

PCR-DGGE AS A USEFUL METHOD FOR BACTERIAL DIVERSITY MONITORING IN COKE WASTEWATER TREATMENT

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ABSTRACT

Increasing environmental pollution caused by inappropriate treatment of coke wastewater is a serious problem in wastewater treatment plants. Coke wastewater, containing phenols, cyanides and thiocyanates, are harmful for activated sludge biocenosis and causes serious technological problems. As a effective method for this kind of wastewater treatment Anammox (**A**naerobic **A**mmonium **O**xidation) process seems to be useful. Wastewater treatment can be carried out in membrane bioreactors (MBRs), regarded nowadays as convenient in the lab experiments, due to their small size, and in most cases an effluent of much better quality than conventional systems in terms of organic matter, suspended solids, and nutrients. In order to monitor the bacteria biocenosis, its development and changeability, two-MBR system of partial nitrification-Anammox was design. The aim of the study was to perform bacterial biodiversity monitoring using polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) method, known to be one of the most effective molecular method in microorganisms' biodiversity research. The 16S rRNA coding gene was used as a molecular marker. On the basics of fingerprint obtained in molecular method Shannon biodiversity index estimations for total bacterial community were performed. The research revealed that two MBRs inoculated with the same activated sludge differ in biocenosis structure, but not at the level of biodiversity.

Key words: activated sludge bacteria, bacterial diversity, coke wastewater, PCR-DGGE

INTRODUCTION

Due to the rapid industrial development in Poland it is necessary to find new, efficient sources of energy. Coke, the solid residue of impure carbon obtained in distillation process after removal of volatile material, is regarded as useful energy source. Nowadays there are 10 coke-plants in Poland, located in the southern Poland. Their infrastructure is nowadays under remodeling because they need to fulfill European Union standards in case both, the coke production and the pollution elimination. Carbon distillation is a process requiring large volumes of water and it release considerable amount of highly contaminated wastewater. Among wastewater recalcitrant cyanides, thiocyanates and phenols are regarded to be the most harmful to the water environment and they have destructive influence on activated sludge microorganisms used for biological wastewater treatment.

Coke wastewater can be treated with the combination of biological and chemical methods, but they are harmful to activated sludge microorganisms [Lim et al., 2002]. Traditional and one of the main nitrogen removal process performed in wastewater treatments is nitrification, ammonia removal in aerobic conditions and then heterotrophic denitrification, reduction of nitrates in anoxic condition. But nitrifiers responsible for this process are highly sensitive towards chemical contaminants. That is the reason why alternative nitrogen removal processes should be introduced, mainly in such contaminated sewage as coke wastewater. Lately, Anammox process (*Anaerobic Ammonium*

Oxidation) is found to be useful in nitrogen removal. Although Anammox bacteria are difficult to analyze using standard microbiological procedure, because they grow very slowly and it is difficult to obtain well established Anammox consortia performing the process effectively. In order to monitor bacterial changes in activated sludge while Anammox adaptation molecular methods are useful. Among molecular biology techniques PCR-DGGE (*Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis*) is regarded to be of the most convenient in complex microbial consortia studies.

Wastewater treatment can be carried out in a variety of technological systems, among which membrane bioreactors (MBR) are regarded as convenient in the lab experiments. They are small in size and in most cases an effluent in much better in quality than in conventional systems in terms of organic matter, suspended solids, and nutrients [Fenu et al., 2010; Lerner et al., 2007; Alvarez – Vasquez et al., 2004]. And in case of very slow growing microorganisms (like Anammox bacteria) MBR ensure very good biomass retentions [Cema, 2009].

The aim of this study was to monitor bacterial diversification in two step membrane bioreactor system of partial nitrification-Anammox, inoculated with activated sludge derived from coke wastewater treatment plant during acclimation period. MBRs were operated in different oxygen conditions and artificial coke wastewater served as medium for MBRA (aerobic membrane bioreactor), while MBRA effluent as medium for MBRB (anoxic membrane bioreactor).

MATERIALS AND METHODS

Experiments settings

In this experiment two membrane bioreactors were used: aerobic membrane bioreactor (MBRA), where the first phase of treatment was performed and anoxic membrane bioreactor (MBRB) were pre-purified medium was directed from aerobic reactor (Anammox bioreactor).

Inoculum originated from coke wastewater treatment plant and synthetic coke wastewater were used (see Table 1). The medium was directed to aerated MBRA, the effluent of MBRA was directed to MBRB maintained in anoxic conditions.

