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Bioaugmentation of microbial communities in laboratory and pilot scale sequencing batch biofilm reactors using the TOL plasmid

S. Venkata Mohan [a,b,*], Christina Falkentoft [a,c], Y. Venkata Nancharaiah [a,d], Belinda S. McSwain Sturm [a,e], Pierre Wattiau [a,f], Peter A. Wilderer [a,g], Stefan Wuertz [a,h], Martina Hausner [a,i]

[a] Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, D-85748 Garching, Germany
[b] Bioengineering and Environmental Centre, Indian Institute of Chemical Technology, Hyderabad 500 007, India
[c] European Patent Office, Erhardtstrasse 27, 80331 Munich, Germany
[d] Water and Steam Chemistry Laboratory, BARC Facilities, Kalpakkam 603102, India
[e] Department of Civil, Environmental and Architectural Engineering, The University of Kansas, Learned Hall, 1530 W 15th Street, Lawrence, KS, USA
[f] Veterinary and Agrochemical Research Institute, Department of Bacteriology, Groeselenberg 99, B-1180 Brussels, Belgium
[g] Institute for Advanced Studies on Sustainability, Amalienstr. 75, 80799 Munich, Germany
[h] Department of Civil and Environmental Engineering, University of California, One Shields Avenue, Davis, CA 95616, USA
[i] Department of Chemistry and Biology, Ryerson University, 350 Victoria Street, Toronto, Ontario, Canada M5B 2K3

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ABSTRACT

The aim of this study was to investigate the effectiveness of bioaugmentation and transfer of plasmid pWWO (TOL plasmid) to mixed microbial populations in pilot and laboratory scale sequencing batch biofilm reactors (SBBRs) treating synthetic wastewater containing benzyl alcohol (BA) as a model xenobiotic. The plasmid donor was a *Pseudomonas putida* strain chromosomally tagged with the gene for the red fluorescent protein carrying a green fluorescent protein labeled TOL plasmid, which confers degradation capacity for several compounds including toluene and BA. In the pilot scale SBBR donor cells were disappeared 84 h after inoculation while transconjugants were not detected at all. In contrast, both donor and transconjugant cells were detected in the laboratory scale reactor where the ratio of transconjugants to donors fluctuated between 1.9×10^{-1} and 8.9×10^{-1} during an experimental period of 32 days. BA degradation rate was enhanced after donor inoculation from 0.98 mg BA/min prior to inoculation to 1.9 mg BA/min on the seventeenth day of operation. Survival of a bioaugmented strain, conjugative plasmid transfer and enhanced BA degradation was demonstrated in the laboratory scale SBBR but not in the pilot scale SBBR.

1. Introduction

Bioaugmentation is the application of indigenous or allochthonous wild type or genetically modified organisms to polluted hazardous waste sites or bioreactors in order to accelerate the removal of undesired compounds (Van Limbergen et al., 1998). Bioaugmentation has been used to improve the start-up of a reactor (Wilderer et al., 1991), to enhance reactor performance (Stephenson and Stephenson, 1992; Jianlong et al., 2002), to protect the existing microbial community against adverse effects (Hajji et al., 2000; Quan et al., 2003; Bathe, 2004; Venkata Mohan et al., 2005a, 2006), to accelerate the onset of degradation (Bathe et al., 2005; Hu et al., 2008; Venkata Mohan et al., 2007; Park et al., 2008) or to compensate for organic or hydraulic overloading (Chong et al., 1997). Bioaugmentation has been also used as a treatment strategy for contaminated soils (Brandon, 1997; Halden et al., 1999; Newby et al., 2000; Roane et al., 2001; Rojas-Avelizapa et al., 2003; Top et al., 1999, 2002; Zhang et al., 2000; Sarma et al., 2006; Venkata Mohan et al., 2006, 2008, 2009; Shailaja et al., 2007; Rodrigo et al., 2008; Jezequel and Lebeau, 2008). Bioaugmentation with genetically modified organisms carrying plasmid-encoded catabolic genes has the potential to enhance the breakdown of xenobiotic compounds by increasing the degradation potential of an indigenous microbial population via horizontal gene transfer. Horizontal gene transfer is considered to be an important mechanism for microorganisms to rapidly adapt to changing environments. Conjugation, the exchange of genetic elements via cell-to-cell contact is thought to be significant for bacteria residing in biofilms and other bioaggregates which represent high cell density environments (Hausner and Wuertz, 1999;Wuertz et al., 2004). Novel catabolic traits can be acquired as a result of horizontal gene transfer and can be directly inherited by future generations (Amabile-Cuevas and Chicurel, 1996).

