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COMPARISON OF PCR-DGGE AND NESTED-PCR-DGGE APPROACH FOR AMMONIA OXIDIZERS MONITORING IN MEMBRANE BIOREACTORS' ACTIVATED SLUDGE

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Abstract: Nitritation, the first stage of ammonia removal process is known to be limiting for total process performance. Ammonia oxidizing bacteria (AOB) which perform this process are obligatory activated sludge habitants, a mixture consisting of *Bacteria*, *Protozoa* and *Metazoa* used for biological wastewater treatment. Due to this fact they are an interesting bacterial group, from both the technological and ecological point of view. AOB changeability and biodiversity analyses both in wastewater treatment plants and lab-scale reactors are performed on the basis of 16S rRNA gene sequences using PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) as a molecular biology tool. AOB researches are usually led with nested PCR. Because the application of nested PCR is laborious and time consuming, we have attempted to check the possibility of using only first PCR round to obtain DGGE fingerprinting of microbial communities. In this work we are comparing the nested and non-nested PCR-DGGE monitoring of an AOB community and presenting advantages and disadvantages of both methods used. The experiment revealed that PCR technique is a very sensitive tool for the amplification of even a minute amount of DNA sample. But in the case of nested-PCR, the sensitivity is higher and the template amount could be even smaller. The nested PCR-DGGE seems to be a better tool for AOB community monitoring and complexity research in activated sludge, despite shorter fragments of DNA amplification which seems to be a disadvantage in the case of bacteria identification. It is recommended that the sort of analysis approach should be chosen according to the aim of the study: nested-PCR-DGGE for community complexity analysis, while PCR-DGGE for identification of the dominant bacteria.

INTRODUCTION

Nitrification is biological oxidation of ammonia to nitrite followed by the oxidation of these nitrites into nitrates. The ammonia removal process consists of two stages: ammonia oxidation, performed by ammonia oxidizing bacteria (AOB) and nitrite oxidation, performed by nitrite oxidizing bacteria (NOB). Both groups of microorganisms are

obligatory activated sludge habitants, a mixture consisting of *Bacteria*, *Protozoa* and *Metazoa* used for biological wastewater treatment. But the first stage of nitrification is found to be rate limiting for total process performance [8]. That is why AOB are an interesting bacterial group in activated sludge, from both the technological and ecological point of view [10].

It is a common knowledge that most of the environmental bacteria are uncultivable in the laboratory [5] and to study their diversity and changeability in the bacterial community culture-independent methods are required [12]. Ammonia oxidizers monitoring in the environmental samples is usually performed with polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). This community monitoring is possible using functional genes (such as *amoA*, responsible for ammonia oxidizing enzymes production), but this approach is found to be difficult to perform for AOB diversity monitoring with DGGE probably due to a primer site degeneration. It is also difficult to identify AOB according to their Amo gene sequence. That is the reason why the most suitable approach for bacterial diversity monitoring is DGGE analysis on the basis of partial 16S rRNA gene amplification. This molecule is known as a prokaryotic universal molecular marker and there is a large database of this gene sequences. However, the usage of the universal bacterial primers leads to the

