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Polyvinyl alcohol biodegradation under denitrifying conditions

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ABSTRACT

Polyvinyl alcohol was biodegraded under denitrifying conditions with a microbial community originated from a municipal wastewater treatment plant. The derived microbial consortium was capable of polyvinyl alcohol degradation under both denitrifying and aerobic conditions. The community dynamics was monitored by temperature gradient gel electrophoresis, and a principal utilizing organism was identified and assigned as *Steroidobacter* sp. PD. The possible role of *Steroidobacter* sp. PD was also investigated by sequencing the 16S rDNA clone library prepared from the degrading community. qPCR analysis showed that the fraction of the microorganism in the community was very low initially (0.02%) and had reached to about 16% by the end of the biodegradation experiment. The study revealed that polyvinyl alcohol can be biodegraded in a water environment not only under aerobic but also under denitrifying conditions.

1. Introduction

Polyvinyl alcohol (PVA) is a water soluble polymer produced on a mass scale. Its annual production exceeds 1 megaton and a steady rise in this figure (IHS Handbook, 2007) is discernible. PVA is used as a thickening, emulsifying or film-forming agent or as an adhesive in many household and industrial applications, especially in the paper, textile and chemical industries. Due to such mass production and utilization a considerable amount is expected to leak from the afore-mentioned processes into the environment, especially into waste-water.

Fortunately, PVA was found to be biodegradable despite its full carbon backbone. It has, however, also gradually become apparent that PVA-degradation capacity is not ubiquitous, and that it is rather sparsely distributed among some bacterial and fungal taxa. Most such bacterial degraders were classified among pseudomonads but especially sphingomonads (Kawai, 1999). Some other bacterial degraders comprise Gram-negative species like *Alcaligenes faecalis* (Matsumura et al., 1994) but also Gram-positive ones, e.g. *Bacillus megaterium* (Mori et al., 1996). Examples of some fungal degraders identified so far include *Penicillium* (Qian et al., 2004), and *Aspergilus* (Jecu et al., 2010; Stoica-Guzun et al., 2011). From the literature it is obvious that substantial biodegradation can mainly be expected in an aerobic aquatic environment, while PVA removal from soils is very limited and can be mostly a result of the non-specific action of lignolytic enzymes (Mejia et al., 1999; Chiellini et al., 2003). There are some indications describing partial anaerobic removal of relatively low molecular weight PVA (Matsumura et al., 1993; Gartiser et al., 1998) or partial anaerobic removal of PVA as a component of a polymer blend with other easily biodegradable constituents (Hrnčiřík et al., 2010). Yu et al. (Yu et al., 1996) have suggested that the addition of nitrates could enhance PVA removal in a sequential anaerobic–aerobic bioreactor. However, none of these reports identified the relevant microorganisms or judged at least whether a specific PVA degradation was observed.

Two principal enzyme systems have been described. The first one, which relies on secondary alcohol oxidase (Shimao et al., 1983) with oxygen as an electron acceptor, can in principle be functional under

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aerobic conditions only. The second, constitutes a PVA degradation pathway beginning with the action of PQQ containing periplasmic dehydrogenase (PVADH), from which electrons are probably conducted to soluble cytochrome c and further toward a terminal acceptor; oxygen, most likely under aerobic conditions (Shimao et al., 1996). The process should be feasible under anaerobic conditions in the presence of an alternative electron acceptor, e.g. nitrogen containing electron acceptors (Zumft, 1997), Reimann et al., 2007). Subsequently, β-hydroxyl ketone products of PVA dehydrogenase can be hydrolyzed in an aldolase like reaction catalyzed by a related hydrolase (Hirota-Mamoto et al., 2006; Kawai and Xiaoping, 2009), and shorter fragments of the polymer can then probably be assimilated.

The authors sought to investigate such a possibility in a series of experiments. The study reports on PVA specific biodegradation under denitrifying conditions and brings to light evidences of PVA consumption related to denitrification, in addition to identifying microorganisms relevant to the process.

2. Materials and methods

2.1. Sampling of the microbial community

Waste-water sludge from the denitrifying compartment of the Zlin-Malenovice municipal waste-water treatment plant (Czech Republic) was sampled in March 2010, and then on two more occasions at about two-month intervals. The sludge was immediately purged with nitrogen, filtered through a screen (4 mm^2) to remove macroscopic particles, washed twice with mineral medium of pH 7.2 (Muchova et al., 2009) by centrifugation (5000 g, 12 min), and purged with nitrogen again.