* Corresponding author. Address: Bioengineering and Environmental Centre, Indian Institute of Chemical Technology, Hyderabad 500 007, India. Tel.: +91 40 27160123x2664.
E-mail address: vmohan_s@yahoo.com (S. Venkata Mohan).

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In open engineered systems such as wastewater treatment plants, cell densities and growth rates are much higher and the impact of horizontal gene transfer may be enhanced by rapid division of transconjugants passing their newly acquired genetic information to the next generation (Hausner and Wuertz, 1999; Wuertz et al., 2002). Conjugation is likely to play a major role in spreading genetic information and can be exploited in bioaugmentation. Catabolic plasmid transfer has been previously studies in model aerobic suspended growth microcosms (Molin and Tolker-Nielsen, 2003; Bathe, 2004; Bathe et al., 2005; Boon et al., 2000; McClure et al., 1991). Sequencing batch reactor (SBR) technology has been developed on the basic scientific assumption that periodic adaptation of microorganisms to defined process conditions is effectively achieved in a batch fed system in which xenobiotic exposure time and the frequency of exposure can be set independently of any inflow conditions (Wilderer et al., 2001). Thus, SBR technology can influence the distribution and physiological state of microorganisms, which are selected to grow in the reactor. Bioaugmentation-mediated enhanced degradation of xenobiotics in a sequencing mode activated sludge system has been shown previously (Bathe, 2004; Bathe et al., 2005). However, bioaugmented species often fail to compete with the indigenous population and may also cause process inhibition (Bouchez et al., 2000a, b). Similarly, possible instability of the catabolic plasmid and varied survival success of the introduced strain in the system do not ensure effective degradation (Lewis et al., 1986; McClure et al., 1991). Therefore, it is imperative to monitor the survival of the introduced strain and plasmid transfer (emergence of transconjugants) along with the performance of the reactor. For this purpose, cultivation independent in situ techniques using fluorescent marker genes along with microscopy are useful as reviewed by Sorensen et al. (2005). One such culture independent approach for in situ tracing of the inoculated strain or evaluating the extent of the spread of catabolic plasmid is to use fluorescent protein genes such as those for the green fluorescent protein (GFP) or red fluorescent protein (DsRed) as markers (Boon et al., 2000; Nancharaiah et al., 2003; Bathe, 2004; Bathe et al., 2005).

The aim of this study was to evaluate process enhancement using bioaugmentation and horizontal gene transfer of the TOL plasmid, which confers degradation capacity for several compounds including toluene and benzyl alcohol (BA) (Harayama et al., 1986), to mixed microbial biofilm communities in biofilms cultivated in laboratory and pilot scale sequencing batch biofilm reactors (SBBRs) treating synthetic wastewater containing benzyl alcohol (BA) as a model xenobiotic compound. Conjugal transfer of the plasmid and the fate of donor cells were investigated in situ with confocal laser scanning microscopy (CLSM) and GFP and DsRed as genetic markers. Studies were performed separately in two independent SBBRs (pilot and laboratory scale) at low and higher volume levels.

2. Methods

2.1. Donor strain and plasmid

The donor organism was a modified *Pseudomonas putida* strain KT2442 (Christensen et al., 1998). The strain was chromosomally labeled with the DsRed gene and carried a *gfp*5 tagged TOL plasmid, as described in detail by Nancharaiah et al. (2003). The donor strain shows both green and red fluorescence, whereas transconjugant cells which received the TOL plasmid fluoresced only green. The differential fluorescence emission was the basis for microscopic tracking and differentiation of introduced donors and transconjugants that had acquired the *gfp*-tagged plasmid upon conjugation.

