ARCHITECTURE CIVIL ENGINEERING ENVIRONMENT
The Silesian University of Technology No. 2/2015

THE ADJUSTMENT OF GRANULAR SLUDGE DNA ISOLATION FOR PCR-BASED METHODS

Aleksandra ZIEMBIŃSKA-BUCZYŃSKA [a*], Barbara KOCIOŁEK [b], Katarzyna ŚLIPKO [b], Anna DANIŁOWICZ [b], Grzegorz CEMA [a], Joanna SURMACZ-GÓRSKA [c]

[a] Dr.; The Silesian University of Technology, Environmental Biotechnology Department, Akademicka 2, 44-100 Gliwice, Poland
*E-mail address: *aleksandra.ziembinska-buczynska@polsl.pl*

[b] The Silesian University of Technology, Environmental Biotechnology Department, Akademicka 2, 44-100 Gliwice, Poland

[c] Prof.; The Silesian University of Technology, Environmental Biotechnology Department, Akademicka 2, 44-100 Gliwice, Poland

Received: 14.01.2015; Revised: 5.03.2015; Accepted: 5.03.2015

Abstract
European Union regulations require very high standards of wastewater treatment due to both, economic and environmental reasons. Thus wastewater treatment plants are searching for new, efficient technologies to obtain the best quality of WWTPs' effluent. Among other biological methods granular sludge is known to be effective and useful way for sewage purification due to its better sedimentation properties. Granular sludge is interesting also from microbial ecology point of view. The composition of the granules is very difficult to be analyzed with traditional cultivation methods so molecular tools usage is advisable. In order to perform any molecular analysis DNA isolation is required. In this article we compared two DNA isolation methods – mechanical method and commercial GeneMatrix Soil DNA Isolation Kit (Eurx) and two ways of granular sludge preparation with PBS washing before isolation to check does the method of isolation and preparation influence further laboratory procedures, such as polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) separation and fingerprint obtainment. It was stated that there is no influence of the DNA isolation method on the amount of PCR products obtained, but it influences qualitative DGGE resolution and bioinformatical analysis of the results.

Streszczenie
Regulacje Unii Europejskiej zaostrzają standardy dotyczące ścieków oczyszczonych, zarówno z powodów ekonomicznych, jak i ochrony środowiska. Dlatego też oczyszczalnie ścieków poszukują nowych, wydajniejszych technologii oczyszczania ścieków. Wśród tych metod osad granulowany wydaje się być efektywną i użyteczną drogą oczyszczania ścieków, ze względu na lepsze niż tradycyjny osad czynny właściwości sedymentacyjne. Osad granulowany jest interesujący również z punktu widzenia ekologii mikroorganizmów. Analiza składu granul jest niezmiernie trudna do wykonania tradycyjnymi metodami mikrobiologicznymi, dlatego też do takich badań niezbędne jest wykorzystanie metod biologii molekularnej. W celu prowadzenia analiz z zakresu biologii molekularnej konieczne jest izolowanie DNA bakteryjnego. W tej pracy podjęto próbę porównania izolacji DNA dwiema metodami – mechaniczną i z użyciem komercyjnego zestawu odczynników GeneMatrix Soil DNA Isolation Kit (Eurx) oraz wykorzystano dwie modyfikacje przygotowania materiału do izolacji poprzez płukanie buforem PBS. Badania miały na celu określenie, czy procedury przygotowawcze i metoda izolacji mają wpływ na efektywność dalszych procedur badawczych, takich jak amplifikacja z użyciem łańcuchowej reakcji polimerazy (polymerase chain reaction, PCR) oraz rozdział w gradiencie czynnika denaturującego (denaturing gradient gel electrophoresis; DGGE) i uzyskanie wzorów struktury genotypowej zbiorowiska. W badaniach wykazano, że wybór metody izolacji nie ma większego wpływu na ilość uzyskanego produktu PCR, ma jednak wpływ na jakość rozdziału DGGE i wynik analizy bioinformatycznej uzyskanych wyników.

Keywords: **Granular sludge; DNA isolation; Mechanical method, Biodiversity measurement; PCR-DGGE.**

1. INTRODUCTION

Biological methods are the most common ways of wastewater treatment. Among these methods granular sludge become more and more popular wastewater treatment technology. Sludge granules are the microbial aggregates (with a diameter up to 2-4 mm) presenting high sedimentation velocity and high microbial activity [1]. They are interesting from both technological and microbial point of view. They represent high structural complexity but this sort of sludge is almost impossible to study with classical microbial approach. Thus molecular tools, such as PCR-DGGE [2, 3] or FISH [4], have to be used.

