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ORIGINAL PAPER

Molecular insight into activated sludge producing polyhydroxyalkanoates under aerobic–anaerobic conditions

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Abstract One of the options enabling more economic production of polyhydroxyalkanoates compared to pure cultures is the application of mixed cultures. The use of a microbial community in a sequencing batch reactor has a few advantages: a simple process control, no necessity for sterile processing, and possibilities of using cheap substrates as a source of carbon. Nevertheless, while cultivation methods to achieve high PHAs biomass concentration and high productivity in wild and recombinant strains are defined, knowledge about the cultivation strategy for PHAs production by mixed culture and species composition of bacterial communities is still very limited. The main object of this study was to characterize on the molecular level the composition and activity of PHAs producing microorganism in activated sludge cultivated under oxygen limitation conditions. PHAs producers were detected using a PCR technique and the created PHA synthase gene library was analyzed by DNA sequencing. The obtained results indicate that PHAs-producers belonged to *Pseudomonas* sp., and possessed genes coding for mcl-PHA synthase. The kinetics of mcl-PHA synthase expression was relatively estimated using real-time PCR technology at several timepoints. Performed quantitative and qualitative analysis of total bacterial activity showed that there were differences in total activity during the process but differential expression of various groups of microorganisms examined by using DGGE was not observed.

Keywords Activated sludge · Denaturant gradient gel electrophoresis · Polyhydroxyalkanoates · *Pseudomonas* sp. · Real-time PCR · Sequencing batch reactor

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Introduction

Polyhydroxyalkanoates (PHAs) are accumulated as carbon/energy storage material in various microorganisms. PHAs are considered to be biodegradable substitutes for conventional polymers. One of the alternatives enabling more economic production of PHAs compared to pure cultures is the application of mixed cultures as a bioplastic factor. The use of mixed cultures, such as activated sludge, has a few advantages: a simpler process control, no requirement of monoseptic processing, and possibilities of using cheap substrates and even wastes as a source of carbon [1].

The ability of PHAs accumulation was also proved in activated sludge operated under anaerobic–aerobic conditions or when exposed to a transient carbon supply. These systems are commonly used in treatment plants in purpose of phosphorus removal from wastewater. Recently, Chua et al. [2] proposed technology production of PHAs by using excess sludge and external carbon substrate in a separate stage of treatment in wastewater plants.

Among various potential systems for PHAs industrial production in wastewater treatment plants, a sequencing batch reactor (SBR) seems to be the most appropriate. Its key characteristic features are the change between feast and famine in the duration of cycle and anaerobic–aerobic conditions imposed. Moreover, a number of operating

strategies can be implemented. However, despite operating strategies in the cycle being very important in the arrangement of the microbial community, such investigations have not been conducted so far.

Microorganisms of activated sludge are able to produce and accumulate PHAs as carbon and energy storage material under unsteady conditions originating from periodic feeding and variation in the presence of an electron acceptor [1]. This unbalanced culture, described as feast and famine conditions, allowed for more effective PHAs accumulation. Under feast and famine conditions, the microorganisms which are able to store the substrate during the initial feast phase have a competitive advantage over other microbes, as they can use the stored polymer as an internal carbon source in the famine phase. Thus, under the feast and famine regime the activated sludge is enriched by those microrganisms and its overall potential to store is increased [3].

Microbial composition, that developed under such a condition influence the quantity and quality of accumulated polymers, thus characterization of this microbiota is required to develop promising strategies for improved process performance. Despite many papers dealing with PHA production by mixed culture, there is a lack of information about microorganisms responsible for PHA production in such an environment. Recently, the molecular biology tools have provided a new insight into microbial ecology allowing one to obtain an encompassing, high-resolution view of microbial structure in natural and semi-natural environments.

However, a more important topic in microbial based systems is the detection of physiological activity, that could be estimated by the measure of key genes expression. One approach for studying specific microbial activities is to investigate the expression of genes by analyzing mRNA transcripts. Regulation at the transcription level almost immediately affects the rate of protein synthesis, and detection of gene expression can be used for the investigation of specific microbial processes [4]. This method could be particularly useful for chasing genes encoding enzymes involved in PHAs synthesis. However, only a small number of studies have reported successful analyses of mRNA isolated from activated sludge [5], which released from technical difficulties related to the isolation of mRNA. These include the misconception that the half-lives of prokaryotic mRNA are too short to allow for isolation and characterization, and mRNA isolated from such as material will be degraded by ribonucleases [6].

Recently, real-time polymerase chain reaction (PCR) has emerged as a robust and widely used methodology for biological investigation because it can detect and quantify very small molecules of nucleic acids in a strongly specific way. As a research tool, a major application of this technology is the rapid and accurate assessment of changes in gene expression as a result of physiology or environmental changes [7].

This research was developed to uncover the bacterial species and their activity in the process of PHAs accumulation in the aim of understanding the relationships between environmental conditions and biopolymers production by microorganisms. In the first, genetic identification of PHAs producers in the activated sludge under oxygen limitation conditions was performed. Then, the expression of the gene coding for medium-chain-length PHAs (mcl-PHA) and 16S rRNA was analyzed during a three-stage cultivation of activated sludge.

