J. Microbiol. Biotechnol. (2012), **22**(3), 371–377
http://dx.doi.org/10.4014/jmb.1106.06039
First published online December 14, 2011
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Mcl-PHAs Produced by *Pseudomonas* sp. Gl01 Using Fed-Batch Cultivation with Waste Rapeseed Oil as Carbon Source

Możejko, Justyna[1]*, Andreas Wilke[2], Grzegorz Przybyłek[1], and Slawomir Ciesielski[1]

[1]*Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Sloneczna 45G, 10-718 Olsztyn, Poland*
[2]*Department of Mechanical and Process Engineering, University of Applied Sciences Offenburg, Badstr. 24, 77652 Offenburg, Germany*

Received: June 20, 2011 / Revised: October 28, 2011 / Accepted: November 5, 2011

The present study describes medium-chain-length polyhydroxyalkanoates (mcl-PHAs) production by the *Pseudomonas* Gl01 strain isolated from mixed microbial communities utilized for PHAs synthesis. A two-step fed-batch fermentation was conducted with glucose and waste rapeseed oil as the main carbon source for obtaining cell growth and mcl-PHAs accumulation, respectively. The results show that the *Pseudomonas* Gl01 strain is capable of growing and accumulating mcl-PHAs using a waste oily carbon source. The biomass value reached 3.0 g/l of CDW with 20% of PHAs content within 48 h of cultivation. The polymer was purified from lyophilized cells and analyzed by gas chromatography (GC). The results revealed that the monomeric composition of the obtained polyesters depended on the available substrate. When glucose was used in the growth phase, 3-hydroxyundecanoate and 3-hydroxydodecanoate were found in the polymer composition, whereas in the PHAs-accumulating stage, the *Pseudomonas* Gl01 strain synthesized mcl-PHAs consisting mainly of 3-hydroxyoctanoate and 3-hydroxydecanoate. The transcriptional analysis using reverse-transcription real-time PCR reaction revealed that the *phaC1* gene could be transcribed simultaneously to the *phaZ* gene.

Keywords: Biopolymers, mcl-polyhydroxyalkanoates, *Pseudomonas* sp., real-time PCR, reverse transcription, waste oil

Polyhydroxyalkanoates (PHAs) are intracellular carbon and energy storage compounds accumulated by a wide range of bacteria that have properties similar to petrochemical materials [1]. The biosynthesis of these polymers is prompted by stress conditions, such as the shortage of an essential nutrient: nitrogen, oxygen, phosphate, magnesium, or potassium [26]. These bacterial biopolymers have been divided into two main groups: short-chain-length PHAs (scl-PHAs) with 3 to 5 carbon atoms per repeat unit, and medium-chain-length PHAs (mcl-PHAs) consisting of 6 to 14 carbon atoms [18]. Scl-PHAs have been studied in depth and have been industrially produced. The physical properties of mcl-PHAs are quite different in comparison with scl-PHAs. They have a low degree of crystallinity with a low melting temperature, low tensile strength, and high elongation to break, and can be modified by chemical reactions. Moreover, they are biodegradable, non-toxic, and biocompatible, and therefore can be used for a variety of applications including agriculture, pharmacy, and medicine that cannot be obtained with scl-PHAs.

However, the introduction of mcl-PHAs to the worldwide market is currently limited, mainly due to the high production cost compared with synthetic plastics. Therefore, it would be useful from the economical point of view that non-toxic and especially cheap carbon sources are taken into account. It is known that a suitable carbon source determines both PHAs content and their monomeric composition, which has an important influence on the final properties of the polymer. Furthermore, the fed-batch fermentation process seems to be a better approach to obtain useful amounts of PHAs because a growth phase is necessary to achieve high-cell-density cultures before starting the subsequent PHAs accumulation phase [17, 25]. The combination of two types of substrate could affect the high synthesis of mcl-PHAs by microorganisms. This is dependent on a substrate feeding strategy to control the essential nutrient concentration [29]. Moreover, the use of waste oils as a carbon source in a nutrient-limited second stage for mcl-PHAs accumulation may be helpful in the creation of a more economical fermentation process.

In the current study, two-step fed-batch fermentation was applied to the production of mcl-PHAs using glucose

**Corresponding author*
Phone: +48 89 5234144; Fax: +48 89 5234131;
E-mail: justyna.mozejko@uwm.edu.pl

and waste rapeseed oil as carbon sources. The cell growth, PHAs production, monomers composition, and expression of key PHA genes were examined during the fermentation process.