In case of PCR-based methods the quality of DNA isolated from the material is a crucial step. The isolation protocol should be relatively fast and inexpensive in order to speed up the laboratory procedures. Most of the researchers working with granular sludge use kits for DNA isolation, which are cost consuming [5-7]. It could be also suspected that depending on DNA isolation method different fingerprints presenting the granule genotypic structure can be obtained.

For years of PCR usage in the standard laboratory protocols several PCR reaction inhibitors were discovered. Their presence causes difficulties in a high quality PCR amplicon obtainment or in some cases, no product is present in post-reaction mixture. Such substances as humic acids [8, 9], polysaccharides [10, 11] or urea [12] are known to interfere with DNA during PCR amplification. In most DNA isolation protocols additive sample washing is required. Especially for environmental samples, that can be contaminated with a long list of substances.

That is why in this experiment we: (1) compared the kit method with a mechanical method based on bead beating for large amount of pure DNA obtainment; (2) compared DGGE fingerprints structure obtained from DNA samples isolated with different methods; (3) checked the necessity of longer PBS washing to obtain better PCR product.

2. MATERIALS AND METHODS

2.1. Granular sludge samples collection and preparation

The samples of granular sludge in triplicate were collected from the fluidized bed reactor located at Poznań University of Technology in volume of 50 ml and frozen for further DNA isolation in -45°C. The samples were washed with 1×PBS buffer (Phosphate Buffered Saline, Sigma) in order to remove potential PCR inhibitors and impurities. The washing procedure was performed in proportion sludge to PBS 1:1 (w/v). Two ways of protocol were used: with mixing 130 rpm overnight at 25°C and thrice directly before DNA isolation, when samples with PBS were vortexed for 30 sec., centrifuged at 13000 rpm, the supernatant was decanted. All samples were grinded in a sterile mortar before isolation procedure.

2.2. DNA isolation

Total genomic DNA was extracted from the grinded granular sludge samples in two ways: with a mechanical method (0.25 g/sample, according to the previous method [13]) and with bead beating and with GeneMatrix Soil DNA isolation Kit (Eurx; 0.3 g/sample according to Manufacturer's instruction).

Samples treated with a mechanical method after two ways of the treatment with PBS washing overnight and thrice directly before the procedure were disintegrated with bead beating (Roth, Germany) in lysis buffer (Tris-HCl 100 mM, EDTA 100 mM, NaCl 1.5 M; pH = 8.0). The samples were incubated 20 minutes in 1400 rpm and 200 µl 10% (w/v) SDS was added. After 30 minutes of incubation in 65°C the samples were centrifuged twice at 13 000 rpm and placed on spin filters (A&A Biotechnology). DNA attached to the filter was washed twice with 70% ethanol solution (A&A Biotechnology).

The samples underwent DNA isolation with GeneMatrix Soil DNA isolation Kit (Eurx) according to Manufacturer's instruction.

The DNA obtained with both procedures underwent electrophoretic separation in 0.8% (w/v) agarose gel containing 2 µl of ethidium bromide (10 mg/ml) in 1 × TBE buffer (Promega) for 30 minutes at 80 V and visualized under UV light. The amount of DNA was measured spectrophotometrically using Nanodrop (Thermo Scientific) and stored at -20°C until PCR amplification. Table 1 resents the scheme of the procedure.

2.3. Granular sludge samples PCR-DGGE analysis

DNA isolated with different methods usually possesses different purity and amount obtained from the same size of the sample. In order to present the potential differences with PCR product qualities and DGGE fingerprints obtainment partial 16S rRNA gene PCR amplification of all the bacteria was performed using primers: 338f-GC and 518r gene frag-

Table 1.
DNA isolation scheme with mechanical method and with GeneMatrix Soil DNA isolation Kit (Eurx)