In addition, the bacterial community structure was characterized by denaturing gradient gel electrophoresis (DGGE) of PCR and RT-PCR amplified 16S rDNA and 16S rRNA fragments, respectively. These two complementary techniques were used to distinguish bacterial populations that were only present from those that were metabolically active at each time-point.

Materials and methods

Enrichment of activated sludge

In this experiment a culture medium consisting of organic and mineral salts solutions was used. The organic solution contained a mixture of molasses and acetic acid at a weighted ratio of 2.9. A portion of 240 g molasses was dissolved in 760 g deionised water and hydrolysed at 100°C and pH 1.5 (adjusted with 98% H_2SO_4) for 30 min. Next, the solution was cooled to about 60°C and neutralized to pH 7.5 with $Ca(OH)_2$. After 12 h, it was centrifuged at 4,500 rpm for 10 min to remove the precipitated $CaSO_4$. The mineral salts solution contained per liter: 0.26 g of NH_4Cl; 0.03 g KH_2PO_4; 0.19 g of $Na_2HPO_4 \cdot 12\ H_2O$; 0.2 g of $MgSO_4 \cdot 7\ H_2O$; 0.06 g of $FeCl_2 \cdot H_2O$; 0.01 g of $CaCl_2 \cdot 2\ H_2O$ was supplemented with 1 mL of trace element solution (0.3 g of H_3BO_3; 0.2 g of $CoCl_2 \cdot 6\ H_2O$; 0.1 g of $ZnSO_4 \cdot 7\ H_2O$; 0.03 g of $MnCl_2 \cdot 4\ H_2O$; 0.03 g of $NaMoO_4 \cdot 2\ H_2O$; 0.028 g of $NiSO_4 \cdot 7\ H_2O$; 0.01 g of $CuSO_4 \cdot 5\ H_2O$ dissolved in 0.5 N HCl per liter). Loading amounts of chemical oxygen demand (COD), ammonia–nitrogen (NH_4–N) and phosphate–phosphorus (PO_4–P) into SBR were 3,000, 67 and 49 mg/L, respectively.

The sample of the activated sludge was obtained from the municipal wastewater treatment plant in Olsztyn (North Poland). To ensure that steady state conditions had been attained, all tests performed were started after 2 months. The criteria for steady state were constancy of the biomass concentration in the reactor, PHAs content of the biomass dry weight and COD amount in the effluent.

The experiment was performed in a laboratory-scale SBR with a working volume of 4 L. The SBR was operated with a cycle of 24 h. Each cycle consisted of anaerobic (1 h), aerobic (21 h) and settling phase (2 h). The cycle began with the addition of 2 L of the culture medium at the start of the anaerobic phase and the effluent was removed at the end settling period, resulting in the hydraulic retention time of 48 h. The sludge retention time was kept to 5 days by withdrawing excess sludge from the reactor at the end of anaerobic phase. During the anaerobic phase, the reactor was continuously stirred (30 rpm). The dissolved oxygen concentration was controlled at 3.0 mg/L at the end of the aerobic period. The temperature of the reactor was maintained at 23°C.

Analytical techniques

For determination of mixed liquor suspended solids (MLSS), activated sludge samples taken from the SBR were filtered on a Whatman glass microfiber filter. The filter was dried at 105°C for 2 h and weighed.

Extraction and estimation of PHAs was performed according to Bormann et al. [8]. The biomass was separated from the culture broth by centrifugation at 21,000 g for 10 min (MPW-350; MPW Med. Instruments, Warsaw, Poland). The obtained pellet was resuspended in an equal volume of Clorox (The Clorox Company, Oakland, CA, USA) and incubated at 37°C for 1 h. The whole mixture was centrifuged once again and the supernatant was discarded. The lysed cell pellet was washed with acetone and ethanol. Finally, PHAs was extracted by hot chloroform. The chloroform was evaporated at room temperature and the PHAs mass was weighed after drying at 105°C for 2 h.

The PHAs content of the cell dry weight (CDW) was calculated by dividing the measured PHAs concentration by overall biomass concentration and expressed as a percentage value. The sludge retention time (SRT) was calculated by dividing the amount of biomass present in the reactor by the amount of excess sludge withdrawn from the reactor per day. The specific growth rate was calculated as the inverse of sludge retention time. The yield coefficient of PHAs on substrate consumed ($Y_{PHA/S}$) was expressed as a ratio between the specific PHAs storage rate (q_{PHA}, in mg PHA/L h) and the specific substrate uptake rate ($-q_S$, in mg COD/L h). The specific PHAs storage rate and the specific substrate uptake rate were determined by adjusting a linear function to the experimental data and calculating the first derivative at time zero (beginning of the experiment). The volumetric productivity of PHAs was expressed as follows:

$$\frac{P_{r,SBR} = C_{PHA} - V_w}{V} \quad (1)$$

where

$P_{r,SBR}$ volumetric productivity of PHA (mg PHA/L cycle)
C_{PHA} final concentration of PHA at the and of the anaerobic phase (mg PHA/L)
V_w volume of excess sludge (L)
V working volume of reactor (L)