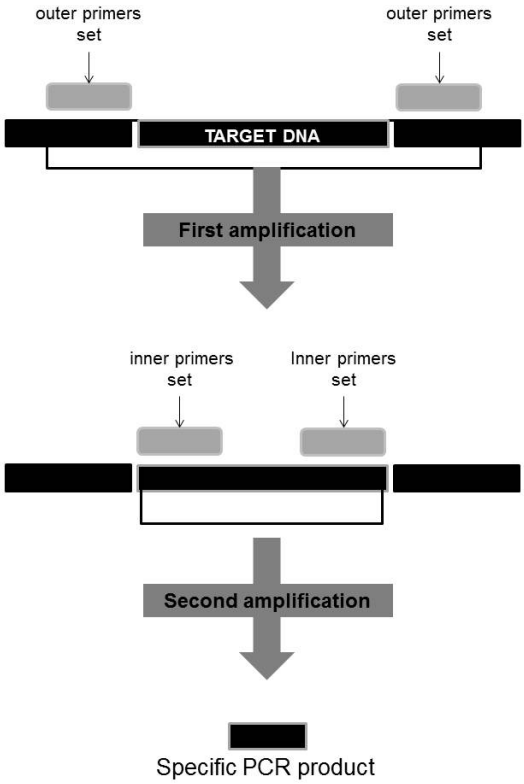

Fig. 1. Nested PCR scheme

dominant microbiota visualization in the fingerprint [12] and it is not suitable for the presentation of a particular bacterial group complexity. In such cases the nested-PCR approach is useful. The technique is based on two-step amplification using two pairs of PCR primers: an outer pair – amplifying a bigger part of the marker gene on the DNA obtained from the sample, and an inner pair – targeting the particular bacterial group amplifying a smaller product on the first-step product as a sample (Fig. 1) [4]. This technique is a precise tool for complex bacterial biocenosis structure monitoring with a particular interest in a functional bacterial group.

In an attempt to obtain a more detailed picture of an AOB community in an activated sludge from two MBRs (Membrane Bioreactors) dealing with polycyclic aromatic hydrocarbons (PAHs) rich medium nested PCR-DGGE was used. Because the application of nested PCR is laborious and time consuming, we have attempted to check the possibility of using only first PCR round to obtain DGGE fingerprinting of microbial communities. In this work we are comparing the nested and non-nested PCR-DGGE monitoring of an AOB community and presenting advantages and disadvantages of both methods used.

MATERIALS AND METHODS

Experimental settings

The analysis was performed on the activated sludge from two membrane bioreactors – control (MBRA – without contaminants) and an experimental one (MBRB – medium with a contamination in concentration range between 200 and 1000 µL/L, increasing gradually during the experiment). The MBRB medium was contaminated with P-30 crude oil fraction obtained from PKN Orlen Refinery, Poland. The reactors, volume of 10 L, were equipped with a Kubota membrane module with a nominal pore size of 0.4 µm. MBRs and were seeded with the activated sludge from municipal wastewater treatment plant from Zabrze, Poland and operated concurrently for 157 days at hydraulic retention time of about 24 h. The activated sludge samples were collected at 2-week interval, pelleted by centrifugation and frozen at -20°C.

DNA isolation and PCR conditions

Total bacterial DNA was isolated from the activated sludge samples with DNA Isolation Kit from Soil (MP Biomedicals). The amount and purity of the DNA samples were measured fluorometrically with Qubit Flourometer (Invitrogen) and kept frozen at -20°C until PCR.

Partial 16S rRNA gene was amplified in a two-round PCR. The first PCR round (non-nested PCR) was performed on bacterial DNA samples isolated directly form the activated sludge samples with AOB specific primers CTO, while primers 338F-GC and 518R were used for the second round of amplification (nested PCR), with the first round PCR product as a sample [7]. The sequences of the primers used are presented in Table 1. The PCR amplification was performed in C-1000 Termocycler (BioRad) in 30 µL of a PCR mixture. The content of the PCR mixture and the programs used in the study are presented in Tables 2 and 3. The products of the amplification were visualized in 0.8% [w/v] agarose gel containing ethidium bromide (10 µg/mL) under UV light and photographed.

Table 1. The sequences of the primers used in the study

primer	sequence 5'–3'	product size	references
CTO189f – ABC – GC	CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA CMA AAG YAG GGG ATC G	465 bp	Kowalchuk et al., 1997
CTO 654r	CTA GCY TTG TAG TTT CAA ACG C		
338f – GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	180 bp	Muyzer et al., 1993
518r	ATT ACC GCG GCT GCT GG		

Table 2. The PCR mixtures for the amplification used in the study

PCR mixture component			volume [µL]	final concentration
MiliQ water			fill up to 30 µL	–
Polymerase buffer			3	1×
$MgCl_2$			1.2	0.2
Primer forward (Oligo.pl)			0.25	5 pmol/µL
Primer reverse (Oligo.pl)			0.25	5 pmol/µL
dNTPs (Promega)			1.3	20 pmol/µL
TAQ Go Flexi polymerase (Promega)			0.3	1.5 U
DNA sample	I PCR round	DNA from the sample	1	0.15–0.2 µg/µL
	II PCR round	I round PCR product	0.5	0.15–0.2 µg/µL

Table 3. The PCR programs for the amplification used in the study

	I PCR round	II PCR round
Primers	CTO189f – ABC – GC and CTO 654r	338f-GC and 518r
Predeanturation	95°C, 10 min	95°C, 10 min
Denaturation	95°C, 1 min	95°C, 1 min
Annealing	57°C, 1 min	53°C, 1 min
Elongation	72°C, 2 min	72°C, 2 min
Final elongation	72°C, 12 min	72°C, 12 min
Number of cycles	35	30

