Use of temperature gradient gel electrophoresis for the investigation of poly(vinyl alcohol) biodegradation

L. Husarova, J. Ruzicka, H. Marusincova and M. Koutny

***Abstract*—** The application of temperature gradient gel electrophoresis (TGGE) as a culture-independent method to monitor changes in the composition of bacterial community during PVA degradation under denitrification conditions is reported. TGGE was used to separate DNA fragments after PCR amplification from total DNA extracted from the acclimated denitrifying sludge with universal bacterial primers targeted the region of the 16S rRNA gene. TGGE proved to be useful and enable to visualize potential degraders and their time dynamics during the biodegradation experiment.

***Keywords*—** poly(vinyl alcohol), waste water treatment sludge, temperature gradient gel electrophoresis (TGGE).

I. Introduction

Poly(vinylalcohol) (PVA) is water-soluble synthetic polymer which has a wide range of application in industrial sectors. Its properties comprise viscosity, flexibility, film forming, dispersing power and adhesive strength. Due to these properties PVA is mainly used in sectors such food, chemical, paper and textile industries. This polymer belongs as well as Polyvinylethylen (PE) to group of biological degradable plastics. Among the most common microorganisms degrading PVA Pseudomonas or Sphingomonads can be found. PVA is biodegradable under aerobic conditions [1] and partially in anaerobic environment [2], [3], but in latter case a longer time for degradation of PVA is apparently needed.

DNA based microbial community fingerprinting techniques like temperature gradient gel electrophoresis (TGGE) or most often denaturing gradient gel electrophoresis (DGGE) have been so far extensively applied to describe the structure of procaryotic communities [4], [5]. They allow simultaneous processing of a larger number of samples and provide an estimate of diversity independent to conventional cultivation techniques. TGGE and DGGE are based on the separation of DNA fragments of the identical size differing in their nucleotide sequence [6], [7]. Sequence separation occurs in acryl amide gels with a denaturing gradient, where each fragment migrates and finally stops at its melting point. Melting point is directly related with its guanine-cytosine (GC) content [7], [8].

Our main goal was to adapt the TGGE technique to monitor changes in the composition of bacterial community during PVA degradation under denitrification conditions.

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II. Materials and Methods

A. Degradation experiments.

Denitrifying sludge was used from a municipal waste water treatment plant. The sludge was kept under nitrogen until its use. PVA (100 $mg.l^{-1}$) and KNO_3 (1000 $mg.l^{-1}$) were dissolved in mineral medium and this solution (culture medium) was purged with a stream of nitrogen. The mineral medium contained ($mg.l^{-1}$): 50 mg $MgSO_4.7H_2O$; 30 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$; 10 mg $CaCl_2.2H_2O$; 50 mg NH_4Cl, 0.086 mg $MnSO_4.5H_2O$; 0.114 mg H_3BO_3; 0.086 mg $ZnSO_4.7H_2O$; 0.074 mg $(NH_4)_6Mo_7O_{24}$; 0.05 mg $Co(NO_3)_2.6H_2O$; 0.08 mg $CuSO_4.5H_2O$; 181 mg K_2HPO_4 and 1912 mg $Na_2HPO_4.12\ H_2O$

The acclimated denitrifying sludge was centrifuged at 4600 RPM for 10 minutes. Then the wet sediment ($1g.l^{-1}$) was suspended in culture medium. This suspension or its dilutions in culture medium (10^{-1},$10^{-2}$and 10^{-3} g l^{-1}) were filled in the test gasproof flasks (110 ml) and bubbled through with nitrogen again. Test flasks were incubated at 25°C and stired at 250 RPM. Samples were taken by injection syringe and centrifuged (10 000 RPM, 20 minutes). The wet sediments were saved for analysis of DNA and concentrations of PVA in supernatants were determined by a iodometric method [9].