Total DNA extraction

Genomic DNA was isolated as follows: 0.02 g as semi-dry weight of aggregated sludge sample was washed in sodium phosphate buffer (0.1 M; pH 8.0), pelleted by centrifugation, suspended in the proteinase K buffer (100 mM Tris–HCl; 10 mM EDTA; pH 8.0) and incubated at 55°C in the presence of sodium dodecyl sulphate, proteinase K and lysozyme. During incubation the activated sludge samples were mechanically disrupted by multiply pippeting. DNA was purified using phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) solution, and precipitated with two volumes of ethanol (96%) and 0.1 volume of sodium acetate (pH 5.2). The pellet was washed with 70% ethanol, dried and re-suspended in 200 µl of TRIS/EDTA (10 mM Tris–HCl,1 mM EDTA, pH 7.5). DNA concentration was determined by spectrophotometry at 260 nm using a Biotech Photometer (WPA, UK).

Total RNA extraction and purification

All reagents and processes were made in/with sterile, disposable, nuclease-free lab ware. For RNA extraction, the sludge pellets had been stored in RNA Later (Q-BIOgene) to maintain the quality of the extracted RNA. RNA was extracted in duplicate from approximately 0.02 g as semi-dry weight of the aggregated sludge sample. The cells were lysed in the solution of guanidine isothiocyanate and phenol. The flocks were dispersed by vigorous pippeting. After that RNA was purified on the silica membrane (Total RNA isolation kit, A&A Biotechnology, Poland). To eliminate contaminating genomic DNAs, 3 U of RQ1 RNase-Free DNase (Promega, USA) was added to each RNA extract before incubation at 37°C for 30 min. Quality and quantity of isolated RNA was measured spectrophotometrically using Biotech Photometer (WPA, UK). Purified RNA samples were immediately used for further molecular analyses.

PCR detection of PHAs producers in activated sludge

To detect microorganism producing polyhydroxyalkanoates, PCR was performed with four primer pairs that were previously described as suitable for recognizing four

different classes of PHAs producers (Table 1). PCR was performed in Eppendorf® Mastercycler Gradient (Eppendorf, Germany). The mixtures used for PCR amplification contained 50 ng of extracted total DNA, 0.5 μM of each primer, 100 μM of deoxynucleoside triphosphate (Promega, WI, USA), 1 U of *Taq* DNA polymerase (Invitrogen, Life Technologies), 5 μl reaction buffer (500 mM KCl, pH 8.5; Triton X-100), 1.5 mM $MgCl_2$ and sterile water to a final volume of 50 μl. The standard PCR amplification was carried out using the following program: 95°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 42–62°C (gradient temperature) for 30 s, extension at 72°C for 1 min and single final elongation at 72°C for 5 min. The presence of PCR products was confirmed by analyzing 5 μl of the product on a 1.2% agarose gel stained with ethidium bromide, and size of PCR products was estimated using øX174 DNA/*Hin*f *I*.

Molecular cloning, sequencing and phylogenetic analysis

The PCR amplified products were fractionated on 1% agarose gel, stained with ethidium bromide, and DNA from appropriate bands was recovered using Wizard®SV Gel and PCR Clean-Up System (Promega, Madison, USA). These fragments were subcloned using the TOPO-TA Cloning® Kit for Sequencing (Invitrogen, Life Technologies) into the pCR4-TOPO vector. Several ampicillin resistant colonies were screened by colony PCR using M13 forward and reverse primers. Plasmid DNA was purified from overnight bacterial cultures and sequenced by an automatic DNA sequencer ABI 373 (Applied Biosystems, CA, USA) using a Dye terminator sequencing kit according to manufacturer protocol at the Institute of Biochemistry and Biophysics in Warsaw, Poland.

A similarity search of nucleotide sequences was performed using the BLASTN non-redundant database at http://www.ncbi.nlm.nih.gov/BLAST. Alignment analysis was carried out by using Clustal W [14]. Genetic relationships were determined by the distance method with the MEGA 2.1 program [15] using nucleotide sequences of the *phaC2* gene. Distance tree was estimated according to the neighbor-joining method of Saitou and Nei [16]. To determine the degree of statistical support for branches in the phylogeny, 1,000 bootstrap replicates of data were analysed. The nucleotide sequences were deposited in GenBank under accession number: DQ869269.

Reverse transcription real-time PCR

The reverse transcription (RT) reaction was carried out using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas) in total volume of 40 μl. Total RNA in amount of 1.0 and 2 μl of random hexamer primer (0.2 μg μl^{-1}) was added to distilled water. After the mixture was incubated at 70°C for 10 min to denature secondary structures of rRNA, the tube was placed on ice and the following mixture was added: 8 μl of 5× reaction buffer [250 mM Tris/HCl (pH 8.3 at 25°C), 250 mM KCl, $MgCl_2$, 50 mM dithiothreitol (DTT)], 2 μl of ribonuclease inhibitor (20 U/μl) and 2 μl of 10 mM dNTP mix. The mixture was incubated at 25°C for 5 min to allow the annealing of the random hexamer primers. After adding 2.0 μl of RevertAid M-MuLV, reverse transcriptase (200 U/μl) reaction was carried out at 25°C for 10 min and at 42°C for an additional 60 min.