PBS washing	pretreatment	isolation method	isolation effectiveness evaluation
1×PBS washing overnight, 130 rpm, 25°C	grinding of 0.5 g granular sludge	Mechanical method with bead beating	DNA evaluation in agarose gel and with Nanodrop
		GeneMatrix Soil DNA isolation Kit (Eurx)	
1×PBS thrice directly before the DNA isolation		Mechanical method with bead beating	
		GeneMatrix Soil DNA isolation Kit (Eurx)	

ment [3]. PCR reaction was performed in 30 µl mixture with 1.5 U GoTAQ G2 Polymerase (Promega), 1× buffer with 2 mM $MgCl_2$, 5 pmol/µl of each primers and 20 pmol/µl of dNTPs. Total bacteria DNA from granular sludge samples obtained in all the procedures was used as DNA template in concentration of 0.2 µg/µl. The amplification was performed in thermocycler T-1000 (Bio-Rad) as previously described [3]. The PCR products were separated in 0.8% (w/v) agarose with 2 µl ethidium bromide (10 mg/ml) in 1 × TBE buffer (Promega) and visualized under UV light.

The DGGE of 25 µl of the PCR products obtained in reactions with 338F-GC/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 16 h at 40 V in a 1 × TAE buffer (Promega) 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 distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad).

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 Biodiversity Index as previously described [14].

3. RESULTS AND DISCUSSION

For any biological material DNA isolation efficacy and the purity of the material obtained in the procedure are crucial for further molecular tools usage. In case of molecular research performed on complex microbial community the issue of DNA obtainment is very important due to the fact that over 95% of environmental bacteria cannot be obtain as pure culture [15]. Thus molecular approach is the only way for these bacteria study.

It is particularly important in case of PCR-DGGE - a method of visualizing bacterial community composition. The fingerprints obtained in this method present genotypic structure complexity of the biocenosis. Every single DNA band in the fingerprint is a particular bacterial genotype amplified on the basis of DNA sample. That is why DNA isolation form environmental sample is crucial point of PCR-DGGE procedure.

Both, natural and engineered biocenoses possess substances known to be PCR inhibitors such as humic acids or polysaccharides. In order to remove them from the sample several methods of sample treatment can be used. Among them, PBS washing is the cheapest and the easiest way of inhibitors removal.

As it can be seen on Figure 1, mechanical method lead to cleaner and less degraded DNA obtainment than GeneMatrix spin Kit for Soil. DNA isolated with GeneMatrix Kit is smeared and probably partially degraded. Also the optical density measurements show that DNA obtained with the first method gave stronger bands (Table 2). This difference can be caused by the difference in granular sludge weight used for isolation. Nonetheless, in both methods the weights: 0.25 g and 0.3 g for used for mechanical and kit isolation, respectively were chosen according to the previous protocols.

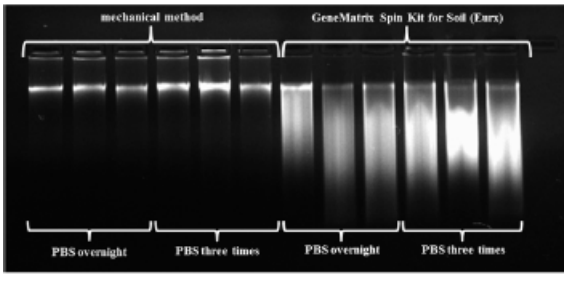

Figure 1.
DNA visualization after isolation in 0.8% (w/v) agarose gel under UV light

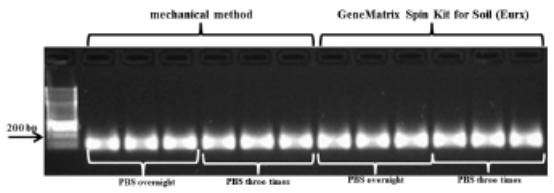

Figure 2.
PCR products visualization in 0.8% (w/v) agarose gel under UV light

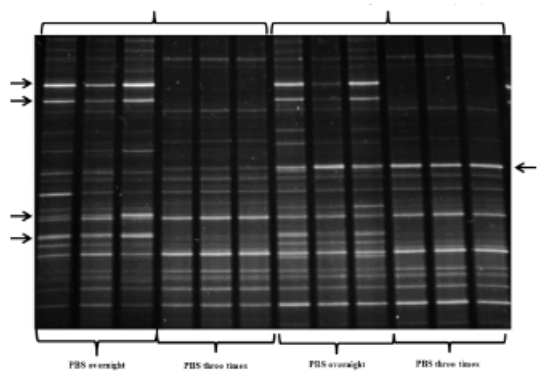

Figure 3.
DGGE results visualized under UV light; arrows point at DNA bands more bright in PBS overnight washed samples

