Asia Pacific Journal of Molecular Biology and Biotechnology, 2008
Vol. 16 (1) : 11-15

RESEARCH NOTE

Evaluation of nitrogen sources for growth and production of medium-chain-length poly-(3-hydroxyalkanoates) from palm kernel oil by *Pseudomonas putida* PGA1

Annuar, M.S.M[1,*], Irene K.P.Tan[1] and K.B. Ramachandran[2]

[1]*Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2]*Department of Biotechnology, Indian Institute of Technology (IIT) Madras, Chennai-600036, India*

Received 5 April 2007 / Accepted 26 October 2007

Abstract. Selected organic and inorganic nitrogen sources were evaluated for growth of *Pseudomonas putida* PGA1 and its production of medium-chain-length poly-(3-hydroxyalkanoates) (PHA_{MCL}). The effect of these nitrogen sources on the cells growth, PHA_{MCL} yield, monomer composition and molecular weight when this bacterium was cultivated on saponified palm kernel oil (SPKO) as the major carbon source was investigated. It was found that bacto-peptone gave significantly higher yield of residual biomass (PHA-free biomass) and PHA_{MCL} as compared to ammonium salt, urea, yeast extract and beef extract. No significant difference in the monomer composition of the PHA_{MCL} produced was observed with the different nitrogen sources. All the PHA_{MCL} produced had high molecular weight, with the weight average (M_w) ranging from 90,000 to 127,000 and poly-dispersities (M_w/M_n) ranging from 1.7 to 1.9.

Keywords. monomer composition; molecular weight, nitrogen sources, PHA_{MCL} yield

Biodegradable polyhydroxyalkanoates (PHA) is an attractive candidate as a substitute for certain applications in place of petrochemical derived plastics. In spite of its potentials, PHA has yet to make a market impact because of its high price compared to synthetic plastics. One way to partially offset this price difference is by increasing the biomass and PHA accumulation in bacterial cells grown on inexpensive substrates.

In most microorganisms, intracellular PHA is accumulated as a response to nutrient(s) limitation such as nitrogen, phosphorus, and oxygen. Nitrogen in the form of ammonium salt is widely used for the growth and production of PHA on a variety of carbon sources by microorganisms. On the other hand, complex nitrogen source such as yeast extract, peptone and beef extract have been shown to improve short-chain-length PHA production (poly-3-hydroxybutyrate; PHB) in bacterial fermentation (Bormann *et al.*, 1998). However, to date no studies have been reported on the effect of different nitrogen sources on cell growth, yield, monomer composition and molecular weight of medium-chain-length PHA (PHA_{MCL}) produced by a *Pseudomonas sp.*

The growth and PHA_{MCL} production by *Pseudomonas putida* PGA1 on saponified palm kernel oil (SPKO) as sole carbon and energy source with ammonium as the limiting nutrient in a defined medium have been described in the previous studies (Annuar *et al.*, 2007). They reported the cultivation conditions that were favorable for a high yield PHA_{MCL} production and its process kinetics in an automated batch and fed-batch bioreactor. Kinetics of ammonium uptake by *P. putida* PGA1 and its relation to the growth of the organism when cultivated on SPKO in a defined medium have also been reported (Annuar *et al.*, 2006).

With the aim to improve biomass and PHA_{MCL} production from SPKO by *P. putida* PGA1, alternative nitrogen sources to ammonium were evaluated for the fermentation process. It is also to investigate whether the monomer composition of the PHA_{MCL} produced and its molecular weight are affected

Abbreviations. PHA, poly(3-hydroxyalkanoates); PHA_{MCL}, medium-chain-length poly(3-hydroxyalkanoates); PHB, poly(3-hydroxybutyrate); SPKO, saponified palm kernel oil; M_n, molecular number; M_w, molecular weight; N, residual nitrogen concentration (g/L); **P, PHA_{MCL}** concentration (g/L); **R, residual cell concentration (g/L); X, total biomass concentration** (g/L); $Y_{R/N}$, yield of residual cell concentration on nitrogen (g R/g N); **μ**, specific growth rate (h^{-1})

*Author for Correspondence.
Mailing address: Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. Tel: 603-7967-6740; Fax: 603-7967-4178; E-mail: suffian_annuar@um.edu.my

when the different nitrogen sources are used.

Pseudomonas putida PGA1 strain used in this study was a gift from Professor G.Eggink from the Agrotechnological Research Institute, Wageningen, The Netherlands.