For real-time amplification of phaC synthase gene of *Pseudomonas* sp. this same primers pair was used as for detection (Table 1). For 16S rRNA amplification primers 341F and 515R were chosen [13]. The specificity of each primers pair was controlled by melt curves analysis.

Reactions were performed using SYBR green master mix (Applied Biosystems) in 25 μl final volume. Primers were used at a final concentration of 50 nM per reaction.

Table 1 PCR primers used in this study

Primers	Nucleotide sequence (5′–3′)	Amplicon/PCR product length	Purpose	References
phaCF1	ATC AAC AAR TWC TAC RTC YTS GAC CT	phaC (I class) ~406 bp	Detection	[9]
phaCR4	AGG TAG TTG TYG ACS MMR TAG KTC CA			
I-179L	ACA GAT CAA CAA GTT CTA CAT CTT CGA C	phaC1/C2 (II class) ~540 bp	Detection/real-time PCR	[10]
I-179R	GGT GTT GTC GTT GTT CCA GTA GAG GAT GTC			
P1	ATN GAY TGG GGN TAY CCN	phaC (III) class ~500 bp	Detection	[11]
P2	RAA DAT CCA YTT YTC CAT			
B1F	AAC TCC TGG GCT TGA AGA CA	phaC (IV class) ~590 bp	Detection	[12]
B1R	TCG CAA TAT GAT CAC GGC TA			
341F[a]	CCT ACG GGA GGC AGC AG	16S rDNA ~174 bp	Real-time PCR/DGGE	[13]
515R	AAT CCG CGG CTG GCA			

[a] To this primer the 33 nt GC clamp was added

Templates were either 1.5 µl of cDNA per reaction or water in no template control (NTC) in this same volume. Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Fluorescence measurement was recorded during each annealing step. An additional step starting from 90 to 60°C (0.05°C/s) was performed to establish a melting curve of each primer pairs. This allowed for the verification the specificity of PCR reaction. Real-time PCR was performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems).

Gene expression was presented using a modification of the $2^{-\Delta\Delta Ct}$ method, described by Livak and Schmittgen [17]. Reaction was normalized by determining the amounts of RNA added to cDNA reaction. All real-time PCR experiments were performed in duplicate and each experiment included triplicate samples. To examine the intrasample variation, the ±SD was determined from three sample replicates. The expression of each gene was presented as $2^{-\Delta C_t}$ where ΔC_t ($C_{t\ \text{time}\ \times} - C_{t\ \text{time}\ 0}$) and time 0 represents the 1× expression of each gene.

DGGE analysis

Extracted DNA and cDNA from RT reactions (see above) were amplified by PCR for DGGE analysis using 16S rRNA primer pair specific for eubacteria (341F, 515R; Table 1), spanned V region of 16S rDNA [18]. A touchdown thermocycling program was used for PCR as described by Murray et al. [19].

DGGE was performed with a D-CODE Universal Mutation System (Bio-Rad, Hercules, USA). The PCR samples (20 µl) were applied directly to 8% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) in a 0.5× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.5) with a denaturing gradient ranging from 30 to 70%. Denaturation of 100% corresponds to 7 M urea and 40% formamide. The gradient gel was cast with a gradient delivery system (Model 475, Bio-Rad, Hercules, USA). Electrophoresis was run at a constant voltage of 60 V at 60°C. After 16 h of electrophoresis, the gel was stained with SYBR gold (Molecular Probes, USA) at 10,000× dilution in 1× TAE buffer for 30 min. Stained gel was viewed with an ultraviolet transiluminator and recorded with CCD camera (Gel Logic 200, KODAK, USA).

Results

Enrichment of activated sludge

Operational conditions and their values are given in Table 2. In brackets confidence interval were reported. The obtained data revealed that consumption of organic matters in the anaerobic phase was 54% and aerobic phase −72%. At average biomass concentration at level of 2.94 mg CDW/L, SRT was 5 days and a specific growth rate 0.2 d^{-1}.

Figure 1 shows the typical profiles of PHAs storage and biomass concentration during the cycle. Two distinct phases can be clearly defined. In the polyhydroxyalkanoates accumulation phase the content of PHA in dry biomass weight increased, whereas in the polyhydroxyalkanoates consumption phase it consequently decreased in the sludge. At the beginning of the cycle, PHAs concentration in the sludge was maintained at 4.7%. After the feed, during anaerobic phase PHAs content started to rapidly increase, achieving a maximum value (24.6% CDW) at the end of this phase. After shifting the reactor to the aerobic conditions, the profile of PHAs content showed a decrease, leading to a minimal value at the end of the aerobic phase (5.9% CDW).