Electrophoretic separation with PCR-DGGE and data analysis

To separate PCR products a mixture from both PCR rounds denaturing gradient gel electrophoresis (DGGE) was used. The electrophoretic separation was performed in 8% [v/v] polyacrylamide gel (Acrylamide:bisacrylamide, 39.5:1, Sigma-Aldrich) with

a denaturant gradient – urea (Fluka) ranging between 30–60%. DGGE was run in Dcode Mutation Detection System (BioRad) in 1 × TAE (pH = 8.0, Tris, acetic acid, EDTA), in 60°C, 55 V for 15 and 9 hours for non – nested and nested PCR products, respectively.

DGGE fingerprints analysis was performed with Quantity One 1D Software (BioRad). Shannon biodiversity index was calculated on the basis of the densitometric measurements as described previously [15].

RESULTS AND DISCUSSION

Nowadays DGGE is one of the most useful bacterial monitoring methods in the complex bacterial biocenoses [6]. The research revealed the difficulties in bacterial diversity monitoring using only AOB specific primers CTO in non-nested PCR approach. The longer PCR products (465 bp) amplified with CTO primers directly from the total bacterial DNA obtained from activated sludge samples were used in PCR-DGGE method but as it is shown in Figure 2 only the dominant bacteria are visualized. The DNA bands from the fingerprint obtained from such a large PCR product should be easier to be excised from the gel and sequenced for the bacteria identification. A direct procedure seems to be more reasonable from the point of the AOB diversity monitoring. Nevertheless, this approach gives only a limited picture of the community and even though combined with further genotype identification by sequencing, it did not lead to the total AOB diversity monitoring. This situation occurred probably due to the lower DGGE detection threshold for the amount of these PCR products.

The nested-PCR approach performed with 338F-GC and 518R primers (Figure 3) gives much more clear information about the diversity and changeability of AOB in two MBR systems. However, this approach possesses also disadvantages. The DNA bands excised from the gel are too short for performing a precise identification of the genotypes. Such short sequences are also impossible to be deposited into National Centre for Biotechnology Information (NCBI) gene database. The two-step procedure can in some cases increase the possibility of PCR mixture contamination. Also, the polymerase error number increases during the second amplification.

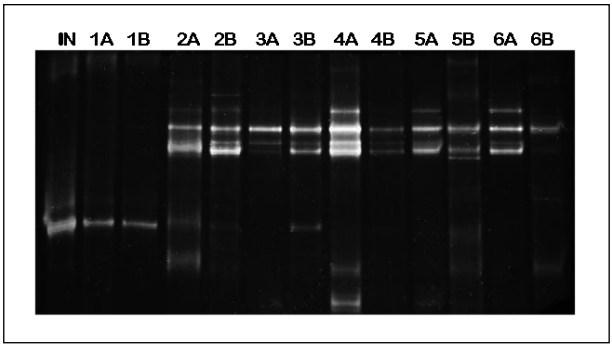

Fig. 2. DGGE analysis of activated sludge AOB community using non-nested PCR with CTO primers; IN-inoculum, 1A–6A – MBRA samples, 1B–6B – MBRA samples collected at 2-week intervals from two MBRs

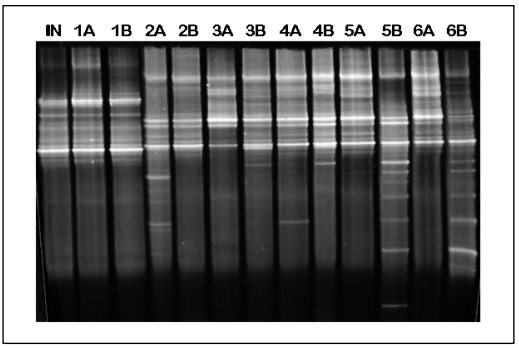

Fig. 3. DGGE analysis of activated sludge AOB community using nested PCR with 338f-GC and 518r primers; IN-inoculum, 1A–6A – MBRA samples, 1B–6B – MBRA samples collected at 2-week intervals from two MBRss