2.2. Biodegradation experiments

Incubations were conducted in glass bottles (total volume, 320 ml) containing 280 ml of the mineral medium (in g l^{-1}: KH_2PO_4 0.09, $Na_2HPO_4 \cdot 12H_2O$ 0.96, NH_4Cl 1.0, $MgSO_4 \cdot 7H_2O$ 0.1, $Fe(NH_4)_2(SO_4)_2.6H_2O$ 0.03, $CaCl_2$ 0.01, KNO_3 0.86, trace element solution (Muchova et al., 2009) 1.0 ml), 100 mg l^{-1} of PVA (POVAL 205, 87–89% of hydrolysis; Kuraray Co. Ltd., Japan), 500 mg l^{-1} of NO_3^- (as KNO_3), 1 g l^{-1} dry weight of the preprocessed sludge from the first instance of sludge sampling. The cultures were purged with nitrogen and sealed with stoppers equipped with a rubber septa and incubated anaerobically on a magnetic stirrer (each bottle contained a magnetic bar; 250 rpm) at 25 °C. The experiments were carried out in three replicates. As an alternative, the PVA biodegradation experiment was also conducted under an aerobic condition in 500 ml flasks equipped with gas permeable stoppers filled with 100 ml of identical media under vigorous shaking (180 RPM).

The PVA concentrations were assayed in microplates (Joshi et al., 1979; Vaclavkova et al., 2007). After removing biomass by centrifugation (15,000 × g, 15 min), 20 μl of a sample, 42 μl of boric acid solution (40 g per liter) and 10 μl of I_2/KI solution (12.7 g I_2 and 40 g KI per liter) were pipetted into a well, and after 5 min an absorbance reading of 660 nm was made. Actual PVA concentration was deduced from a calibration curve.

Nitrates were assayed potentiometrically with an ion-selective electrode (Perfection NO_3 combination, Mettler Toledo) according to the manufacturer's instructions. KNO_3 solutions in above described mineral medium were used for calibration.

2.3. Isolation procedures

2.3.1. Preparation of enriched PVA degrading consortia

Fresh media of the described composition were inoculated with preprocessed sludge from the second instance of sludge sampling and cultivated for 60 days under identical conditions. PVA consumption was verified and the culture was used to inoculate another fresh medium, and this cultivation was held for 30 days. Then the microorganisms were collected by centrifugation (5000 × g, 12 min) and used for inoculating fresh medium with increased concentrations of nutrients (PVA, 150 mg l^{-1}; NO_3^-, 1000 mg l^{-1}); the initial dry weight of the inoculum was set to 100 mg l^{-1}. After four weeks of cultivation the culture was diluted ten times with fresh medium of the latter composition and cultivated again for another four weeks. Following this, the culture was supplemented three times in three week intervals in a manner that the PVA concentrations were set to 200 mg l^{-1} and nitrate concentrations to 1000 mg l^{-1}; this culture was designated "B". In an identical way the sludge sample from the third instance of sampling was processed and the final culture designated "D". After every manipulation, cultures were always purged with nitrogen and sealed.

2.3.2. Isolation attempts

After the enrichment procedure, microorganisms from "Culture B" were collected by centrifugation, carefully resuspended, and a series of dilutions were prepared and transferred onto agar plates containing mineral agar with nitrate (BMA, control), mineral agar with nitrate and 500 mg l^{-1} PVA (BMA-PVA), and mineral agar with nitrate, succinate and ethanol (BMA-SE, 500 mg l^{-1} of each carbon source). Variants of the described solid media containing 20 μg l^{-1} of pyrroloquinolino quinone (PQQ) were also used. The plates were incubated at 25 °C anaerobically (anaerostat MERCK, Anaerocult A) or under aerobic condition.

2.4. DNA isolation, PCR and TGGE conditions

DNA from denitrifying cultures was extracted using a commercial DNA extraction kit (PowerSoil, MoBio) according to the manufacturer's instructions, involving an initial bead-beating step. The primers fD1 and rD1 (AGAGT TTGAT CCTGG CTCAG and AAGGA GGTGA TCCAG CC, respectively) were used to amplify nearly a full-length 16S rRNA gene (Weisburg et al., 1991). Each 25 μl PCR reaction contained 12.5 μl of GoTaq Green hot start master mix (Promega), 1 μl of each primer solution (12.5 pmol), 9.5 μl of water for molecular biology, and 1 μl (5–10 ng) of bacterial DNA. All amplifications were carried out in Piko Thermal Cycler (Finnzymes); the temperature profile was as follows: initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Subsequently, 1 μl of the first PCR product was used as a template for nested PCR amplifying the V3-hypervariable region of the 16S rRNA gene with the primer pair 341fGC and 518r (ATTAC CGCGG CTGCT GG and CCTAC GGGAG GCAGC AG, respectively), where the GC clamp (CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG) was covalently attached to the 5′ end of the forward primer (Muyzer et al., 1993). The temperature program consisted of 1 min at 94 °C and 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min. TGGE separation of the amplified PCR products was achieved on a TGGE Maxi system (Whatman-Biometra) in an isocratic denaturing gel (8% acrylamide, 20% deionized formamide, 1× TAE, 2% glycerol and 8 M urea). Two to five micro liters of PCR products (60–100 ng DNA) were loaded into each well. A 100-bp DNA ladder (NEB) was loaded as a marker (Das et al., 2007). Electrophoresis was performed at the constant voltage of 130 V for 18 h and the optimal gradient was found to be from 35 °C to 55 °C. Afterward, the electrophoresis gels were stained with GelStar (Cambrex), according to the manufacturer's instructions, and documented. The whole procedure was repeated twice with identical results.