Biomass concentration is an important parameter, so it was measured throughout the cycle. An expected, biomass concentration decreased during the anaerobic phase from 3.16 to 2.5 g of cell dry weight per liter (g CDW/L). At the beginning of aerobic phase biomass concentration started

Table 2 Operational conditions of activated sludge (confidence interval of the mean is reported in brackets)

	Symbols	Units	Values
Concentration of organic matters (COD) at the start of the cycle	C_0	mg COD/L	1,605 (±19.8)
Concentration of organic matters (COD) at the end of anaerobic phase	C_{an}	mg COD/L	738 (±51.8)
Concentration of organic matters (COD) at the end of the cycle	C_e	mg COD/L	209 (±39.6)
Average biomass concentration in the reactor	X_{av}	mg CDW/L	2,940 (±213.0)
Ratio of organic matters concentration (COD) and biomass			
Concentration at the start of the cycle	C_0/X_0	mg COD/mg CDW	0.55
Sludge retention time (SRT)	Θ	d	5.0 (±0.13)
Specific growth rate	μ	d	0.20

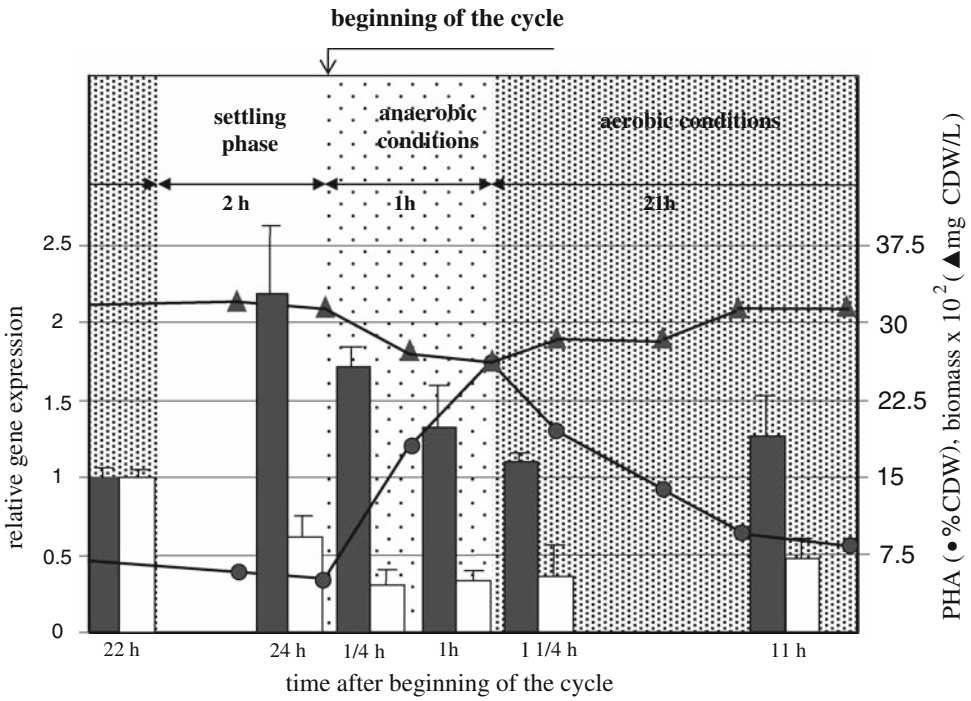

Fig. 1 The relative levels of 16S rRNA (*filled square*) and *phaC* (*open square*) transcripts during cycles in the SBR. Genes expression was quantified using relative PCR, and $2^{-\Delta\Delta Ct}$ method, described by Livak and Schmittgen [17]. Expressions of studied genes in the end of growing phase (24 h) were used as the calibrator. *Vertical bars* indicate SD. Profiles of PHAs (*filled circle*), % CDW, and biomass (*filled triangle*, $10^2 \times$ mg CDW/L) concentration in the course of time

to increase reaching a level of 3.34 g CDW/L at the end of this phase.

The effectiveness of PHA accumulation was evaluated on the basis of yield coefficient of PHA on substrate consumed ($Y_{PHA/S}$) and volumetric productivity ($P_{r,SBR}$). Yield coefficient of PHA was calculated as the ratio $q_{PHA}/-q_S$. For calculations, it was assumed, that PHA synthesis and substrate consumption proceeds with a zero order rate. From obtained data, it results, that the specific PHA storage rate and the specific substrate uptake rate amounted to 484.5 mg PHA/L h and 907.2 mg COD/L h, respectively. On this basis, the calculated value of $Y_{PHA/S}$ was 0.53 mg PHA/mg COD. The volumetric productivity was defined as the amount of PHA produced per reactor volume per cycle. At maximum PHA concentration of 707 mg PHA/L and working volume of SBR 4 L, the productivity calculated from Eq. 1 amounted to 141,4 mg PHA/L·cycle.