Materials and Methods

Strain and Culture Medium

The *Pseudomonas* sp. Gl01 strain isolated from mixed microbial communities utilized for PHAs synthesis has been identified as a new strain producing medium-chain-length polyhydroxyalkanoates (mcl-PHAs) [5, 6]. The strain from long-term storage tubes (in nutrient broth containing 15% glycerol) were grown in Luria–Bertani broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% NaCl] at 30°C with 220 rpm shaking for 24 h before inoculation into the bioreactor. The culture medium contained the following components per liter: 3.5 g $Na_2HPO_4{\cdot}12H_2O$, 5.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4{\cdot}7H_2O$, 10.0 g glucose (according to the feeding strategy), and 2.5 ml of trace element solution. Each liter of trace element solution contained, 0.3 g of H_3BO_3, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.03 g of $MnCl_2{\cdot}4H_2O$, 0.03 g of $NaMoO_4{\cdot}2H_2O$, 0.028 g of $NiSO_4{\cdot}7H_2O$, and 0.01 g of $CuSO_4{\cdot}5H_2O$ dissolved in 0.5 N HCl. After the consumption of glucose, waste frying rapeseed oil was used as a substrate in the production media. $MgSO_4{\cdot}7H_2O$ solution was sterilized and added separately.

Fermentation Conditions

Before the fermentation experiment, flask cultures were conducted in 250 ml Erlenmeyer flasks containing 100 ml of a mineral medium (described above) plus from 5 g/l to 40 g/l of glucose. The flasks were incubated for 24 h at 30°C in a rotary shaker at 220 rpm. The cells were harvested at several time intervals to determine the optical density value and cell dry weight. Maximum specific growth rate was determined as an equation for the linear trend line for the exponential phase. A linearization had been made where the slope of the linear regression gives the maximum specific growth rate μ_{max} in 1/h.

In order to determine the effect of waste rapeseed oil concentration on PHAs content, flasks experiment were carried out. The 100 ml of mineral media (described above) were supplemented with varying concentrations of waste rapeseed oil from 5 to 100 ml/l. Cells were cultivated for 24 h at 30°C in a rotary shaker at 220 rpm.

The fermentation study was carried out at 30°C with a 5 l working volume in a fermentor (BioFlo 110; New Brunswick Scientific) equipped with a pH controller. The temperature was maintained by a thermostatic jacket. The production of mcl-PHAs was performed by a two-stage cultivation. First, the cultivation was conducted in the culture medium containing glucose (10 g/l) for the first 4 h. In the second step, when this substrate was depleted, waste rapeseed oil was added two times into the broth as a carbon source, first at 4 h (15 ml/l) and then at 22 h (15 ml/l).

Parameters like the dissolved oxygen, pH value, and ammonium concentration were controlled during the experiment. pH was maintained at 7.0 through the modulated addition of an aqueous solution of 1 N NaOH and 1 N HCl. The dissolved oxygen was monitored during the whole cycle with an O_2-electrode (InPro 6800; Mettler Toledo GmbH, Switzerland) and 80% air saturation maintained by adjusting the agitation rate from 300 rpm to 1,000 rpm automatically. The total fermentation time was 48 h.

Analytical Procedures

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}). During the study, samples of culture broth were periodically removed for analysis. The cell dry weight of the lyophilized biomass was determined by centrifugation of 100 ml culture samples at 11,200 ×*g* for 10 min and washing twice with hexane and once with distilled water to remove hydrophobic rapeseed oil. The concentration of ammonium in the medium was determined by the modified titrimetric method with the preliminary distillation step described in Standard Methods [2]; in this case, 0.01 N HCl was applied instead of 0.02 N H_2SO_4. Nitrogen was expressed as ammonium concentration. The PHAs content was defined as the percentage of the ratio of PHAs concentration to total cell concentration (g/l).

PHAs Extraction

Quantitative and qualitative analyses of the obtained polymer were carried out. Firstly, quantitative determination of PHAs was done gravimetrically. PHA polymers were extracted by shaking the freeze-dried cells in chloroform at 50°C for 3 h. The mixture was filtered through No. 1 Whatman filter paper by simple filtration. PHAs dissolved in a small volume of chloroform were precipitated with 4 volumes of 70% solution of chilled methanol and then allowed to evaporate for at least 2 days at room temperature.