Table 1. Feed medium used for MBRs during acclimation period

compound	concentration [g/l]
NH ₄ Cl	3,45
KH ₂ PO ₄	0,17
CH ₃ COONa	2
C ₆ H ₅ OH	0,3
NaHCO ₃	70

DNA extraction and PCR conditions for activated sludge samples

Activated sludge samples (volume of 10 ml) from the two MBRs were collected at 2-week interval, pelleted by centrifugation (5 000 × g, 10 min, 4°C) and stored at -20°C. Total genomic DNA was extracted from 0.2 g of the activated sludge samples using mechanical method described previously [Machul and Ziemińska, 2012]. The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at -20°C until PCR amplification.

In this study partial 16S rRNA gene amplification was performed using primers 338F with GC clamp and 518R, which amplified a partial (ca. 180 bp) 16S rRNA gene fragment of all the bacteria [Muyzer i in., 1993]. PCR procedure was described previously [Ziembińska et al., 2009].

Denaturing gradient gel electrophoresis conditions and DNA bands extraction

The DGGE of the PCR products obtained in reactions with 338F-GC and 518R primers underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% for 16S rRNA gene, 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30 – 60% denaturant was prepared according to the manufacturer's instruction. The gel was run for 10 h at 70 V in a 1 × TAE buffer (Tris, acetic acid, EDTA, pH = 8.0) at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 min and destained in MiliQ water for 40 min, then visualized under UV light and photographed using a Quantity One 1D Software (BioRad).

Numerical analysis of the DGGE fingerprints obtained in the experiment

The analysis of DGGE fingerprints was performed using a Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and Shannon diversity index for the samples was calculated, according to the equation [Li et al. 2011]:

$$H' = -\sum P_i \ln P_i$$

with:

$$P_i = n_i / N_i$$

where:

P_i is a relative probability of DNA band appearance in the fingerprint,

n_i is densitometrically measured intensity of DNA band,

N_i is the amount of DNA bands in the fingerprint.

RESULTS AND DISCUSSION

As it is seen at figure 1 the diversification of the bacterial community started from second sampling time after inoculation. The primary activated sludge biocenosis diversified into two genotype groups, with high and low GC content (Fig. 1, frames), with low GC content bacteria more diverse in MBRA, while high GC content bacteria in MBRB.

Some of low GC contents bacteria seem to dominant in both systems, while genotype A appeared in the second sampling time, and is maintained in the system for the total length of acclimation period. In case of their identification DNA sequencing is required. Genotype B band is stronger in bioreactor A with oxic condition while in bioreactor B – anoxic environment, seems to disappearing while the process. Interestingly, high GC content bacteria are more diverse in case of anoxic conditions. Such situation may occur due to the ecological microniche presence in bioreactor B, presenting microaerophilic conditions. It could be also connected with the feed medium. Artificial coke wastewater could eliminated some of more sensitive genotypes, while in MBRB the effluent of MBRA was the feed medium, probably less harmful than the primary wastewater. Probably the diversification of the inoculum could appeared also because of other physio-chemical changes (such as the medium content), not only because of the differences in oxic conditions.

Biodiversity of activated sludge bacteria is maintained at the similar level in both bioreactors, ca. 2,5-3,0 (Figure 2), in MBRA Shannon index is slightly lower, probably caused also by the medium composition. It could be also a result of the increase of a high GC content genotypes number.

As it is presented in this experiment biodiversity index analysis should always be compared with the qualitative analysis of DGGE. Although the level of biodiversity is comparable the changes in the genotype composition are more drastic. Probably they are caused by oxic conditions as well as the composition of the medium.

