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Performance and spatial community succession of an anaerobic baffled reactor treating acetone–butanol–ethanol fermentation wastewater

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ABSTRACT

An anaerobic baffled reactor with four compartments (C1–C4) was successfully used for treatment of acetone–butanol–ethanol fermentation wastewater and methane production. The chemical oxygen demand (COD) removal efficiency was 88.2% with a CH_4 yield of 0.25 L/(g $COD_{removed}$) when organic loading rate (OLR) was 5.4 kg COD m^{-3} d^{-1}. C1 played the most important role in solvents (acetone, butanol and ethanol) and COD removal. Community structure of C2 was similar to that in C1 at stage 3 with higher OLR, but was similar to those in C3 and C4 at stages 1–2 with lower OLR. This community variation in C2 was consistent with its increased role in COD and solvent removal at stage 3. During community succession from C1 to C4 at stage 3, abundance of *Firmicutes* (especially OTUs ABRB07 and ABRB10) and *Methanoculleus* decreased, while *Bacteroidetes* and *Methanocorpusculum* became dominant. Thus, ABRB07 coupled with *Methanoculleus* and/or acetogen (ABRB10) may be key species for solvents degradation.

1. Introduction

Butanol has many industrial applications and has more recently been considered as a next generation biofuel with many advantages over ethanol (Durre, 2008). There exists a high annual global demand for butanol production, which will likely increase in the future. Solventogenic clostridia produce butanol, acetone and ethanol (designated as "solvents" in this study and used elsewhere henceforth unless stated otherwise) by acetone–butanol–ethanol (ABE) fermentation using various raw materials (e.g., molasses sugar or corn starch) (Ezeji et al., 2007). Recent innovations in ABE fermentation have included genetic strain improvements to increase solvents resistance and product yield; advanced fermentation and downstream processing techniques to enhance efficiency; and alternative substrates to reduce cost (Lee et al., 2008). Thus, butanol production has the potential to compete economically with the petrochemical industry, which relies on nonrenewable fossil fuels and increases CO_2 emissions (Durre, 2008). Unfortunately, ABE fermentation produces a large volume of high-strength wastewater containing residual solvents that are toxic to humans (Jones and Woods, 1986) and cause severe environmental pollution (Baltrenas and Zagorskis, 2009). To our knowledge, only a few studies concerning the treatment of ABE fermentation wastewater have been conducted.

An anaerobic baffled reactor (ABR) consists of several compartments, which are separated by a series of vertical baffles to increase biomass retention. Compared with other high-rate anaerobic reactor designs, an ABR has many advantages, including lower construction cost and higher resilience to hydraulic and organic shock loads, as summarized by Barber and Stuckey (Barber and Stuckey, 1999). One of the most significant advantages of an ABR is the phase separation that results from organic component concentration gradients from the first compartment to the last one and allows spatial community succession for the development of different microbial populations (Barber and Stuckey, 1999). This protects more sensitive populations (e.g., methanogens) from exposure to toxic materials and enhances their resistance to changes in environmental factors such as pH, and fatty acid and heavy metal levels (Barber and Stuckey, 1999). For this reason, ABRs have been applied for the treatment of a variety of high-strength wastewaters with xenobiotic components (Boopathy and Tilche, 1992; Grover et al., 1999; Ji et al., 2009; Liu et al., 2009). Owing to the toxicity of butanol and acetone in ABE fermentation wastewater, ABR technology was used in this study.

Spatial community succession in ABRs has been studied extensively, based mostly on cultivation techniques and morphological descriptions (Ji et al., 2009; Uyanik et al., 2002). Culture-independent methods targeting phylogenetic markers such as 16S rRNA genes may provide more detailed information on microbial community structure and dynamics (Kaplan and Kitts, 2004). Recently, the PCR-denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA genes has been used to rapidly monitor shifts in microbial

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community compositions in ABRs (Liu et al., 2009). Studies using fluorescence *in situ* hybridization and 16S rRNA gene clone libraries have provided more detail information on population compositions and dynamics in ABRs (Lalbahadur et al., 2005; Plumb et al., 2001).

Little is known about the diversity of microbial communities involved in anaerobic butanol and acetone degradation. Although syntrophic anaerobic bacteria and methanogens with the ability to degrade butanol have been isolated (Imachi et al., 2009, 2002; Schink, 2006), whether these strains are present and functional in natural communities is unknown. Moreover, microorganisms with the capacity to degrade acetone under anaerobic conditions (free of sulfate and nitrate) have not yet been reported (Schink, 2006). A methane-producing, acetone-enriched culture was obtained by Platen (Platen and Schink, 1987), but its phylogenetic composition was not determined.

In this study, the relationship between spatial community succession and ABR treatment of ABE fermentation wastewater was investigated using PCR-DGGE, clone library and real-time quantitative PCR based on 16S rRNA gene. Moreover, an analysis of spatial community succession disclosed the microbial diversity and possible key species involved in the anaerobic degradation of butanol and acetone.