However, nested PCR possesses several advantages. The amplification is more specific for a particular target DNA and the sensitivity of the PCR reaction is higher, also the procedure requires less biological material than the first PCR round. With a proper primer concentration in a PCR reaction mixture, there is no primer or primer-primer dimers appearance. PCR product usage as a template eliminates the possibility of PCR inhibitors present in the sample [2, 11, 13]. The fingerprint structure (community structure) obtained is more reliable when the artifacts of non-specific DNA bands are not appearing in the gel. Biodiversity indexes calculated on the fingerprints are more adequate, as can be seen in Figure 4. In some cases, as in samples 2 and 3 in CTO, non-nested amplification gave only one visible band in DGGE fingerprint. This is the reason why the biodiversity index calculated equals zero. Such a situation seems to be impossible in the case of this sort of material, especially in comparison with Shannon index calculated for nested-PCR-DGGE in which the values are in the range of 2.6–2.9. Interestingly, the comparison of Shannon index calculated from non-nested and nested-PCR-DGGE seems to be proportional. This could suggest that the information about changeability and biodiversity of AOB obtained from both gels could be treated as complimentary.

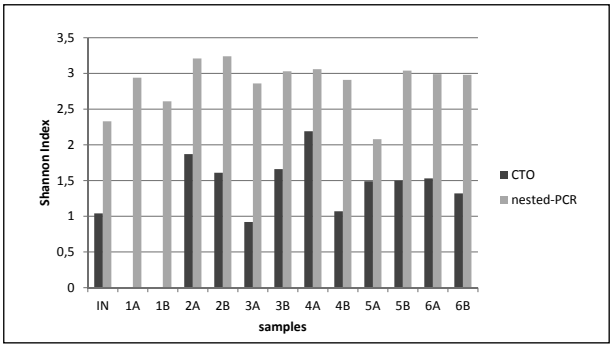

Fig. 4. The comparison of Shannon biodiversity index calculated on the basis of DGGE pattern obtained from the first (non-nested/CTO) and the second (nested) PCR amplification; IN-inoculum, 1A–6A – MBRA samples, 1B–6B – MBRA samples collected at 2-week intervals from two MBRs

It is worth mentioning that nested-PCR approach was used successfully in several experiments for AOB monitoring [1, 3, 9, 14].

CONCLUSIONS

PCR is found to be a very sensitive tool for the amplification of even a minute amount of DNA sample. In the case of nested approach, the sensitivity is higher and the template amount could be even smaller. The nested PCR-DGGE seems to be a better tool for AOB community monitoring and complexity research in activated sludge, despite shorter fragments of DNA amplification which seems to be a disadvantage in the case of bacteria identification. It should be stated that the sort of analysis approach should be chosen according to the aim of the study, nested PCR for community complexity analysis, while non-nested PCR for identification of the dominant bacteria.

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ZALETY I WADY WYKORZYSTYWANIA TECHNIKI NESTED-PCR W MONITORINGU BAKTERII UTLENIAJĄCYCH AMONIAK W OSADZIE CZYNNYM BIOREAKTORA MEMBRANOWEGO

Nitiritacja – pierwszy etap nitryfikacji, jest uznawany za krok limitujący przebieg całości procesu utleniania amoniaku. Bakterie utleniające amoniak (ang. ammonia oxidizing bacteria, AOB), które prowadzą ten proces są stałymi mieszkańcami osadu czynnego – mieszaniny bakterii, *Protozoa* i *Metazoa,* wykorzystywanych do biologicznego oczyszczania ścieków. Z tego powodu są one interesujące zarówno z punktu widzenia technologii, jak i ekologii mikroorganizmów. Analizy zmienności i bioróżnorodności bakterii utleniających amoniak, zarówno w oczyszczalni ścieków, jak i w reaktorach w skali laboratoryjnej, są prowadzone w oparciu o sekwencje genu kodującego 16S rRNA z użyciem metody biologii molekularnej, jaką jest PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis). Analizy te są zazwyczaj prowadzone techniką tzw. nested-PCR. Ze względu na fakt, że metoda ta wymaga większego nakładu pracy i czasu, niż tradycyjny jednoetapowy PCR (ang. non-nested PCR) podjęto próbę sprawdzenia możliwości zastosowania techniki jednoetapowego PCR do uzyskania wzorów prążkowych DGGE bakterii utleniających amoniak. W tej pracy zaprezentowano wyniki analizy PCR-DGGE z użyciem technik nested i non-nested PCR oraz podjęto próbę wykazania ich wad i zalet.