All the chemicals used were general purpose reagent grade. Experiments were carried out in shake flasks using Hotech (Model 178) orbital shaker incubator. Cells were first grown in rich medium consisting of ($g\ L^{-1}$): nutrient broth 10, yeast extract 15, and ammonium sulfate 5. The flasks were incubated at 30°C and 250 rpm rotation. The cells did not accumulate PHA when grown in this rich medium and hence was solely used to produce sufficient biomass for transfer to the production medium. After 24h, the cells were harvested aseptically by centrifugation, washed with saline and re-suspended in mineral salt solution. The composition of the mineral salt solution has been described in previous studies (Annuar *et al.*, 2007). The re-suspended cells were transferred to the production medium containing 1%w/v SPKO as the major carbon source and 0.5%w/v of each of the respective nitrogen sources in mineral salt solution. All experiments were carried out in triplicate flasks and samples were taken at 24h, 48h and 72h. The pH of the production medium was adjusted to 7.0 prior to starting the experiments. Saponification of palm kernel oil (PKO) was carried out as previously described (Tan *et al.*, 1997). PKO consists of a mixture of C6-C18:2 fatty acids with approximately 82% saturated fatty acids and 18% unsaturated fractions. The nitrogen sources tested were $NaNH_4HPO_4.4H_2O$ (Fluka-Chemika), yeast extract (MERCK), beef extract (MERCK), bacto-peptone (OXOID) and urea (Ajax Chemicals). To avoid precipitation during autoclaving, solutions of magnesium salt, trace elements and SPKO (pH 7.0) were sterilized separately before adding to the rest of the medium components.

Total biomass concentration was estimated by first spinning down the cells in a pre-weighed centrifuge tubes. The cells were then washed twice with 0.83%w/v NaCl solution and dried at 90°C till constant weight. Growth is represented by residual cell concentration (R) which is obtained by subtracting the PHA_{MCL} (P) mass from the total biomass (X) [R=X-P].

Nitrogen concentration in the culture supernatant was determined as ninhydrin-reactive nitrogen (Rowell, 1994).

The residual free fatty acids from SPKO were estimated *via* titration using cell-free culture liquid (Annuar *et al.*, 2007).

To obtain PHA_{MCL} time-profile, the method described in the previous studies of Annuar *et al.* (2007) was used whereby 8.0 mg of dried cells were subjected to acid-catalyzed methanolysis and the PHA_{MCL} amount and composition were determined by gas chromatography (GC) using benzoic acid methyl ester as the internal standard. The gas chromatograph used was Varian Star 3400CX equipped with a fused silica capillary column (30m x 0.53mm ID) (Supelco SPB™-608) and a flame ionization detector. 3-hydroxyalkanoic methyl ester standards (C_8-C_{16}) for GC analysis were obtained from Sigma Chemicals. For molecular weight determination, the PHA_{MCL} was first extracted by refluxing 1.0 g cells (dry weight) in 100 mL chloroform for 4 h. The mixture was filtered to remove cell debris and the filtrate was concentrated by rotary evaporation. The concentrated chloroform was added slowly to a rapidly stirred cold methanol to precipitate the PHA_{MCL}. The film was re-dissolved in chloroform and precipitated again in cold methanol to purify the polymer. The PHA_{MCL} film obtained was then air-dried and subsequently used for molecular weight determination by gel-permeation chromatography (GPC). Full details of the GPC analysis have been described previously (Annuar *et al.*, 2007).

Specific growth rate, μ (h^{-1}) was calculated according to equation (1):

$$\mu = \ln R_{t2} - \ln R_{t1} / (\Delta t) \quad (1)$$

where R_{t2}, R_{t1} : residual cell concentration at time t_2 and t_1, respectively; Δt: time interval of sampling.

Yield ($Y_{R/N}$)of residual cell concentration (R) on nitrogen source (N) was calculated according to equation (2):

$$Y_{R/N} = [R]_{max} - [R]_0 / ([N]_0 - [N]_t) \quad (2)$$

where $[R]_{max}$: maximum residual cell concentration; $[R]_0$: residual cell concentration at t=0; $[N]_t$: nitrogen concentration at time t; $[N]_0$: nitrogen concentration at t=0.