Secondly, the PHAs composition was determined by a gas chromatographer (GC Varian CP-3800) equipped with a capillary column Varian VF-5 ms (30 m × 0.25 mm) with a film thickness of 0.25 μm (Varian, Lake Forest, USA). At the beginning, 1 mg of extracted polymer was transferred into a 10 ml glass tube. Then, PHAs were suspended in 2 ml of acidified methanol containing 3% (v/v) H_2SO_4 and an equal volume of chloroform. Next, the tube was placed in the oven for 20 h at 100°C temperature to perform esterification. Afterwards, 1 ml of distillated water was added and the mixture vortexed. After phase separation, the upper phase was discarded and the lower phase was dried by sodium sulfate and used for GC analysis [3]. The obtained methyl esters were determined according to the method described by Furrer *et al.* [9]. For quantification purposes by FID, the known amounts of pure 3-hydroxyhexanoic (HHx), -octanoic (HO), -nonanoic (HN), -decanoic (HD), -undecanoic (HUD), and -dodecanoic (HDD) acids were derivatized and measured in the same way as the analyzed samples, and measured to calculate their response factors. 3-Hydroxy acids (purity 98%) were obtained from Larodan Fine Chemicals (Sweden).

Molecular Biology Procedures

RNA*Later* (Sigma) stabilized cells were utilized for total RNA isolation using a commercial RNA extraction kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. Prior to reverse transcription, DNA-free total RNA was obtained by sample incubation at 37°C for 30 min with RQ1 RNase-Free DNase (Promega, USA). The concentration of RNA was measured fluorometrically using the Quant-iT RNA Assay Kit (Invitrogen, Carlsbad, NM, USA).

Total RNA was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Canada). The cDNA reaction for each sample contained 1 μg of total RNA and 0.2 μg/μl of random hexamer primer. Samples without reverse transcriptase (RT) were used as the negative control. The synthesized

Table 1. Real-time PCR primers used in the study.

PCR primer	Nucleotide sequence	Amplicon	Reference
GlC1 179R	5'-aag gtc aac gcc ctg acc ctg ctg gt-3' 5'-ggt gtt gtc gtt gtt cca gta gag gat gtc-3'	*phaC1*	[5, 28]
RT-phaZ-F RT-phaZ-R	5'-atc ctc cac agc acc ttg ggc ttg-3' 5'-tgg aag tca tcg cct tcg atg tcc-3'	*phaZ*	This study
341F 515R	5'-cct acg gga ggc agc ag-3' 5'-aat ccg cgg ctg gca-3'	16S rRNA	[21]

first-strand cDNA was suspended in sterile water and stored at −20°C until used.

Real-time PCR reaction was performed using SYBR Green technology in an ABI 7500 real-time PCR system (Applied Biosystems, USA) in MicroAmp Optical 96-well reaction plates (Applied Biosystems, USA). Primer pairs used for real-time amplification of *phaC1, phaZ*, and 16S rRNA genes are given in Table 1. The reactions were run using the universal thermal cycling parameters as follows: 95°C for 3 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. After performing a run, a dissociation stage was included.

Gene expression was estimated using a modification of the $2^{-\Delta\Delta Ct}$ method [20]. Data obtained from the assay were used to compute the expression ratio of the *phaC1* and *phaZ* genes relative to 16S rRNA as the endogenous control. The ΔCt value of the control sample (time 0) was used as the calibrator. Data were reported as relative units.

RESULTS

Flask Cultures

A shake flask experiment was conducted to determine the effect of the glucose concentration on cell growth. In order to find a suitable value of glucose, the culture medium was supplemented with varying concentrations of this sugar, from 5 g/l to 40 g/l. Using 10 g/l, the maximum specific growth rate (0.52 h^{-1}) and maximum cell concentration (2.3 g/l) were obtained (Fig. 2). However, further increase of glucose to 40 g/l started to inhibit the growth of *Pseudomonas* Gl01 (Fig. 1). Therefore, during the first step of the fed-batch cultivation, the appropriate amount of glucose was set at 10 g/l. The yield of biomass to total substrate was estimated at a level of 0.36 g CDW/g glucose.

A shake flask experiment was also done with different waste rapeseed oil concentrations, varied from 5 ml/l to 100 ml/l. With increasing of rapeseed oil concentration up to the level of 20 ml/l, the amount of PHAs extracted from biomass also increased. When 20 ml/l of waste rapeseed oil was used, the PHAs content and PHAs productivity reached 11% of CDW and 9.63 mg/l·h, respectively. However, the further increase of rapeseed oil to 100 ml/l inhibited PHAs production by *Pseudomonas* Gl01, as shown in Fig. 3.

