenin-labeled DMP-INT probe (Fig. 3). Positive hybridization signals were observed in lanes A3–5 and B1–3 corresponding to PCR-amplified products from days 10, 20, 27, 34, 38, and 44 respectively. A positive band was also observed in lane B4, which contained 1 μg purified DNA from *P. putida* ATCC 11172. No bands were observed in lane A2, which contained amplified reaction mixture from SBR 1 on day 1, 3 days prior to bioaugmentation of the reactor, or in A1, which contained day-20 amplified reaction mixture from unagumented SBR 2.

Evaluation of *dmpN* expression in SBR 1

Total nucleic acids from activated sludge samples were isolated by rapid freeze/thaw lysis and *dmpN* mRNA was amplified by RT/PCR. DNA in the samples was eliminated by treatment with DNase. Following the addition of *P. putida* ATCC 11172 to SBR 1 on day 4, *dmpN* mRNA was observed weekly in the activated sludge of SBR 1 (Fig. 4). Activated sludge samples examined on days 10, 27, 34, and 44 contained *dmpN* mRNA (lanes D, F, H, and J respectively). No *dmpN* mRNA was observed in the unaugmented SBR 2 on identical days (lanes E, G, I, and K respectively) or in either reactor prior to bioaugmentation (day 1; lanes B and C representing samples from SBR 1 and SBR 2 respectively). In addition, no products were observed in PCR-amplified DNase-treated controls, in RT/PCR-amplified RNase-treated controls (representative samples from SBR 1 obtained on day 20 are shown in lanes L and N respectively) or in the reagent blank (lane P), confirming that *dmpN* RNA was the target nucleic acid in RT/PCR.

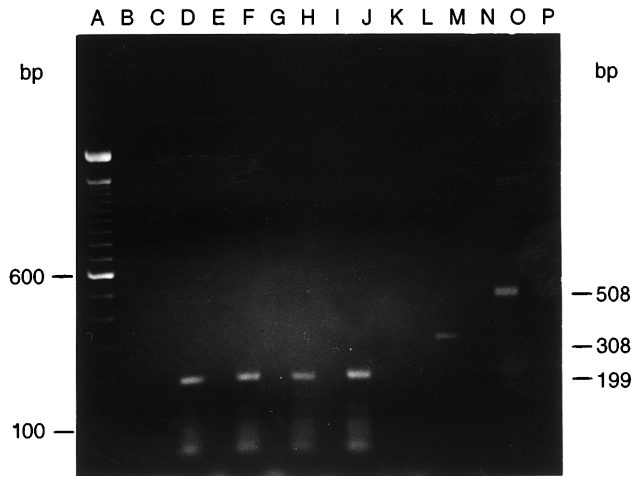

Fig. 4 Nucleic acids from 6-mg activated sludge samples were amplified by reverse transcriptase(RT)/PCR with primers for the *dmpN* gene. *A* 100-bp DNA ladder size marker; *B* day 1 sample from SBR 1; *C* day 1 sample from SBR 2; *D, F, H, J* SBR 1 samples from days 10, 27, 34, and 44 respectively; *E, G, I, K* SBR 2 samples from days 10, 27, 34, and 44 respectively; *L* RT/PCR-amplified RNase-treated control from SBR 1 (day 20); *M* positive mRNA control for RT/PCR; *N* PCR-amplified DNase-treated control from SBR 1 (day 20); *O* positive control lambda DNA for PCR; *P* RT/PCR reagent blank

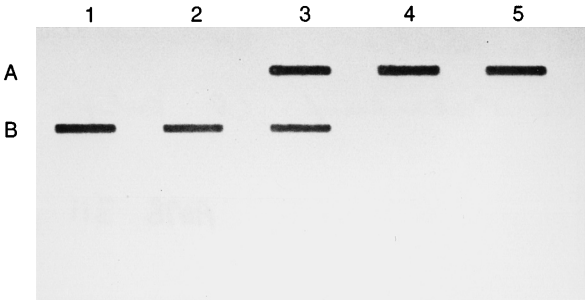

Fig. 5 RT/PCR mixtures containing nucleic acids from activated sludge samples were analyzed by slot-blot hybridization with the digoxigenin-labeled DMP-INT probe. *A1* RT/PCR reaction mixture from SBR 2 obtained on day 20; *A2* RT/PCR reaction mixture from SBR 1 obtained on day 1; *A3–5*, *B1–3* PCR reaction mixtures from SBR 1 obtained on days 10, 20, 27, 34, 38, and 44 respectively; *B4* RT/PCR reagent control

RT/PCR products were verified by slot-blot hybridization with the digoxigenin-labeled DMP-INT probe (Fig. 5). Positive signals were observed for the SBR 1 reaction mixtures from days 10, 20, 27, 34, 38, and 44 (lanes A3–5 and B1–3 respectively). No signals were observed in the SBR 2 reaction mixture from day 20 (lane A1) or in lane A2, corresponding to the SBR 1 RT/PCR reaction mixture from day 1.

Discussion

One of the factors that determines the need for bioaugmentation is the presence or absence of a bacterial

population that is capable of degrading the target pollutant. To date, most bioaugmentation practices have been accomplished by enhancing the growth of indigenous microorganisms, or by augmenting the existing microbial population with exogenous microorganisms capable of degrading the pollutant of interest. In bioreactors, conditions can be effectively optimized to achieve maximum microbial degradation of wastes and pollutants by bioaugmentation. However, one of the major constraints of this application is the ability to follow the added microorganism effectively and the ability to predict whether this organism will persist in the treatment system. The use of molecular techniques like PCR and RT/PCR coupled with biochemical analyses such as HPLC provides a powerful set of tools with which to monitor the survival of an introduced microorganism and its specific metabolic activities through gene expression in bioaugmented and other environmental systems. The molecular methods utilized in this investigation demonstrate not only the presence or absence but also the physiological status of the added *P. putida* as indicated by the results from monitoring the *dmpN* gene. The presence of *dmpN* mRNA in activated sludge of SBR 1 suggests that mRNA synthesis from this gene is stimulated by basal levels of phenol present in the bioreactor, coupled with aeration. This verifies previous observations of Selvaratnam et al. (1995), who noted that *dmpN* expression in pure culture and during a single cycle of sequencing batch reactor operation was stimulated by a combination of phenol and aeration. The evidence that enhanced phenol biodegradation was a result of microbial action, as indicated by the persistence and expression of the *dmpN* gene for over 41 days, clearly shows that monitoring with molecular techniques can successfully assess the state of a bacterium used in bioaugmentation and that bioaugmentation can be effectively used to enhance the degradation of a taget pollutant in sequencing batch reactors.

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