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Determining Microbial Dynamics of Polyhydroxyalkanoates – Producing Consortium in Waste Glycerol using RISA Technique

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Abstract. Ribosomal intergenic spacer analysis (RISA) technique was used to analyze dynamics of microbial community capable of converting waste glycerol to polyhydroxyalkanoates (PHA) under cultivation on 5% $v.v^{-1}$ crude glycerol as a carbon source. Three dominant band patterns were observed during 55 days cultivation. 16S rRNA sequences of dominant RISA bands showed bacterial community cultivated in waste glycerol were closely related to *Azoarcus* sp., *Bacillus cereus*, *Bacillus pseudofirmus*, *Flavobacterium columnare* and *Thauera* sp.

Keywords: Polyhydroxyalkanoate, glycerol waste, Ribosomal intergenic spacer analysis, mixed culture

1. Introduction

Polyhydroxyalkanoates (PHA) is biopolyesters produced mainly by bacteria. The PHA has attracted industrial interest as biodegradable plastics not only their compatible material properties like synthetic thermoplastics but also could PHA be synthesized from renewable carbon resources (e.g. agriculture and industrial wastes [1]. In recent, glycerol has become an inexpensive and abundant carbon source due to its generation as an inevitable by-product of biodiesel production. The tremendous growth of the biodiesel industry created a glycerol surplus that had resulted in a dramatic decrease in crude glycerol prices, making it even a waste stream with a disposal cost. The development of processes that could convert raw glycerol into more valuable products seems to be necessary to bio-refinery industry exponentiation. One of the many promising applications for the use of glycerol wastes was its bioconversion through microbial fermentation to high-value compounds such as biopolymers [2]. Clearly, the growth of processes to convert crude glycerol into higher-value products is an urgent need. The industrial production of PHA was based on their synthesis by microbial isolates in either their wild- or genetically modified strains. The PHA production processes based on mixed microbial populations was one of the alternative technologies that could decrease production costs, since no sterilization is required and bacterial adaptation is quite easy, even for complex substrates that may be present in the waste stream [3]. High substrate costs decrease the profitability of PHA production, and thus low-cost carbon substrates such as agricultural and industrial residues or crude glycerol by-products of biodiesel production process seems to be the most promising source of carbon have been tested for the production of these polyesters [3].

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The majority of molecular methods being used for community analysis examine nucleic acids. Some of these methods directly examine nucleic acids, whereas PCR amplification have been used to increase copies of a target gene for easier detection. Examples of methods being used to directly analyze nucleic acids are DNA: DNA reassociation kinetics, nucleic acid hybridization, fluorescent in situ hybridization, micro-arrays and metagenome sequence analysis. The most common PCR-dependent approaches are DGGE, terminal fragment length polymorphism (T-RFLP), single strand conformational polymorphism, ribosomal intergenic spacer analysis (RISA) and sequence analysis of 16 rRNA gene clone libraries. All of these approaches show strengths and weaknesses that needed to be considered when choosing the most appropriate method for an application. In general, genetic fingerprinting techniques such as DGGE, T-RFLP, and RISA allow higher throughput and the comparative profiling of many samples, and thus facilitate the spatial and temporal analysis of microbial communities in ecosystems [4]. The RISA technique uses the ribosomal 16S-23S intergenic spacer length heterogeneity between taxa to distinguish members of the community. This profiling approach relies on a variable intergenic spacer that was amplified, and then it was resolved on polyacrylamide gel accordingly to the length of this region [5]. The samples for isolation were taken when the functional stability of the mixed microbial community was ascertained. Isolated strains were molecularly distinguished and identified. Nowadays, molecular methods had been applied to identify PHA-accumulating organisms in an aerobic dynamic feeding reactor. Nevertheless, a few of previous studies provided details of the mixed culture in PHA production [6]. The current study investigated microbial dynamics of PHA-producing bacteria capable of converting glycerol wastes to PHA using RISA technique, and identified the bacterial species using 16S rDNA sequencing.