B. DNA extraction and PCR amplification

DNA was isolated from using a commercial DNA extraction kit according to the instructions of the manufacturer (Mo Bio). Eubacterial 16S rRNA gene was amplified with universal primers rD1 and fD1 [10]. In the second nested PCR the primer pair 341f and 518r (5´-ATT ACC GCG GCT GCT GG-3´ and 5´-CCT ACG GGA GGC AGC AG-3´) with a GC clamp (5´-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G-3´) [11] attached to the forward

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primer was used. Hot-start PCR was carried out in a 25 µl reaction mixure containing 12.5 µl of GoTaq green hot start master mix (Promega), 1 µl of each primer solutions (12.5 pmol), 8.5 µl of water and 2 µl (5-10 ng) of bacterial DNA solution. Samples were amplified in Piko Thermal Cycler (Finnzymes) using the following program: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Subsequently, 1 µl of the first PCR product was used as a template for nested PCR, with the primer pair 341fGC and 518r. Temperature program consisted of 1 min at 94°C and 30 cycles of 1 min at 94°C, 1 min annealing at 55°C, 1 min extension at 72°C and final extension at 72°C for 10 min. The size and amount of the PCR products was confirmed by agarose gel electrophoresis.

C. TGGE conditions

TGGE Maxi system (Whatman-Biometra) was used for the separation of nested PCR products. Electrophoresis was performed in 1 mm thick polyacrylamide gel (at constant current 30mA). Denaturating gels (8% acrylamide, 20% deionized formamide, 1x TAE (Tris-acetate-EDTA buffer), 2% glycerol and 8M urea) were made with 2x TAE buffer. After it, the gel was polymerized by adding 110 µl of N,N,N´,N´-tetramethylenediamine (TEMED) and 50 µl of 10% ammonium persulfate (APS) to 50 ml of the gel components mixture. Then, the gel was let to polymerize at least for 2 hours. Consequently, two to five micro liters of PCR products (60-100 ng DNA) were loaded into each well and run at 130 V for 18h. For our purpose, the optimal thermal gradient was found to be from 40 to 60°C. Finally, gels were developed by silver staining following a modification of a protocol [12], [13]. The gel was fixed in the solution of 10% (v/v) ethanol and 0.5% acetic acid for 1h. Then, it was impregnated with 0.2% (wt/v) silver nitrate for 5 min. After two thorough washes with distilled water, the freshly prepared developing solution containing sodium hydroxide (15 $g.l^{-1}$) and 37% HCOH (2 $ml.l^{-1}$) was poured onto the gel. After 5 min the solution was removed and the gel was dried. The gel image was then taken using a digital camera (Syngene).

III. RESULTS AND DISCUSSION

It was found in some previous experiments that PVA is degraded under anaerobic denitrification conditions by the bacterial community present in the sludge from denitrification compartment of the local municipal waste water treatment plant. To investigate the bacterial community and visualize its dynamics TGGE technique was chosen and applied to monitor changes in the community during a biodegradation experiment. Denitrification supporting media containing PVA as sole source of carbon and energy were inoculated with series of dilutions of the bacterial community from the waste water sludge previously acclimated under denitrification conditions with PVA.

Course of PVA biodegradation was followed in these cultures for about 30 days and the results are depicted in figure 1.

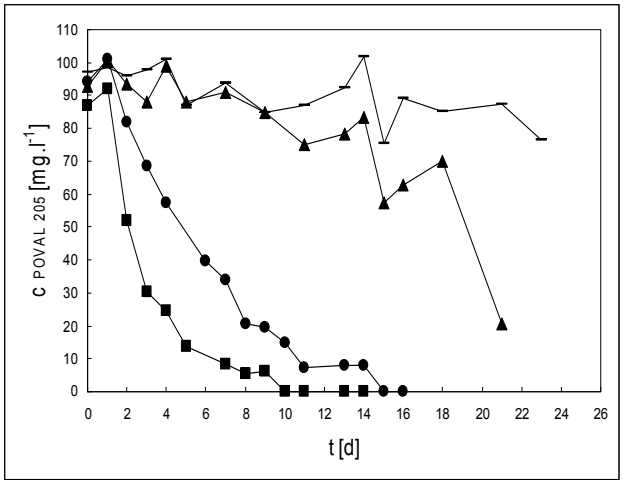

Figure 1. Monitoring of gradual decrease of PVA during denitrifying conditions, culture inoculum with ■ 1 $g.l^{-1}$, ◆ 0.1 $g.l^{-1}$, ▲ 0.01 $g.l^{-1}$, ▬ 0.001 $g.l^{-1}$ of sludge.

It is evident that for two highest inoculations PVA was consumed very fast without any lag phase on the beginning of the observation period. However for the medium inoculation density (0.01 g of sludge per liter of medium) the rate was strongly retarded with an evident lag phase. For the highest dilution the active microorganisms seemed to be diluted out. Samples taken from cultures inoculated with 0.1 and 0.01 g of the sludge per litter were chosen for further experiments.