PCR detection of PHA producers in activated sludge

After successful DNA purification (with average yield of 95 μg/g of semi-dry biomass), the polymerase chain reaction was carried out with the set of four primers pair appropriate for recognizing four different classes of the PHA producers. Two of them reacted positively and the amplicon sizes observed were consistent with expectations. The phaCF1/phaC4 primers amplified a 406-bp region, whereas primers I-179L and I-179R amplified 540-bp fragment. Annealing temperatures in the interval between 50 and 65°C were evaluated, and the optimum was

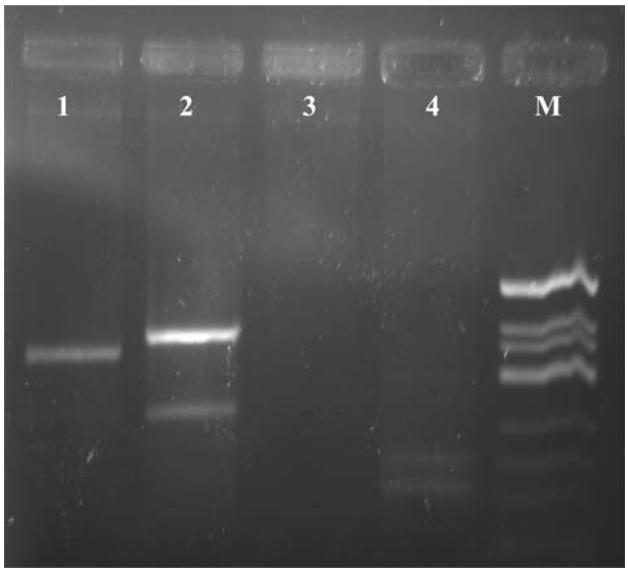

Fig. 2 The result of polymerase chain reaction aimed at detection of genes coding for all known classes of polyhydroxyalkanoate polymerase. *Lane 1* presents detection of I or II class of *pha* genes, whereas *lane 2* demonstrates presence of gene coding for II class of pha polymerase. There was lack of signal characteristic for genes coding for III and IV classes of pha polymerse. M – ØX 174/*Hin*fI DNA size marker (*Promega*)

determined to be 55°C for phaCF1/phaC4 primers, and 52°C for the second pair. Whereas, P1/P2 and B1F/B1R primer pairs did not amplify any DNA fragments, that could testify the presence of members of III and IV class in the activated sludge (Fig. 2). PCR fragments, which were

obtained by using phaCF1/phaC4 and I-179L/I-179R primers pairs were cloned and sequenced. The obtained sequences were identical and were referred to as KBOS06. The sequence was further characterized by a comparison with the sequences published in the GenBank database. The neighbor-joining phylogenetic tree (Fig. 3) demonstrated that the analyzed sequence was most similar to *phaC2* gene of *Pseudomonas* sp. KBOS 04 (AY7903280) [20] with identity/similarity of 95.2/94.6%.

Real-time PCR

The applied procedure of RNA purification allowed to obtain the RNA yield on the level of 5 μg/g of semi-dry biomass with purity ratio of 1.7. The expression of the second class PHA synthase (*phaC*) was examined using these same primers as for detection (I-179L and I-179R) [10]. Overall metabolic activity of bacterial cells during all PHA production phases was estimated by analysis of 16S rRNA using primers 341F and 515R [13]. The primers sets used generated the expected amplicons, without any non-specific products. Figure 1 presents the experimental results for PHA synthase gene and total, eubacterial RNA activities.

The transcript level was measured by relative real-time PCR; as a calibrator genes expression at the end of the aerobic phase was taken. The levels of RNA as well as *phaC* transcripts showed similar trends during all phases of culture: maximum values were observed in during settling phase and diminished with time, reaching the lowest values in the middle of anaerobic phase (*phaC*) or in the very beginning of aerobic conditions (16S rRNA). Variations obtained for *phaC* transcript levels in the different conditions are weak; it has relatively constant transcript level compared to rRNA gene. The relative levels of 16S rRNA gene expression show different patterns, increasing more than twofold upon settling phase. After that, level of this gene expression gradually decreased reaching the starting level in the beginning of aerobic phase.

DGGE

The bacterial community structure of activated sludge was determined by DGGE analysis of 16S rRNA gene fragments amplified by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) from extracted DNA and RNA, respectively. DNA-DGGE indicates total abundance off all bacteria present while RNA-DGGE provides greater sensitivity, targets active members of the community. The activated sludge showed the presence of a heterogeneous microbial community, rich in different bacterial species, with some dominant, whose members are represented by the strongest bands. More than 25 distinct DGGE bands were obtained per sample, six of which were dominant (Fig. 4, Lane 1). There were six bands that differentiated DNA and RNA pools (A, B, C, D, E and F bands). The bands A, B and C were present only in DNA profile, and bands E and D were characteristic only for profiles made on the base of RNA. There was no detectable difference between RNA-DGGE profiles in the course of time (Fig. 4, lanes 2–7).