One-way ANOVA of the results was carried out using MATLAB 6.1 (The MathWorks, Inc.) software. The fermentation profiles from the shake flask experiments are shown in Figure 1(A), (B), (C) and (D). There was a lag period of 24 h after inoculation due to the different medium composition used to grow the inoculum. The residual cell concentration (PHA_{MCL}-free biomass; R), which represents growth, peaked at 48 h after which it started to level off or decline (Figure 1A). PHA_{MCL} accumulation paralleled the growth pattern; the maximum concentration obtained at 48h for all nitrogen sources except for cultivation using beef extract (Figure 1B). Nitrogen utilization and free fatty acids consumption reflected the growth pattern (Figure 1C & 1D). For comparison of the effect of the different nitrogen sources, only maximum growth and PHA_{MCL} concentration obtained at 48h were considered (Table 1). At P=0.05, there was no significant difference in the residual cell concentration produced using ammonium salt and bacto-peptone. A significant difference of means however, was observed when this group was compared with yeast extract, beef extract and urea. The residual cell concentrations obtained were not significantly different between yeast extract and beef extract as nitrogen source. Cultivation with bacto-peptone yielded the highest PHA_{MCL} concentration (2.34±0.20 $g\ L^{-1}$) (Table 1). This can be explained by the fact that PHA_{MCL} accumulation is usually enhanced by nutrient limitation such as nitrogen. The nitrogen (N) content of bacto-peptone (0.12 $g\ N\ g^{-1}$ bacto-peptone) is low compared to the N-content

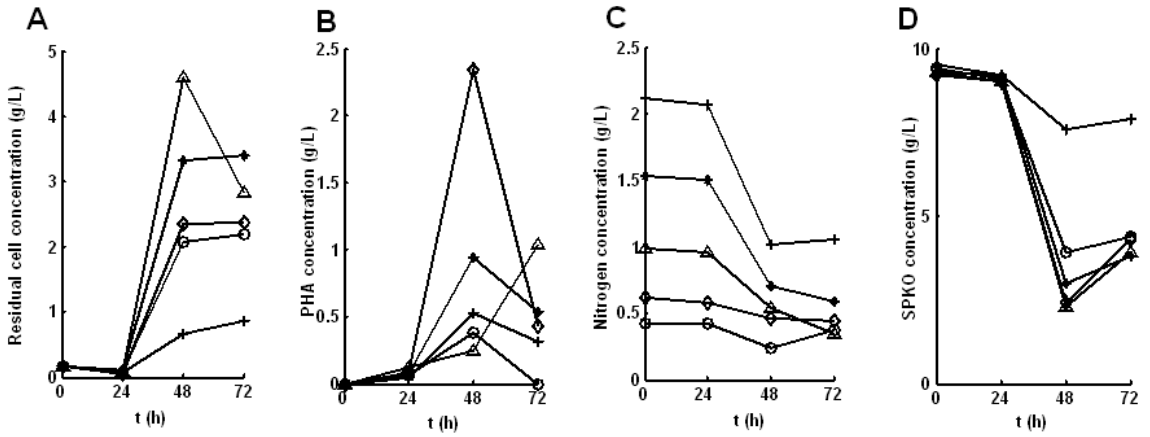

Figure 1. Cultivation of *P. putida* PGA1 supplied with different nitrogen sources with 1.0% (w/v) SPKO as a major carbon source. Profiles of growth (A), PHA$_{MCL}$ concentration (B), nitrogen concentration (C) and free fatty acids (D).

Each data point is an average of triplicate measurements. Keys: $NaNH_4HPO_4.4H_2O$ (○); urea (+); yeast extract (∗); beef extract (Δ) and bacto-peptone (◊).

of yeast extract (0.31 g N g^{-1} yeast extract) and beef extract (0.20 g N g^{-1} beef extract) (Table 1). The higher N-content of yeast extract and beef extract may be the reason that growth was favored over the PHA accumulation. Urea had the highest N-content (0.42 g N g^{-1} urea) among the different N-sources tested but surprisingly a low amount of residual cell concentration was produced, which resulted in a similarly lower PHA$_{MCL}$ concentration. As the yield of PHA$_{MCL}$ is dependent upon the yield of cells, urea is not a preferable nitrogen source for *P. putida* PGA1 growth under the cultivation conditions described. As for ammonium salt, a fairly similar amount of residual cell concentration was obtained (Table 1). However, the PHA$_{MCL}$ production from cultivation using bacto-peptone was higher by six-fold as compared to cultivation using ammonium salt. The specific growth rate, μ of *P. putida* PGA1 was relatively similar for all the nitrogen sources tested (i.e. 0.14-0.16 h^{-1}), except for urea where it was lower at 0.10 h^{-1} (Table 1). The yield of residual cell concentration on nitrogen source, ($Y_{R/N}$) was highest when bacto-peptone was used in the fermentation (13.7 g R g^{-1} N), which indicated that the type of nitrogen present in bacto-peptone was more efficiently utilized as compared to the others (Table 1).