2.2. Sequencing batch biofilm reactors (SBBRs)

2.2.1. Pilot scale SBBR

Pilot scale glass SBBR with 38 l of total volume and 21 l of working volume was used in the experiments. Clay beads (Biolith clay beads, Phillip Muller, Germany) with a diameter of 4.5–8 mm and porosity of approximately 0.33 were used as carrier material for biofilm formation. The reactor was operated in plug flow mode without back washing. A sludge retention time (SRT) of 14 days was maintained throughout the study by daily removal of a fixed amount of carrier beads and replacement with fresh beads. The water exchange ratio was 50%. A data acquisition and control system with a computer program written in the graphical programming language Lab View 5.1 (National Instruments, Germany) was used to control cycle sequence and duration, air supply, substrate addition, effluent withdrawal and recirculation. The reactor was operated in a sequencing batch mode with a total cycle period/hydraulic retention time (HRT) of 8.2 h (cycle configuration: fill phase – 15.5 min, aeration phase – 480 min, draw phase – 4.5 min).

2.2.2. Laboratory scale SBBR

A glass laboratory scale SBBR with a volume of 1.6 l and an exchangeable water volume of 1 l was used. Glass beads with a diameter of 7–10 mm (average diameter ~8.5 mm) and porosity of approximately 0.34 were used as carrier material for biofilm formation. The reactor was operated in a completely mixed configuration with an SRT of 10 days by daily removal of 160 ml of glass beads. The reactor was operated with an 8 h cycle (HRT) comprised of 16 min filling phase, 450 min aeration with recirculation phase and 14 min drawing phase with 100% water exchange. Two control reactors were operated separately under the same operating conditions without bioaugmentation and bioaugmentation with BA. Therefore, control reactor received BA but not the donor strain and was used to evaluate the effect of bioaugmentation on BA degradation and COD removal, whereas control reactor received neither BA nor the donor strain and was used to assess the effect of BA on COD removal.

2.3. Inoculum and operation of reactors

To develop biofilm in the pilot scale SBBR, an inoculum collected from a biofilm reactor operating in the laboratory treating municipal wastewater was used. The laboratory scale SBBRs were inoculated with wastewater collected from the aeration tank outlet (TSS = 1630 mg/l; MLVSS = 1108 mg/l) of a municipal wastewater plant (WWTP Grueneck, 23 Munich, Germany). Biomass was harvested by centrifugation, washed twice with PBS [in g/l, NaCl, 8, KCl, 0.2, Na_2HPO_4, 1.44, NaH_2PO_4, 0.2, pH 7.0], and stored as a glycerol culture at −80 °C until further use. Before inoculation, the stored culture was washed twice in PBS, resuspended in synthetic wastewater, incubated overnight at 30 °C, harvested by centrifugation and resuspended in the synthetic wastewater before being added to the laboratory scale SBBR.

After inoculation, all reactors were fed with synthetic wastewater without benzyl alcohol (BA) and operated until stable operating conditions with respect to carbon removal were achieved. The composition of the synthetic wastewater was as follows (in mg l^{-1}): sodium acetate, 61.7; citric acid, 66.7; D-(+)-glucose, 46.7; sodium gluconate, 61.7; BA, 108.2; yeast extract, 1.0; NH_4Cl, 26.7; $NaH_2PO_4 \cdot 2H_2O$, 75.5; $MgSO_4 \cdot 2H_2O$, 90; $CaCl_2$, 14; $NaHCO_3$, 275.4; $FeCl_3 \cdot 6H_2O$, 0.45; H_3BO_3, 0.045; $CuSO_4 \cdot 5H_2O$, 0.009; KI, 0.054; $MnCl_2 \cdot 4H_2O$, 0.036; $Na_2MoO_4 \cdot 2H_2O$, 0.018; $ZnSO_4 \cdot 7H_2O$, 0.036; $CoCl_2 \cdot 6H_2O$, 0.045; and disodium EDTA, 3.0. The organic substrate and nutrient stock solutions were prepared separately prior to experimentation and stored in refrigerator and subsequently used by further diluting with tap water. The substrate oxidation in stocks