Discussion

The idea of polyhydroxyalkanoates (PHAs) production using activated sludge arose from a recognition of the PHA function as an intermediate metabolic product in wastewater treatment processes. Especially, it is well known that PHAs are the energy and carbon reserve material in polyphosphate accumulating organisms (PAO) in the enhanced biological phosphorus removal processes with sequencing

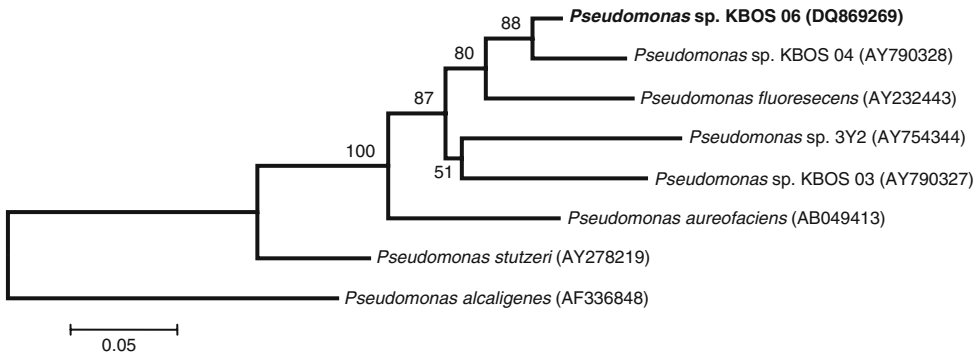

Fig. 3 Phylogenetic position of identified strains of *Pseudomonas* species (KBOS 06) based on 441 bp long fragment of *phaC2* gene. Tree was constructed by using neighbor-joining algorithm and analysis was performed by including most similar *phaC2* gene sequences deposited in GenBank. The tree was rooted by using sequence of phaC1 gene of *Pseudomonas alcaligenes*. Bootstrap values for branches are given at the *nodes*, whereas accession numbers are given in *parentheses*. *Bar* 0.05 estimated substitution per sequence position

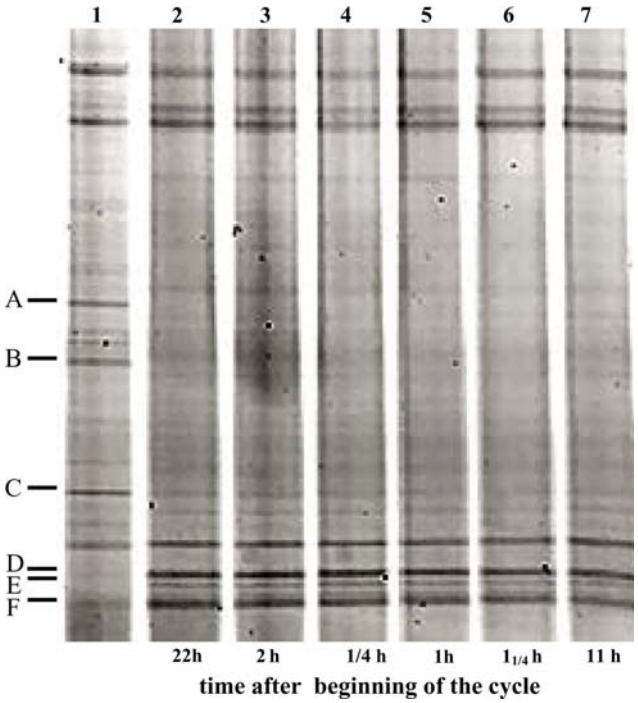

Fig. 4 DGGE fingerprints of (*1*) PCR amplified and (*2–7*) RT-PCR amplified 16S rDNA and 16S rRNA, respectively, of the microbial community in activated sludge operated under aerobic–anaerobic conditions. RNA samples were taken at different time from the beginning of cycle

anaerobic and aerobic conditions (anaerobic–aerobic processes). In anaerobic conditions, PAO take up organic substrates and accumulate them as PHAs. When the condition turns aerobic, PHAs are utilized for growth and polyphosphate regeneration [2, 21]. Our results agree well with the described scheme (Fig. 1); during anaerobic period PHAs concentration dramatically increase in reaction to lack of oxygen. Then, it decreased contrary to biomass, which started to increased when the operation was switched to aerobic conditions. The maximal amount of PHAs accumulated by the microbial population (24.8% CDW) was observed after 30 min of anaerobic phase and is comparable with the other values reported in the literature [2].

The recent development of molecular biological tools such as fluorescent in situ hybridization (FISH) or methods based on PCR provides sensitive and rapid detection of single genes and could be successfully applied for bacterial diversity. However, these techniques are not capable of analyzing cells activities or of indicating a direct response to changes in the environmental conditions of the bioreactor [5]. Analysis of mRNAs as indicators of gene expression should significantly enhance the understanding of mechanisms of PHAs production in activated sludge.

mRNA molecules are gene copies used for synthesis of specific proteins by the cell and concentration of mRNA is correlated with the protein synthesis rate and with the activity of the microorganism. Therefore, the content of mRNA molecules will give very accurate pictures of the in situ function and activity of the microbial community. Detection and quantification of a specific mRNA molecule can be done by reverse transcription PCR. Recently, developed real-time PCR in combination with reverse transcription offers the possibility of utilizing real-time reverse transcription PCR to investigate the expression of a function gene in a variety of environments. This technique has the potential of real-time monitoring of specific functional activity in a complex community with high sensitivity and specificity. Although some studies on mechanism of PHA-related genes have been reported using a pure culture of *Paracoccus denitrificans* [22], *Ralstonia eutropha* [23], and *Pseudomonas corrugata* [24] there is a lack of information concerning the activity of PHA-related such genes in complex microbial communities.