Two-Stage Fed-Batch Culture of *Pseudomonas* Gl01 Strain

The medium-chain-length PHAs production by the *Pseudomonas* Gl01 strain was evaluated in a two-step culture. Cells were grown initially in a mineral medium containing glucose as the only carbon source in order to speed up the cell growth (first step) and subsequently cultivated with waste rapeseed oil in the following step. During the second step, the Gl01 strain accumulated mcl-PHAs under the nitrogen-limited conditions. The samples were taken at time intervals to carry out the analysis. Fig. 4 shows the ammonium concentration, biomass value, and the PHAs content related to the cellular dry weight. During the whole fermentation process, the dissolved oxygen was maintained at a level of 80%. The concentration of ammonium

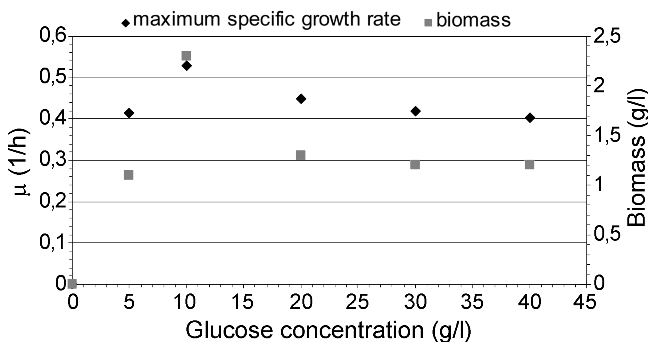

Fig. 1. Maximum specific growth rate (μ_{max}) and biomass value of the samples fed with different glucose concentrations.

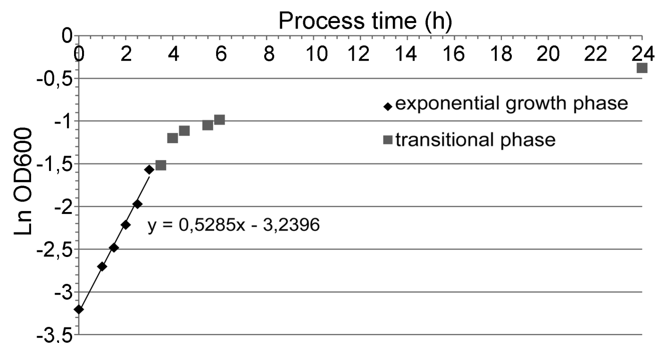

Fig. 2. Determination of μ_{max} using 10 g/l of glucose as a substrate.

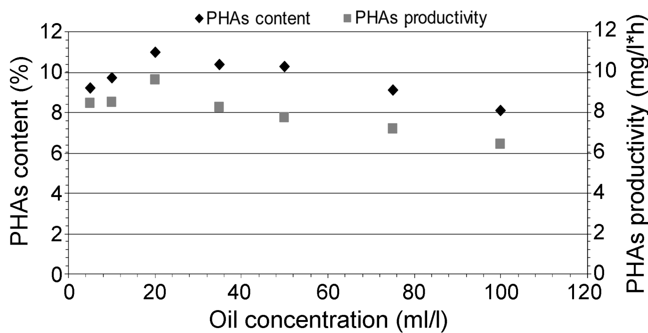

Fig. 3. PHAs content and PHAs productivity obtained by cultivation with different waste rapeseed oil concentrations.

was estimated during the two stages of fermentation. The obtained data show that the ammonium value decreased from 212.8 mg NH_4/l to 14 mg NH_4/l within the first stage of the cultivation and was exhausted to 0.0 mg/l at 17 h of the experiment, being no longer available for the bacterial strain. Under the experimental conditions and under nitrogen limitation, the synthesized PHAs value started to increase. During the growth phase, the PHAs content amounted to 3% of the cell dry weight, while in the second step, when the bacterial cells were fed by waste rapeseed oil, the PHAs concentration increased from 3% to 20% of the cell dry weight.

As shown in Fig. 4, the biomass value increased to 1.7 g/l when glucose was used as the sole carbon source. After the addition of waste oil (at 4 h), the biomass value increased rapidly and reached a maximum amount of 3.2 g/l at 17 h, and then decreased to 2.5 g/l at 22 h. The

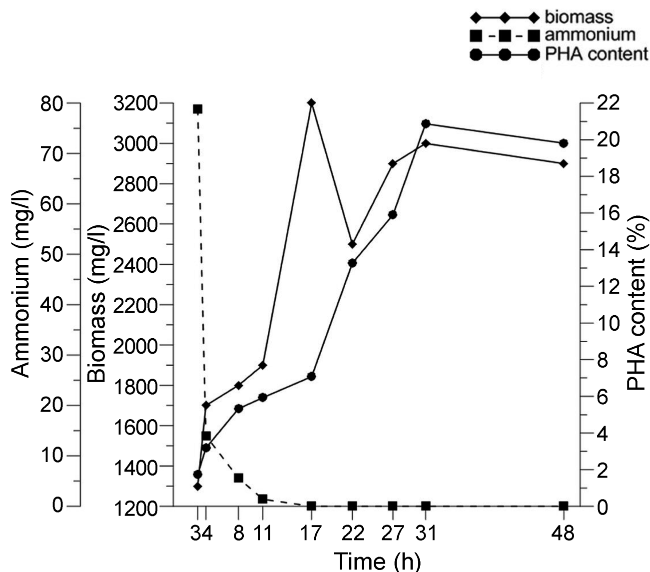

Fig. 4. Cell concentration, mcl-PHA content, and ammonium value in *Pseudomonas* sp. strain Gl01 during two-step fed-batch cultivation.