2. Methods

2.1. Bioreactor design

The studies were performed in a lab-scale ABR ($280 \times 93 \times 445$ mm, LWH) (Supplementary Fig. S1) made of transparent plexiglass and having an effective volume of 8 L. The ABR was separated into four equal compartments, and each compartment was further divided by vertical baffles into downflow and upflow sections with a volume ratio of 1:4. A bottom edge slanted at 45° produced effective mixing and contact between the wastewater and anaerobic sludge (Uyanik et al., 2002). The first, second, third, and fourth compartments were denoted as C1, C2, C3, and C4, and each contained three sample pots (Supplementary Fig. S1). The total biogas produced from all four compartments was collected by the gas–liquid displacement method.

2.2. ABE fermentation wastewater

ABE fermentation wastewater was obtained from batch fermentation of 8% corn meal by *Clostridium acetobutylicum* (ATCC 824) at 37 °C. When ABE fermentation was finished, the fermentation liquor was autoclaved at 121 °C for 30 min to vaporize solvents, passed through a stainless steel sieve (1 mm) twice to remove solid particles, and then used as raw wastewater. The raw wastewater had a chemical oxygen demand (COD) ranging from 45 to 63 kg L^{-1} and residual solvents concentrations of 0.7–0.8 g L^{-1} acetone, 0.56–0.63 g L^{-1} ethanol, and 4.9–5.6 g L^{-1} butanol. The raw wastewater was diluted according to the organic loading rate using effluent from C4 and tap water in a 1:1 ratio, and trace elements were added. To maintain a COD:N:P ratio of 200–300:5:1, NH_4Cl and $Na_2HPO_4{\cdot}12H_2O$ were also added.

2.3. Reactor operation and sampling

The reactor was maintained at 40 ± 1 °C and inoculated with 10% fresh slurry from a mesophilic lab-scale biogas digester in our laboratory that was fed with pig manure and rice straw. During the initial 32-day start-up period, the ABR was kept running at a low organic loading rate (OLR) of 0.96 kg COD m^{-3} d^{-1} and a 60-h hydraulic retention time (HRT) until performance stabilized. The OLR was then gradually increased in three stages, to 2.67, 3.22, and finally 5.4 kg COD m^{-3} d^{-1}, and the HRT was maintained at 40 h. The OLR at each stage was not increased until a stable methane production level was achieved.

At each stage, samples were taken from the top port of each compartment, and the pH, volatile fatty acids (VFAs), and COD were assayed. The bottom sample ports were used to collect samples for microbial community analysis. The volume and CH_4 concentration of biogas produced from all four compartments were measured periodically.

2.4. DNA extraction

Genomic DNA was extracted following the method of Zhou (Zhou et al., 1996) with minor modifications. Sample pellets were suspended in 7.25 mL of extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, and 1% (w/v) cetyltrimethylammonium bromide), and then 50 μL of proteinase K (10 mg m L^{-1}) and 320 μL of 25% (w/v) SDS were added. After the cells were lysed for 2 h at 65 °C, the crude lysate was clarified by centrifugation at 6000*g* for 10 min. The supernatant was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), followed by extraction with chloroform: isoamyl alcohol (24:1) to remove residual phenol. The aqueous phase was precipitated with 0.6 volume of isopropanol for 30 min at room temperature, and the DNA was collected by centrifugation at 12,000*g* for 5 min. The DNA was dissolved in 200 μL of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), treated with 2 μL of RNase (10 mg mL^{-1}) for 30 min at 37 °C, and stored at −20 °C until needed.

2.5. 16S rRNA gene amplification and denaturing gradient gel electrophoresis (DGGE)

The V3 region of the 16S rRNA gene was amplified by PCR using the primers EubacVf (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and Vr (5′-ATT ACC GCG GCT GCT GG-3′) for the bacteria, or the primers PARCH340f (5′-CCC TAC GGG GYG CAS CAG-3′) and PARCH519r (5′-TTA CCG CGG CKG CTG-3′) for the archaea. PCR amplification was performed as described by Muyzer (Muyzer et al., 1993) and Ovreas (Ovreas et al., 1997). Reconditioning PCR was performed (Thompson et al., 2002) to reduce heteroduplexes and single-stranded DNA in the PCR products. The concentration of the reconditioned PCR product was assayed by agarose gel electrophoresis.

DGGE was performed using a DCode Universal Mutation Detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products of 16S rRNA gene V3 region (500 and 400 ng for bacterial and archaeal PCR products, respectively) were loaded on 8% (w/v) polyacrylamide gels in 1×TAE, with a denaturing gradient ranging from 25–60% for bacterial DGGE or 35–65% for archaeal DGGE (where 100% denaturant contains 7 M urea and 40% deionized formamide). After electrophoresis at 200 V and 60 °C for 5 h, the gels were silver stained. The migration and intensity of each band was analyzed using Quantity One software (Bio-Rad) as described by the manufacturer, and the intensity of each band was normalized. Principal component analysis (PCA) was performed based on the normalized band density and variance–covariance matrix using the PAST software package (Palaeontological Statistics, version 1.88).

2.6. Construction and sequencing of 16S rRNA gene clone libraries

Bacterial and archaeal 16S rRNA gene clone libraries were constructed using the bacterial universal primer set 27f (5′-GAGAGTTT GATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTACGA-3′)