P. putida PGA1 is known to produce a medium-chain-length PHA from SPKO with constituent monomers of C_8, C_{10}, C_{12}, C_{14} and C_{16}-3-hydroxyacyl esters (Tan *et al.*, 1998; Annuar *et al.*, 2007). Except for cultivation using urea, morphologically all purified PHA$_{MCL}$ films from the cultivations with different nitrogen sources were soft, flexible and transparent. These characteristics were similar to those reported in earlier studies (Tan *et al.*, 1998; Annuar *et al.*, 2007). The PHA$_{MCL}$ from cultivation using urea could not be cast into film due to insufficient amount of samples that could be extracted from the cells.

It was hypothesized that the monomer compositions of the PHA$_{MCL}$ produced would remained relatively constant irrespective of the nitrogen sources used. It was also assumed that only different types of carbon sources would influence

Table 1. Effect of different nitrogen sources on residual cell growth and PHA$_{MCL}$ production at 48h.

Nitrogen Source (N) (g N/g compound)	Residual cell concentration (R) (g/L) [initial=0.17(±0.03)]	PHA$_{MCL}$ (g/L) [initial=0]	PHA$_{MCL}$ content (% cell dry weight) [initial=0]	Yield, $Y_{R/N}$ (g R/g N)	Specific growth rate μ(h^{-1})
$NaNH_4HPO_4.4H_2O$ (0.07)[a]	2.08 (±0.26)	0.38 (±0.05)	15.7 (±0.7)	10.6	0.14
Urea (0.47)[a]	0.67 (±0.12)	0.53 (±0.14)	43.9 (±2.4)	0.5	0.10
Yeast extract (0.31±0.01)[b]	3.32 (±0.06)	0.94 (±0.09)	22.1 (±1.9)	3.8	0.15
Beef extract (0.20±0.01)[b]	4.59 (±0.18)	0.25 (±0.03)	5.1 (±0.4)	9.8	0.16
Bacto-peptone (0.12±0.01)[b]	2.36 (±0.12)	2.34 (±0.20)	49.7 (±3.4)	13.7	0.16

[a] calculated; [b] assayed (Materials and Methods section)

Table 2. Molar fractions of PHA$_{MCL}$ monomers (C_8-C_{16}) produced by *P. putida* PGA1 from cultivation using different nitrogen sources.

Nitrogen sources	Molar fraction (mole%)				
	C_8	C_{10}	C_{12}	C_{14}	C_{16}
Ammonium	56.6 (±0.8)	22.4 (±0.3)	4.5 (±0.1)	4.7 (±0.3)	12.0 (±0.7)
Urea	63.7 (±2.0)	25.1 (±1.5)	4.5 (±0.4)	3.2 (±0.1)	3.5 (±0.5)
Yeast extract	50.2 (±0.6)	28.8 (±0.3)	7.0 (±0.3)	4.8 (±0.3)	9.2 (±0.6)
Beef extract	51.6 (±0.9)	35.3 (±0.3)	10.0 (±0.8)	1.8 (±0.0)	1.3 (±0.1)
Bacto-peptone	56.0 (±0.3)	32.0 (±0.4)	7.9 (±0.3)	2.3 (±0.0)	1.8 (±0.1)

Table 3. Relative molecular masses and polydispersities of PHA$_{MCL}$ obtained on cultivation with different nitrogen sources at 48h.

Nitrogen source	Molecular weight (M_w)	Molecular number (M_n)	Polydispersity (M_w/M_n)
NaNH4HPO4.4H2O	127 601.0	67 383.5	1.89
Yeast extract	106 852.0	61 309.9	1.74
Beef extract	93 354.8	50 371.4	1.85
Bacto-peptone	112 341.0	60 178.0	1.87

the monomer compositions of the PHA$_{MCL}$ as previously reported (Brandl *et al.*, 1988; Gross *et al.*, 1989; Preusting *et al.*, 1990). The results obtained from this study showed the presence of C_8, C_{10}, C_{12}, C_{14} and C_{16}-3-hydroxyacyl esters as monomer constituents of the PHA$_{MCL}$ produced regardless of the type of nitrogen sources used for the fermentation (Table 2). Although there were slight variation in the relative molar proportions of each of the monomer constituent with the different nitrogen sources used (Table 2), it was also found that the monomer compositions of the extracted PHA$_{MCL}$ from the shake-flasks cultivation using different nitrogen sources shared similar range to those of extracted biopolymer obtained from batch and fed-batch bioreactor operations in which SPKO was used as the sole carbon and energy source and ammonium as the nitrogen source (Annuar *et al.*, 2007).