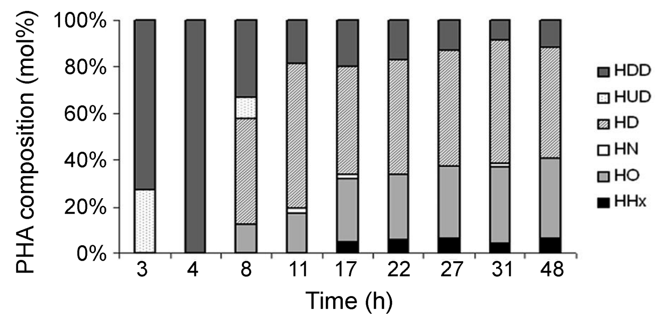

Fig. 5. The monomeric composition of mcl-PHAs (mol%) obtained from *Pseudomonas* strain Gl01 synthesized during fermentation with glucose and waste rapeseed oil, determined by gas chromatography.

HHx, 3-hydroxyhexanoic acid; HO, 3-hydroxyoctanoic acid; HN, 3-hydroxynonanoic acid; HD, 3-hydroxydecanoic acid; HUD, 3-hydroxyundecanoic acid; HDD, 3-hydroxydodecanoic acid.

next portion of the oily carbon source resulted in a rise in CDW to 3.0 g/l.

PHAs Characterization

The purified mcl-PHAs synthesized by the *Pseudomonas* G01 strain were analyzed by gas chromatography (GC) to determine their monomeric composition. The molar fractions of all the detectable constituents in the PHA repeat units composition are shown in Fig. 5. During the growth phase, 3-hydroxydodecanoic acid was detected as the main constituent. When waste rapeseed oil was supplied to accumulate PHAs (second step), the composition of polyesters was found to consist mainly of 3-hydroxyoctanoate and 3-hydroxydecanoate, lesser amounts of 3-hydroxydodecanoate, and trace amounts of both 3-hydroxyhexanoate and 3-hydroxynonanoate. As may be observed from the obtained data, 3-hydroxyundecanoate was not detected in the composition of mcl-PHAs.

Real-Time PCR Assays

In order to quantify the expression levels of the *phaC1* gene, which played a significant role in mcl-PHAs production, and the *phaZ* gene involved in PHAs degradation, reverse-transcriptase real-time PCR was performed. The 16S rRNA gene was used as a reference gene for the relative quantification of the *phaC1/phaZ* transcripts. As may be observed from the reported data (Fig. 6), a higher expression of both analyzed genes was estimated in the second step of the cultivation. When glucose was supplied as the sole substrate (step 1), no change was observed in either genes. As shown in Fig. 6, the expression of the *phaC1* and *phaZ* genes appeared to be induced in the second step of the cultivation, during the PHAs accumulation phase. As a result, *phaC1* gene expression increased at 10 h (14-fold), and then decreased 5.2-fold at the end of the PHAs accumulation phase, whereas, the highest expression level

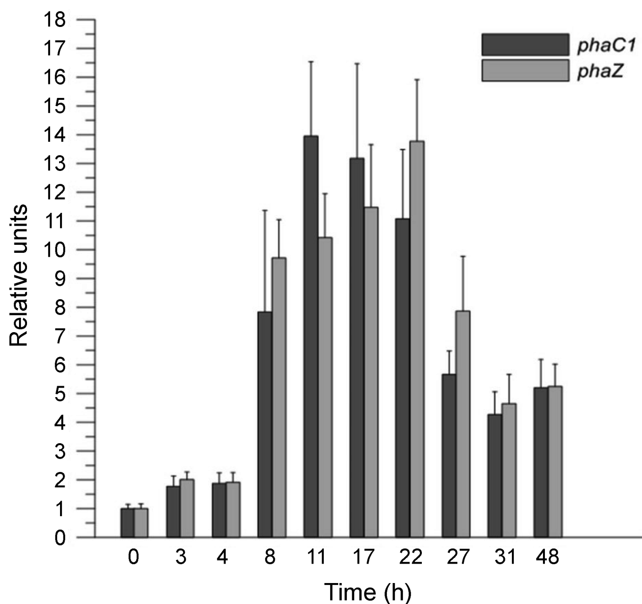

Fig. 6. Relative quantification by RT real-time PCR of *phaC1* and *phaZ* gene expression in *Pseudomonas* sp. strain Gl01. Mean values are calculated from triplicate samples.

of the *phaZ* gene was reached at 22 h (13.8-fold) with a decrease in transcripts at the end of the fermentation.