There is a lack of universal PCR primer pair that could specifically recognize all known classes of genes coding for PHA synthase [25] therefore for PCR detection four primer pairs were used. The positive signals were obtained for PCR primers pairs, which were specific for I and II class of PHAs producers. DNA sequencing revealed that both pair of primers has amplified this same gene fragment, characteristic for pseudomonads. It is widely known that bacteria producing P(3HB) are the most abundant, and occur in almost any kind of environment. Therefore, the existence of I type of PHA synthase in the activated sludge cannot be excluded and lack of their trace could be the result of the low specificity of PCR primers. In order to elucidate this confusion fluorescence in situ hybridization (FISH) with probes specific for *Pseudomonas* sp. should have been performed.

Clones sequencing revealed that the main polyhydroxyalkanoates producers in activated sludge belongs to *Pseudomonas* sp. therefore in gene expression analysis we applied primers specific to these species genes. The PHA operon of *Pseudomonas* sp. is responsible for mcl-PHA production and contains two synthase genes: *phaC1* and *phaC2* disjoined by the gene coding for the PHA depolymerization (*phaZ*) [26]. Applied PCR primers, described by Solaiman et al. [10] are able to bind to both *phaC* genes, and our study does not show which one was active in the created conditions. These results demonstrate that *phaC* mRNA level was quite constant, although small, differences within 2 h in response to the changes of environmental conditions were observed. It seems that expression of this gene is steady throughout the whole cycle but could be sensitive to changes in environment. It is worth pointing out that the level of this gene expression was proportionally high in relation to the total bacterial 16S

rRNA gene expression, especially if we assume that there is an average of 3.6 copies of the 16S rRNA gene per genome [27]. The expression pattern of *phaC* gene expression was not strictly correlated to PHAs storage that validates the complexity of interpreting transcriptional data from species in complex communities. In order to explain the relation between *phaC* expression and kinetics of PHAs accumulation it is necessary to know the half-life of it transcripts that could be in the order of seconds but could reach even hours.

The expression of rRNA is tightly dependent on the physiological status of bacteria [28]. In this study, the profile of rRNA expression was quantitatively analyzed by using eubacterial PCR primers [13]. Applying such a sensitive approach as real-time PCR enabled the discovery of considerable changes during the process. Generally, the differences in the RNA level are correlated well with biomass formation rate [29]. Our results (Fig. 1) reflect tightly this rightness. Similar to *phaC* gene expression the highest value of rRNA activity during settling phase was noticed, when the value of biomass was very high. The lowest 16S rRNA expression, similar to biomass concentration was at the boundary of anaerobic and aerobic phases. Thus positive correlation between biomass concentration and rRNA activity was observed. This observation proved that real-time PCR analysis could be useful for monitoring total bacterial activity in semi-natural bacterial ecosystems.

In order to know the bacterial richness and dynamics of their activity in different stages of the process PCR-DGGE technique was applied. The numbers of PCR products, reflected as bands migrating in each DGGE gel represents the number of 16S rRNA gene sequence similarity groups or operational taxonomic units, which usually are called species for simplicity [30]. RNA-derived DGGE banding patterns, generated by RT-PCR showed a similar diversity to those derived from DNA, but not all dominant bands present in DNA-based profiles were observed in those derived from RNA (Fig. 4; A, B and C bands). Species, demonstrated by these bands were present in activated sludge but their physiological activity was at an undetectable level. The presence of this species suggests that process of selection was not finished at this stage of experiment, and process of acclimatization should be extended. In contrary, there were bands characteristic only for RNA-based profiles (D, E and F bands), these species exhibited incomparable activity in proportion to their cells number in environment. There was a lack of difference between fingerprints based on RNA taken at various stages of cycle, which suggests that all groups of microorganisms present in bioreactor work with this same activity through the whole process, without dependence on environment conditions.

The obtained results revealed that the PHAs producer in analyzed activated sludge belonged to *Pseudomonas* sp. whose *phaC2* gene structure was closest to the previously published sequence of *Pseudomonas* sp. KBOS04 [20]. In both cases inoculum was taken from this same municipal wastewater treatment plant in Olsztyn, what suggests that nucleotide composition of *phaC2* gene could be geographically determined.

In conclusion, the applied PCR based detection allowed for the discovery that a significant PHAs producer in activated sludge fed with mixture of molasses and acetic acid belonged to *Pseudomonas* species. In this study, a minimal value of total rRNA as well as biomass concentration in the end of anaerobic conditions was observed, this concordance evidenced that applied methodological approach could be valuable for understanding the bacterial growth dynamics of mixed cultures. The results of *Pseudomonas* sp. *phaC* gene expression analysis showed that in the activated sludge it is occurring during the whole cycle and thus PHAs synthesis is not controlled by this gene, at least at the transcriptional level. Performed quantitative and qualitative analysis of the total bacterial activity showed that there were differences in the total bacterial activity during the process but a differential expression of various groups of microorganisms detected by using DGGE was not observed. The real-time PCR method that was applied here allowed for an accurate quantification bacterial RNA targets with high sensitivity. However, in order to understand physiological activity of PHAs producing bacteria in such a complex environment as activated sludge there is a need to develop other primer targeting genes coding other proteins involved in this biopolymer production.

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