The results showed that the polymers produced from cultivations using ammonium salt, bacto-peptone and yeast extract are quite similar in their M_w, i.e., 100,000 to 120,000 and in their M_n range, i.e., 60,000 to 67,000 (Table 3). The M_w and M_n for the polymer produced from the cultivation using beef extract (approx. 93,000 and 50,000, respectively), however, are slightly on the lower side of the ranges produced by the other nitrogen sources. This could be due to the amount and content of PHA$_{MCL}$ obtained from this cultivation is the lowest among all. The M_w/M_n ratios for all the polymers tested are within the narrow range of 1.7-1.9 (Table 3). The polymer from the cultivation employing urea as nitrogen source could not be tested for M_w and M_n determination due to insufficient sample that could be extracted from the cells. The values of M_w, M_n and M_w/M_n were within similar range as those obtained in batch and fed-batch bioreactor operation when SPKO was used as the sole carbon and energy source and ammonium as the nitrogen source (Annuar *et al.*, 2007).

This study reported the effect of different nitrogen sources on cell growth and production of PHA$_{MCL}$ by *P. putida* PGA1 utilizing SPKO as major carbon source in shake-flasks cultivation. Complex nitrogen substrate like bacto-peptone has a good potential to be used in the fermentation as it helped to enhance PHA$_{MCL}$ production significantly compared to other nitrogen sources tested. Although the low nitrogen content of bacto-peptone (or any type of nitrogen source) may play a role in enhancing PHA$_{MCL}$ production, it is also likely that the organic constituents in bacto-peptone contributed to this enhancement. Irrespective of the type of nitrogen sources used, whether it is a heterogeneous mixture of organic origin or a chemically defined compound, no significant effect can be observed on the relative molar proportion of monomer constituents of the PHA$_{MCL}$ produced. The different type of nitrogen sources used also did not exert any significant influence on the molecular weight, molecular number nor polydispersity of the extracted PHA$_{MCL}$. This is invaluable information from a quality control perspective in that the composition and quality of the nitrogen source used has no controlling effect on the PHA$_{MCL}$ biosynthesis mechanism, and this resulted in a consistent biopolymer chemical make-up and molecular weight characteristics. On the other hand, the type and quality of the nitrogen source used may affect the cell growth and PHA$_{MCL}$ yield.

It is also suggested that regardless of the type of culture conditions the micro-organism is subjected to e.g. highly transient conditions of the shake-flasks cultivation, or in a

controlled environment of an automated bioreactor (batch or fed-batch) (Annuar *et al*., 2007), monomer constituents of the PHA$_{MCL}$ are quite consistent in their relative molar proportions. Similar observation was made for the molecular weight data of the biopolymer. The results obtained in this study may justify attempts to use industrial proteins for the production of PHA$_{MCL}$ by *Pseudomonas* sp. as there is a lot of nitrogen compound obtainable from renewable resources that can be used as cheap nitrogen substrates.

In conclusion, industrial proteins such as bacto-peptone as a nitrogen source gave significantly high PHA$_{MCL}$ production in this study and that the use of different nitrogen sources has no significant influence on the PHA$_{MCL}$ monomer composition and molecular weight.

ACKNOWLEDGEMENTS

This study was funded by University of Malaya research, grant no. Vot F0156/2001A.

REFERENCES

Annuar, M.S.M., Tan, I.K.P., Ibrahim, S., and Ramachandran, K.B. 2007. Production of medium-chain-length poly(3-hydroxyalkanoates) from crude fatty acids mixture by *Pseudomonas putida. Trans IChemE, Part C, Food and Bioproducts Processing* 85(C2): 104-119.

Annuar, M.S.M., Tan, I.K.P., Ibrahim, S., and Ramachandran, K.B. 2006. Ammonium uptake and growth kinetics of *Pseudomonas putida* PGA1. *Asia Pacific Journal of Molecular Biology and Biotechnology* 14: 1-10.

Bormann, E.J., Leiβner, M., and Beer, B. 1998. Growth-associated production of poly(hydroxybutyric acid) by *Azotobacter beijerinkii* from organic nitrogen substrates. *Applied Microbiology and Biotechnology* 49: 84-88.

Brandl, H., Gross, R.A., Lenz, R.W., and Fuller, R.C. 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied and Environmental Microbiology* 54: 1977-1982.

Gross, R.A., DeMello, C., Lenz, R.W., Brandl, H., and Fuller, R.C. 1989. Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. *Macromolecules* 22: 1106-1115.

Preusting, H., Nijenhuis, A., and Witholt, B. 1990. Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. *Macromolecules* 23: 4220-4224.

Rowell, D.L. 1994. Soil Science, Methods and Application. UK: Longman.

Tan, I.K.P., Sudesh, K., Theanmalar, M., Gan, S.N., and Gordon III, B. 1997. Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1. *Applied Microbiology and Biotechnology* 47: 207-211.