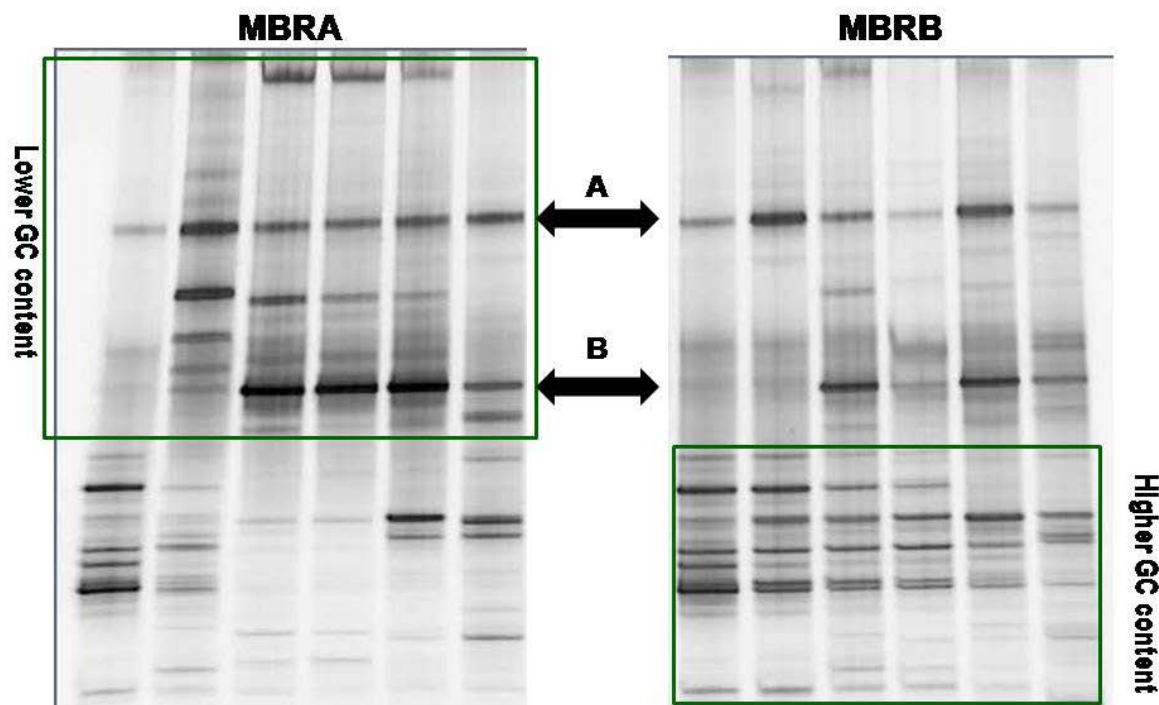


Fig. 1. DGGE patterns for MBRA and MBRB activated sludge bacteria

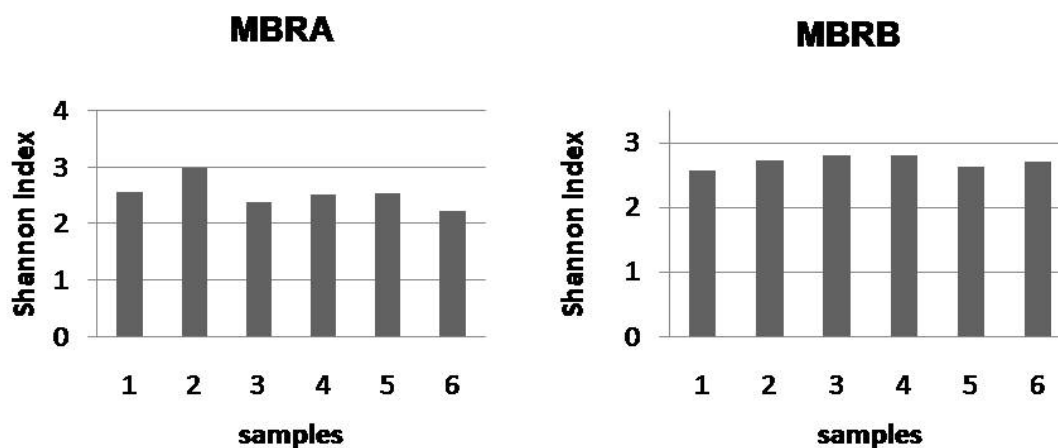


Fig. 2. Shannon biodiversity index of for MBRA and MBRB activated sludge bacteria performed on the basis of DGGE fingerprints

CONCLUSION

The research performed in this experiment revealed that:

- oxic bioreactor (A) presents slightly higher biodiversity than anoxic (B) and mainly bacteria with low and medium level of GC content in DNA,
- anoxic bioreactor (B) possesses similar dynamic changeability and MBRB bacteria represents a group of genotypes with medium and high level of GC content in DNA,

- there is a group of sequences common for both MBRs with lower GC content. It could suggest that there is a group of bacteria insensitive towards physiochemical parameters variations,
- Shannon diversity index gives only a brief picture of the total bacterial diversity in both MBRs. It should be also stated that biodiversity changes are not always linked with serious physio-chemical variations and it does not inform about qualitative changes which are presented by DGGE fingerprint patterns,
- PCR-DGGE is an excellent tool for monitoring of complex bacterial biocenosis in activated sludge, even if the bioreactors treat difficult wastewater (such as coke wastewater).

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