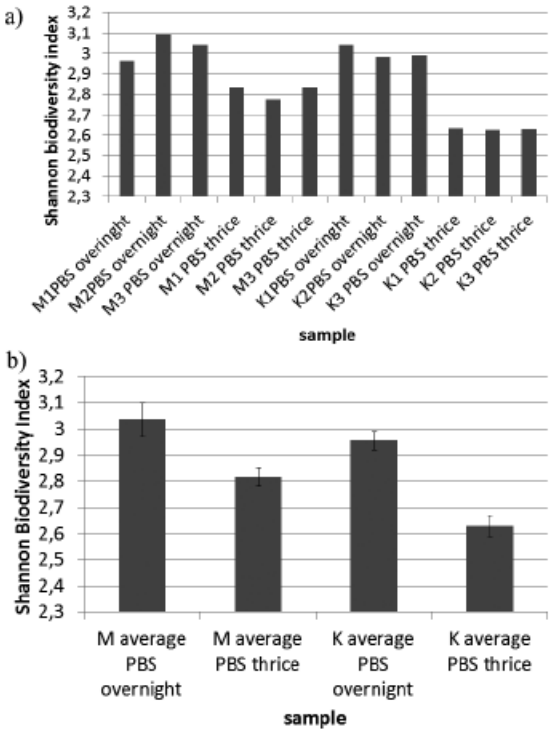

Figure 4.
Shannon Biodiversity Index calculated on the basis of densitometric analysis of DGGE gel; a) separate fingerprint calculation; b) average from 3 samples

Interestingly, according to NanoDrop measurements DNA in larger amount and cleaner – with a higher A 260/280 proportion – is obtained with GeneMatrix Kit. In case of mechanical method overnight washing with PBS gave better results in DNA amount, while its purity is comparable to that with a thrice PBS washing directly before the isolation. In case of GeneMatrix Kit isolation the result is different – overnight washing gave less DNA but with similar purity as thrice PBS washing (Table 2). However, PCR products visible in agarose gel (Figure 2) are similar in the optical density and the amount of DNA in the amplicons measured with NanoDrop is also similar, regardless to DNA isolation method (Table 3). These results suggest that the DNA quality has no significant influence on the amount of PCR product obtained during the sample amplification.

Nonetheless, in DGGE separation of PCR products several DNA bands are more visible than in the other fingerprints, thus it seems that maybe the DNA purity does not influence the PCR products quantitatively, but qualitatively (Figure 3, arrows). It should be mentioned that more bands are visible in DGGE fingerprints for DNA isolated with a mechanical and kit method after overnight washing. It is possible that after longer PBS washing more PCR inhibitors were rinsed out and some of DNA bands were amplified better than with only thrice PBS washing.

In the next step of the procedure PCR products were separated in DGGE gel. The result of the separation is presented in Figure 3. On the basis of DGGE resolution densitometric analysis was performed and Shannon Biodiversity Index, as an example of ecological biodiversity index which could be calculated on the basis of densitometric data, was calculated (Figure 4 a, b).

It is important to underline that DGGE is qualitative method. It means that the fingerprints should be analyzed mainly as genotypic community composition, to lesser degree as qualitative measurement of biodiversity. It is also necessary to point that for the samples with a comparable biodiversity the genotypic structure of the community could be totally different, as it has been previously stated [13]. Thus, it is necessary to analyze biodiversity data together with the fingerprint structures picture.

Shannon Biodiversity Index is the lowest for the samples washed thrice with PCR and isolated with GeneMatrix kit. The highest biodiversity is presented

by the samples washed with PBS overnight and with mechanical method (Figure 4b). It could be suspected, that overnight washing removed enough inhibitors to enable PCR amplification for more genotypes, that thrice PBS washing. It also should be underlined that in contrast to activated sludge flocs (data not published), the granular sludge seems to be relatively heterogenic in its composition, when we compare the fingerprints obtained from three repetition of DNA isolation performed on the sample sludge sample (Figure 3). It also causes slight differences in biodiversity measurements (Figure 4a). It would be wise to perform these analyses in several repetitions and present biodiversity as the average with standard deviation.