Whole bacterial DNA was isolated from the samples taken in selected days of the degradation experiment and subsequently fragments of bacterial 16S rDNA gene were PCR amplified and resolved with help of TGGE. Patterns of bands visible in individual lines represent bacterial communities in a given day of incubation (Fig. 2.). Virtually, each band should represent an individual bacterial strain. For both dilutions samples at beginning (day 0), in the middle and at the end of PVA consumption were investigated. Main attention was paid to a search for an intensive band not present or having weak appearance on the beginning of the experiment (day 0) and becoming intensive during the phase of intensive biodegradation. Such band was identified and designated in Figure 2 as “d”. Additional new bands appeared in the late phase of biodegradation (days 6 or 21 and bands a, b, c, f). We speculated that respective bacterial strains probably utilized some degradation products of PVA formed by the action of the strain related to the band “d” which we assumed was the principal degrader of PVA in the described experiment. Some changes in band intensities (bands e, g) remain more difficult to interpret.

The study proved that TGGE is suitable and could be extremely helpful method in further investigation of PVA biodegradation.

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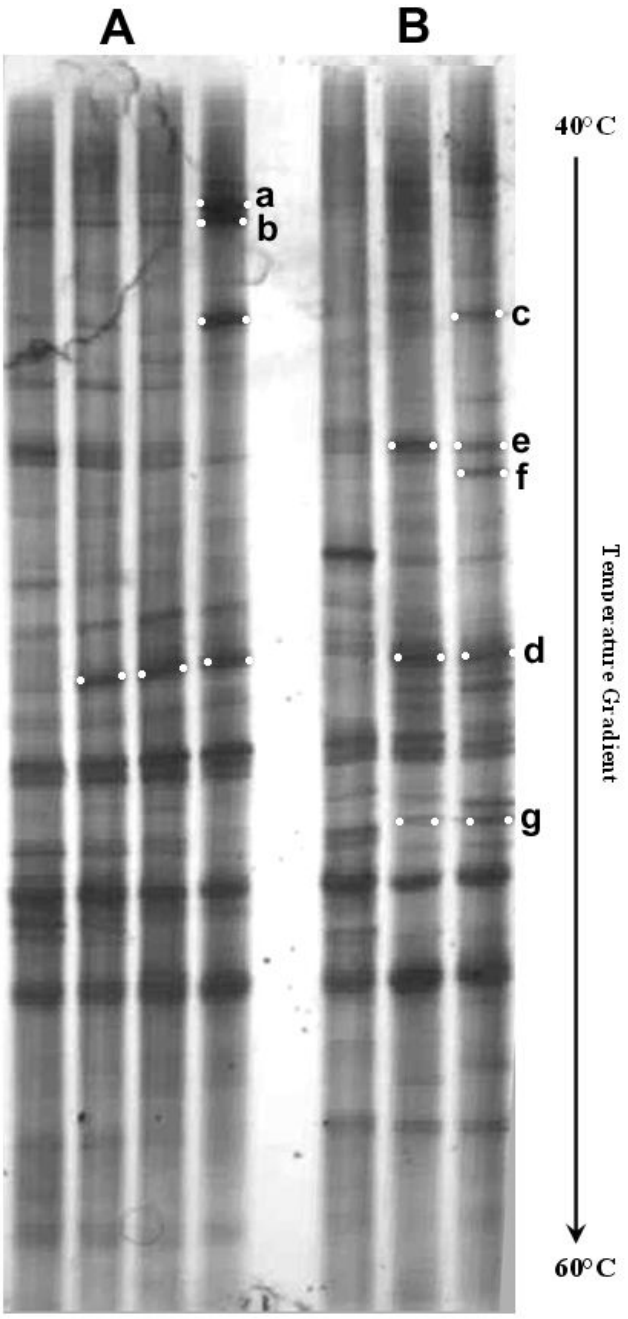

Figure 2. Temperature gradient gel electrophoresis profile of PCR products of 16S rDNA of changing bacterial communities during denitrifying biodegradation of PVA, A (cultured inoculum with 0.1 $g.l^{-1}$ of sludge), B (cultured inoculum with 0.01 $g.l^{-1}$ of sludge).

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