DISCUSSION

In this report, PHAs production and transcriptional analysis of the PHA synthase (*phaC1*) and PHA depolymerase (*phaZ*) genes were performed using the *Pseudomonas* Gl01 strain. The fed-batch culture was divided into two parts: a cell growth stage and a PHAs accumulation stage. Two different carbon sources were used in each stage. Glucose was used as the sole carbon source in the growth phase, while the PHAs accumulation stage was started with a change of the carbon source to waste rapeseed oil. The present study demonstrates that mcl-PHAs could be produced using inexpensive waste frying rapeseed oil, and in the process its usage may reduce high PHAs production costs.

Two-stage fed-batch fermentation is often performed to achieve both high-cell-density and a high concentration of a desirable product. The biomass value and mcl-PHAs concentration increased up to 31 h. In the 48 h of the fermentation process, 20% of PHAs was produced by the *Pseudomonas* Gl01 strain. Mcl-PHAs started to be synthesized in significant amounts when the cell concentration reached 3.0 g/l. The polymer started to accumulate when glucose was totally consumed. The production of mcl-PHAs in two-step and single-step fed-batch cultivations has been described for other *Pseudomonas* strains. Kim *et al*. [15] studied PHAs production with *Pseudomonas putida* by combined use of glucose and octanoate. They reached about 40% of PHAs, whereas cells were grown only on glucose and up to 65.5% of PHAs after supplying octanoate in the first cell-growing stage. *Pseudomonas putida* KT2440 cultivated in single-stage fed-batch fermentation by co-feeding nonanoic acid and glucose accumulated 56% of PHA [32]. Moreover, using also a single stage but with nonanoate as the sole carbon source, 67% of PHAs could be obtained [30].

Furthermore, when a carbon source was changed from glucose to waste frying rapeseed oil, cell growth was minimal for the next 7 h and increased rapidly to 3.2 g/l. This may indicate that bacteria needed an adaptation period for utilizing the second carbon source. It seems likely that the addition of waste rapeseed oil during the cell growth stage would allow to utilize the frying oily carbon source in the second stage more efficiently. Another reason could be the incorporation and accumulation of PHAs in existing cells. No division of the cells occurs then, although the CDW increases. Kim *et al*. [15] also suggested that the adaptation time for utilizing the new carbon source can be shortened. During a two-step fed-batch cultivation designed with glucose and octanoate as the main substrate for cell growth and PHAs accumulation, respectively, they proved that by supplying octanoic acid in the glucose-utilizing cell-growing step, the mcl-PHAs production was enhanced.

Furthermore, based on the previous data, PHAs can be synthesized under unbalanced nutrient conditions, such as nitrogen or phosphate limitation [13, 15, 16, 19, 23]. As can be seen in Fig. 4, in the present study, mcl-PHAs accumulation by the *Pseudomonas* sp. Gl01 was stimulated under nitrogen limitation in the culture broth during the second step of fermentation. Polyester content increased rapidly after 17 h, when nitrogen reached 0.0 mg/l. Other results also suggested that PHAs synthesis occurred mainly during the nutrient-depletion stage [7, 15, 19]. In this study, the average ammonium consumption rate was 0.0497 g/l·h. It is a higher value in comparison with cultivation on crude canola oil as a substrate in the PHAs-accumulating stage (0.0217 g/l·h) [21]. The influence of nitrogen limitation on PHAs concentration was previously tested in *Pseudomonas* strain Gl01 using three different carbon sources. The incremental increase in response to nitrogen starvation was observed only when oleic acid was applied as a substrate [5]. However, some authors reported that different *Pseudomonas* strains growing on octanoate do not need nutrient limitation for effective mcl-PHAs synthesis [4, 8, 14, 22]. The presented data imply that the correlation between biopolymer accumulation and nutrient limitation could depend on the carbon source used for mcl-PHAs synthesis, the cultivation conditions, or the bacterial host, or all of these factors.