2. Materials and methods

2.1. Microbial inoculum

Activated sludge collected from industrial process of wastewater treatment (Olsztyn, Poland) was used in as an inoculum, and was acclimated in the cultivation medium containing 5 % v v^{-1} crude glycerol. The crude glycerol, obtained from the Elstar Oils Company (Malbork, North Poland), was used as a carbon source. The acclimation process was conducted in a 10 L fermentor (Bioflo 3000; New Brunswick, USA) with fed-batch mode operation for 55 days. The cultivation medium contains (per L) 2.3 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.3 g $NaHCO_3$, 0.1 g $CaCl_2.2H_2O$, and 1 mL trace element solution (0.58 g $ZnSO_4.7H_2O$, 3.96 g $MnCl_2.4H_2O$, 0.6 g H_3BO_3, 5.56 g $FeSO_4.7H_2O$, 5.62 g $CoSO_4.7H_2O$, 0.34 g $CuCl_2.2H_2O$, 0.04 g $NiCl_2.6H_2O$, 0.06 g $NaMoO_4.2H_2O$ dissolved in 0.5 N HCl per liter). Crude glycerol was added to the medium to obtain final concentration of 5% $v.v^{-1}$. $(NH_4)_2SO_4$ was used to adjust and keep the carbon-to-nitrogen ratio at a constant level (C/N = 10). During the experiment, biomass was collected for extraction of PHA [7].

2.2. DNA extraction

Genomic DNA was isolated as follows: 50 mg semi-dry weight of aggregated sludge sample was washed with sodium phosphate buffer (0.1 M; pH 8.0), pelleted by centrifugation, sonificated, 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. DNA was purified using genomic DNA Mini Kit (A&A Biotechnology, Poland) and stored 20˚C until DNA PCR amplification.

2.3. PCR amplification and sequencing

The partial 16S rRNA gene was amplified using the primers 8F: 5'-GTG CTG CAGAGA GTT TGA TCC TGG CTCAG-3' and 536R: 5'-CAC GGA TCC GTA TTA CCG CGG CTG CTG-3' [8].

2.4. RISA analysis

The bacterial ISR (intergenic spacer region) located between the small and large subunit of rDNA was amplified with primers 1 (5'-TGC GGC TGG ATC CCC TCC TT-3') and 2 (5'-CCG GGT TTC CCC ATT CGG-3'). 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, Winsconsin, USA), 1 U of *Taq* DNA polymerase (Invitrogen, Life Technologies), 5 mL of reaction buffer (500 mM KCl, Triton X-100, pH 8.5), 1.5 mM $MgCl_2$ and sterile

H_2O to a final volume of 50 mL. The standard PCR amplification was carried out using the following program: 95˚C for 10 min; 35 cycles of denaturation at 94˚C for 30s, annealing at 42˚C for 30s, extension at 72˚C for 1 min and single final elongation at 72˚C for 5 min. The quality of PCR products was confirmed by analyzing 5 mL of the product on 1.0% ($w.v^{-1}$) agarose gel stained with ethidium bromide. Amplified fragments contained ISR plus approximately 200 bp corresponding to flanking regions of genes coding for 16S and 23S rRNA. After successful DNA amplification, the PCR products were separated in 5% polyacrylamide gel (29:1 acrylamide: bisacrylamide). Electrophoresis was carried out at 60V for 100 min in 1×TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA; pH 8.0). Sizes of PCR products were estimated using 1 Kb molecular weight marker (Promega, USA). After appropriate time of electrophoresis, the gel stained with SYBR Gold (Invitrogen) was viewed with an ultraviolet transiluminator and recorded with a CCD camera (Gel Logic 200, Eastman Kodak Company, USA). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA). The sequences of 16S rDNA genes were compared with the GenBank database using the NCBI Blast program. Sequences were aligned using the ClustalW program [9]. Genetic relationships were determined by the neighbor-joining method with the MEGA2 program [10] using nucleotide sequences of the 16S rDNA gene.

3. Results and Discussions

3.1. Dynamics of microbial community

Numerous discrete RISA bands, resulting from differences between the intergenic spacer region lengths of different bacterial species, were apparent. Each band represented at a unique ribotype (i.e. RNA complement of a cell by analogy with phenotype or genotype). Three different band patterns regarding the operation time were observed. The results suggested that microbial compositions in the community was changed with respect to the cultivation time (Fig. 1).

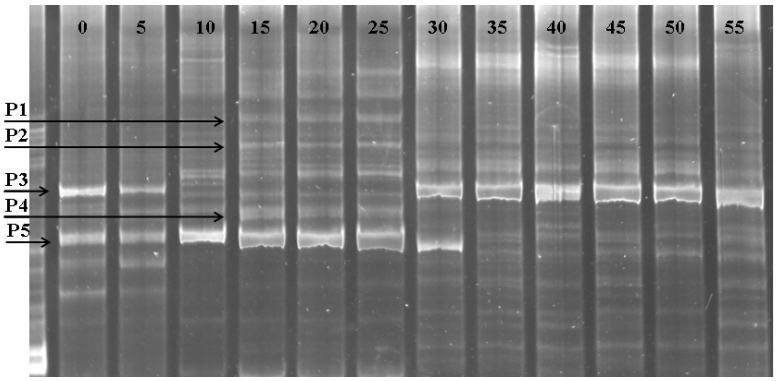

Fig. 1: RISA band patterns of the microbial samples collected from the reactor every 5 days