We also shouldn't forget about a certain dose of imperfection in densitometry analysis performed on the basis of the fingerprints. The results obtained as DNA fingerprint are analyzed as a quantitative measurement of optical density in comparison to the background. The computer programs calculate densitometry according to their standards set by the researcher. Nonetheless, each analysis performed by the computer are set and corrected by the human being so there is always room for error. The more calculation repetition being statistically analyzed the better for the experiment.

4. CONCLUSION

On the basis of the experiment it can be stated that:

- mechanical method lead to cleaner and less degraded DNA obtainment than GeneMatrix spin Kit for Soil according to electrophoretic evaluation and densitometric analysis, but the spectophotometrical measurements differ from electrophoretic and densitometric. More DNA was obtained after overnight PBS washing, regardless to the isolation method;
- there is no influence of the DNA isolation method on the amount of PCR products, but it influences qualitative DGGE resolution;
- granular sludge is a heterogeneous material, so the research in PCR-DGGE granular sludge field should be performed in statistically analyzed repetitions.

ACKNOWLEDGEMENT

The research leading to these results has received funding from the Polish-Norwegian Research Programme operated by the National Centre for Research and Development under the Norwegian Financial Mechanism 2009-2014 in the frame of Project Contract No BARITECH Pol-nor/197025/37/2013. Authors would like to thank coworkers from Poznań University of Technology for research material.

REFERENCES

[1] *Díaz E., Amils R., Sanz J.L.*; Molecular ecology of anaerobic granular sludge grown at different conditions. Water Science and Technology, Vol.48, No.6, 2003; p.57-64

[2] *Muyzer G., Smalla K.*; Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Mini Review. Antonie van Leeuwnhoek, Vol.73, 1998; p.127-141

[3] *Muyzer G., De Waal E.C., Uitterlinden A.G.*; Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, Vol.59, No.3, 1993; p.695-700

[4] *Amann R.I.*; In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: Akkermans, D.L., van Elsasand, J.D. de Bruijn, F.J. (eds.) Molecular Microbial Ecology Manual. Kluwer Academic Publishers: Dordrecht, 1995

[5] *Abdullah N., Yuzir A., Curtis T.P., Yahya A., Ujang Z.*; Characterization of aerobic granular sludge treating high strength agro-based wastewater at different volumetric loadings. Bioresource Technology, Vol.127, 2013; p.181-187

[6] *Liu H., Tang D., Li G., Zhang M., Du G., Chen J.C.*; A comparable study of microbial community in aerobic granular sludge and activated sludge for wastewater treatment. Journal of Environmental Science and Engineering, Vol.1, No.1, 2007; p.69-77

[7] *Cheng M., Cook A.E., Fukushima T., Bond P.L.*; Evidence of compositional differences between the extracellular and intracellular DNA of a granular sludge biofilm. Letters in Applied Microbiology, Vol.53, 2011; p.1-7

[8] *Tsai Y.L., Olson B.H.*; Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Applied and Environmental Microbiology, Vol.58, 1992; p.2292-2295

[9] *Watson R.J., Blackwell B.*; Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Canadian Journal of Microbiology, Vol.46, 2000; p.633-642

[10] *Monteiro L., Bonnemaison D., Vekris A., Petry K.G., Bonnet J., Vidal R., Cabrita J., Mégraud F.*; Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. Journal of Clinical Microbiology, Vol.35, 1997; p.995-998

[11] *Demeke T., Adams R.P.*; The effects of plant polysaccharides and buffer additives on PCR. Biotechniques, Vol.12, 1992; p.332-334

[12] *Khan G., Kangro H.O., Coates P.J., Heath R.B.*; Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. Journal of Clinical Pathology, Vol.44, 1991; p.360-365

[13] *Ziembińska-Buczyńska A., Żabczyński S., Folkert J., Meresta A., Cema G.*; Diversity and changeability of activated sludge bacteria in two stage nitritation-anammox membrane bioreactor treating coke wastewater. Architecture, Civil Engineering, Environment, Vol.4, 2013; p.67-72

[14] *Ziembińska A., Ciesielski S., Miksch K.*; Ammonia oxidizing bacteria community in activated sludge monitored by denaturing gradient gel electrophoresis (DGGE). Journal of General and Applied Microbiology, Vol.55, 2009; 375-380

[15] *Bramucci M., Kane H., Chen M., Nagarajan V.*; Bacteria diversity in an industrial wastewater bioreactor. Applied Microbiology and Biotechnology, Vol.62, 2003; p.594-600