Data in the literature show that the monomeric composition of PHAs depends on the substrate supplied [11]. It was

speculated that a bacterial strain growing on glucose produces an even carbon number of repeat units, especially 3-hydroxyoctanoate and 3-hydroxydecanoate [31]. However, our results demonstrated that the polymer accumulated during the cell growth phase was found to be mainly 3-hydroxydodecanoate. Haba *et al*. [10] reported that *Pseudomonas* sp. synthesized PHAs consisting of 8-carbon to 10-carbon atoms cultivating on fatty acids containing from 13- to 18-carbon atoms. The obtained results proved that the analyzed strain showed a tendency to accumulate large amounts of 3-HO and 3-HD growing on waste rapeseed oil. Moreover, the same monomers were detected in a composition of mcl-PHAs produced by *P. putida* IPT046, and *P. aeruginosa* PT169 and IPT171 in a two-step experiment, when different plant oils were used as the sole carbon source in the PHAs-accumulating step [27]. The observed differences in the repeat-units structure of the mcl-PHAs produced from glucose and waste rapeseed oil reflected on the different metabolic pathways of PHA biosynthesis.

The transcriptional analysis revealed that two analyzed genes (*phaC1* and *phaZ*) were up-regulated during growth. These results are in accordance with the data reported for *Pseudomonas aeruginosa* [12]. Moreover, in this work, it was observed that the polymer content did not decrease, even during the highest *phaZ* expression at 22 h of the experiment. This could suggest that PHA synthesis and degradation followed simultaneously. These results are consistent with the data of Ren *et al*. [24] that demonstrated parallel synthesis and degradation of PHAs by measurement of PhaC and PhaZ activity in crude cell extracts of *Pseudomonas putida* U. As can be seen in Fig. 4 and 6, the ammonium value could also influence on the expression of the analyzed genes. From the timepoint when nitrogen was exhausted, the expression rate of both analyzed genes increased.

In conclusion, this is the first report of a *Pseudomonas* strain able to use waste rapeseed oil for PHAs synthesis during two-step fed-batch cultivation. The obtained results confirm that *Pseudomonas* strain Gl01 is capable of utilizing waste rapeseed oil as a carbon source. This strategy of using waste oils could reduce the cost of production of this polyester. The use of glucose in the first step allows obtaining the cell growth and additionally PHAs accumulation on quite a high level.

Acknowledgments

This study was financially supported by the Ministry of Science and Higher Education, Project No. N N523 610839 and by the European Union within the European Social Fund. The authors thank Mr. Mariusz Dubicki for technical assistance and for helping with the analysis of the obtained results.

References

1. Anderson, A. J. and E. A. Dawes. 1990. Occurence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54:** 450−472.
2. American Public Health Association. 1992. *Standard Methods for the Examination of Water and Wastewater*, 20th Ed. American Public Health Association, Washington.
3. Braunegg, G., B. Sonnleitner, and R. M. Lafferty. 1978. A rapid gas chromatographic method for the determination of poly-b-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol. Biotechnol*. **6:** 29−37.
4. Carnicero, D., M. Fernández-Valverde, L. M. Cañedo, C. Schleissner, and J. M. Luengo. 1997. Octanoic acid uptake in *Pseudomonas putida* U. *FEMS Microbiol. Lett.* **149:** 51−58.
5. Ciesielski, S., J. Możejko, and G. Przybyłek. 2010. The influence of nitrogen limitation on mcl-PHA synthesis by two newly isolated strains of *Pseudomonas* sp. *J. Ind. Microbiol. Biotechnol.* **37:** 511−520.
6. Ciesielski, S., T. Pokoj, and E. Klimiuk. 2010. Cultivation-dependent and −independent characterization of microbial community producing polyhydroxyalkanoates from raw-glycerol. *J. Microbiol. Biotechnol*. **20:** 853−861.
7. Diniz, S. C., M. K. Taciro, J. G. C. Gomez, and J. G. da Cruz Pradella. 2004. High-cell-density cultivation of *Pseudomonas putida* IPT 046 and medium-chain-length polyhydroxyalkanoates production from sugarcane carbohydrates. *Appl. Biochem. Biotechnol.* **119:** 51−69.
8. Durner, R., M. Zinn, B. Witholt, and T. Egli. 2001. Accumulation of poly[(*R*)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth in batch and chemostat culture with different carbon sources. *Biotechnol. Bioeng*. **72:** 278−288.
9. Furrer, P., R. Hany, D. Rentsch, A. Grubelnik, K. Ruth, S. Panke, and M. Zinn. 2007. Quantitative analysis of bacterial medium-chain-length poly([*R*]-3-hydroxyalkanoates) by gas chromatography. *J. Chromatogr. A* **1143:** 199−206.
10. Haba, E., J. Vidal-Mas, M. Bassas, M. J. Espuny, J. Llorens, and A. Manresa. 2007. Poly 3-(hydroxyalkanoates) produced from oily substrates by *Pseudomonas aeruginosa* 47T2 (NCBIM 40044): Effect of nutrients and incubation temperature on polymer composition. *Biochem. Eng. J.* **35:** 99−106.
11. Hartmann, R., R. Hany, E. Pletscher, A. Ritter, B. Witholt, and M. Zinn. 2006. Tailor-made olefinic medium-chain-length poly[(*R*)-3-hydroxyalkanoates] by *Pseudomonas putida* GPo1: Batch versus chemostat production. *Biotechnol. Bioeng*. **93:** 737−746.
12. Hoffman, N. and B. H. A. Rehm. 2004. Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **237:** 1−7.
13. Huijberts, G. N. M. and G. Eggink. 1996. Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures. *Appl. Microbiol. Biotechnol*. **46:** 233−239.
14. Huisman, G. W., E. Wonink, G. Koning, H. Preusting, and B. Witholt. 1992. Synthesis of poly(3-hydroxyalkanoates) by