The original seed exhibited rich in different bacterial species, with some dominant, whose members were represented by the dominant bands. The band pattern was changed after 5 days cultivation. Major changes in the microbial structure were observed after 10–day cultivation, related to the maximal percentage content of PHA and reached 47.6% of cell dry weight according to previous study [11]. After 25-day cultivation, less RISA bands were observed. The RISA pattern was converted to a binary matrix, using presence-absence data and checked with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis with bootstrap confidence testing (1,000 resembling) for validation results, which interpreted values of distance between samples. The similarity among the isolates was estimated by UPGMA indices. The program could illustrate cluster of samples, which confirmed the trend of progressive community transition in the reactor (Fig. 2).

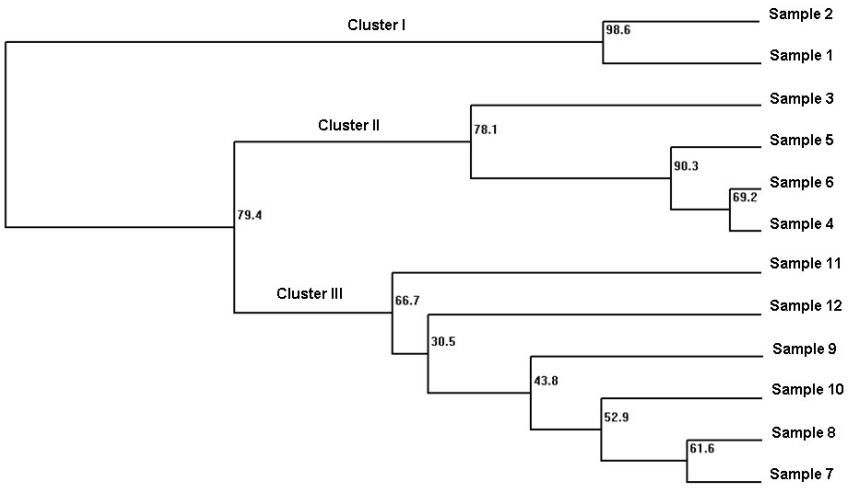

Fig. 2: Phylogenetic tree generated by the neighbor-joining method showing the phylogenetic relationships among bacteria isolated from biomass utilizing the glycerol

Result of phylogenetic analysis based on the 16S rDNA sequences from evolutionary distances by neighbor-joining was shown in Fig. 2. The closeness of bacteria community in each sample with respect to the cultivation time could be separated into two groups, including group I was samples in range of 0 – 5 incubation days (cluster I) whereas group II comprised of two clusters being cluster II and cluster III. The cluster II represented cultivation bacteria culture between 10^{th} – 25^{th} days. While, the cluster III was noticed in the last period of fermentation (between the 30^{th} and the 55^{th} days).

3.2. Identification of polyhydroxyalkanoate-producing bacteria

Dominant RISA bands, P1, P2, P3, P4 and P5 (Fig. 1) were cut and performed 16S rRNA gene sequencing. The sequences showed similarities greater than 91% with sequences obtained from the NCBI GenBank database. Two sequences were closely related to *Bacillus* sp. (P4 and P5), *Azoarcus* sp. (P1), *Flavobacterium columnare* (P2) and *Thauere* sp. (P3). The fingerprints produced by RISA had been shown to be highly reproducible and sufficient to classify both closely and distantly related species and subspecies. *Bacillus* strains were vividly appeared during 0-30 day cultivation but high intensity during 10 – 30 day cultivation, in which the highest percentage content of PHA was observed, *Bacillus* sp. was previously reported its capability of PHB accumulation [12, 13, 14]. *Thaurea* (P3), *Azoarcus* sp. (P1) and *Flavobacterium columnare* (P2) were not directly associated with the PHB accumulation, but were cultivable in the experimental conditions [15].

4. Conclusion

The dynamics of mixed culture occurred during PHA accumulation in the waste glycerol containing media was successfully examined by RISA method. The result of RISA targeting the 16S rRNA gene indicated a significant shift in the microbial community with operation times. The applied approach made possible the isolation and molecular identification of 5 different strains engaged directly or indirectly in PHA production. Knowledge about the species selected in the reactor could be utilized in the construction of defined mixed communities, and used for the conversion of complex feedstock into biopolymers in the future.

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