mutant and recombinant *Pseudomonas* strains. *Appl. Microbiol. Biotechnol*. **38:** 1−5.

15. Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shin, and Y. H. Park. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. *Enzyme Microb. Technol*. **20:** 500−505.
16. Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt. 1988. Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* **54:** 2924−2932.
17. Lee, J., S. Y. Lee, S. Park, and A. P. Middelberg. 1999. Control of fed-batch fermentations. *Biotechnol. Adv.* **17:** 29−48.
18. Lee, S. Y. 1996. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* **49:** 1−14.
19. Lee, S. Y., H. H. Wong, J. Choi, S. H. Lee, S. C. Lee, and C. S. Han. 2000. Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of *Pseudomonas putida* under phosphorus limitation. *Biotechnol. Bioeng.* **68:** 466−470.
20. Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25:** 402−408.
21. López-Cuellar, M. R., J. Alba-Flores, J. N. Gracida-Rodríguez, and F. Pérez-Guevara. 2011. Production of polyhydroxyalkanoates (PHAs) with canola oil as carbon source. *Int. J. Biol. Macromol.* **48:** 74−80.
22. Ramsay, B. A., I. Saracovan, J. A. Ramsay, and R. H. Marchessault. 1991. Continuous production of long-side-chain poly-β-hydroxyalkanoates by *Pseudomonas oleovorans*. *Appl. Environ. Microbiol*. **57:** 625−629.
23. Ramsay, B. A., I. Saracovan, J. A. Ramsay, and R. Marchessault. 1992. Effect of nitrogen limitation on long-side-chain poly-beta-hydroxyalkanoate synthesis by *Pseudomonas resinovorans*. *Appl. Environ. Microbiol*. **58:** 744−746.
24. Ren, Q., G. de Roo, B. Witholt, M. Zinn, and L. Thöny-Meyer. 2010. Influence of growth stage on activities of polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida* U. *BMC Microbiol*. **10:** 254−262.
25. Riesenberg, D. and R. Guthke. 1999. High-cell-density cultivation of microorganisms. *Appl. Microbiol. Biotechnol.* **51:** 422−430.
26. Schlegel, H. G., G. Gottschalk, and R. von Bartha. 1961. Formation and utilization of poly-β-hydroxybutyric acid by knallgas bacteria (Hydrogenomonas). *Nature* **191:** 463−465.
27. Silva-Queiroza, S. R., L. F. Silva, J. G. C. Pradella, E. M. Pereira, and J. G. C. Gomez. 2009. PHA_{MCL} biosynthesis systems in *Pseudomonas aeruginosa* and *Pseudomonas putida* strains show differences on monomer specificities. *J. Biotechnol*. **143:** 111−118.
28. Solaiman, D. K., R. D. Ashby, and T. A. Foglia. 2000. Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Appl. Microbiol. Biotechnol.* **53:** 690−694.
29. Sun, Z., J. A. Ramsay, M. Guay, and B. A. Ramsay. 2007. Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. *Appl. Microbiol. Biotechnol*. **71:** 423−431.
30. Sun, Z., J. A. Ramsay, M. Guay, and B. A. Ramsay. 2007. Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440. *Appl. Microbiol. Biotechnol.* **74:** 69−77.
31. Sun, Z., J. A. Ramsay, M. Guay, and B. A. Ramsay. 2007. Increasing the yield of mcl-PHA from nonanoic acid by co-feeding glucose during the PHA accumulation stage in two-stage fed-batch fermentations of *Pseudomonas putida* KT2440. *J. Biotechnol*. **132:** 280−282.
32. Sun, Z., J. A. Ramsay, M. Guay, and B. A. Ramsay. 2009. Enhanced yield of medium-chain-length polyhydroxyalkanoates from nonanoic acid by co-feeding glucose in carbon-limited, fed-batch culture. *J. Biotechnol*. **143:** 262−267.