HOKKAIDO UNIVERSITY

Title	Isolation of a thermotolerant bacterium producing medium-chain-length polyhydroxyalkanoate
Author(s)	Satoh, Yasuharu; Tajima, Kenji; Nakamoto, Sho et al.
Citation	Journal of Applied Microbiology, 111(4), 811-817 https://doi.org/10.1111/j.1365-2672.2011.05093.x
Issue Date	2011-10
Doc URL	https://hdl.handle.net/2115/50148
Rights	The definitive version is available at www.blackwell-synergy.com
Type	journal article
File Information	JAM111-4_811-817.pdf



Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Isolation of a Thermotolerant Bacterium Producing Medium-chain-length Polyhydroxyalkanoate

Yasuharu Satoh[1*], Kenji Tajima[1], Sho Nakamoto[1], Han Xuerong[1], Tokuo Matsushima[2], Takeshi Ohshima[3], Shin Kawano[4], Tomoki Erata[1], Tohru Dairi[1*], Masanobu Munekata[1]

[1] Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, Japan

[2] Agri Bio Industry Inc., Sapporo, Japan

[3] Hokusei Kigyou Co., Ltd, Sapporo, Japan

[4] Database Center for Life Science (DBCLS), Tokyo, Japan

[*]Corresponding authors:
Address: Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan
E-mail: syasu@eng.hokudai.ac.jp, dairi@eng.hokudai.ac.jp
Tel & Fax: +81-11-706-7118

Running headline: Thermotolerant bacterium producing PHA

Keywords: Polyhydroxyalkanoate, Thermotolerant bacterium, Pseudomonas, Biodiesel fuel by-product

ABSTRACT

Aims: The aim of the present study was to isolate a thermotolerant microorganism that produces polyhydroxyalkanoates (PHAs) composed of medium-chain-length (mcl)-HA units from a biodiesel fuel (BDF) by-product as a carbon source.

Methods and Results: We successfully isolated a thermotolerant microorganism, strain SG4502, capable to accumulate mcl-PHA from a BDF by-product as a carbon source at a cultivation temperature of 45˚C. The strain could also produce mcl-PHA from acetate, octanoate, and dodecanoate as sole carbon sources at cultivation temperatures up to 55˚C. Taxonomic studies and 16S rRNA gene sequence analysis revealed that strain SG4502 was phylogenetically affiliated with species of the genus *Pseudomonas*. This study is the first report of PHA synthesis by a thermotolerant *Pseudomonas*.

Conclusions: A novel thermotolerant bacterium capable to accumulate mcl-PHA from a BDF by-product was successfully isolated.

Significance and Impact of the Study: A major issue regarding industrial production of microbial PHAs is their much higher production cost compared with conventional petrochemical-based plastic materials. Especially significant are the cost of a fermentative substrate and the running cost to maintain a temperature suitable for microbial growth. Thus, strain SG4502, isolated in this study, which assimilates BDF by-product and produces PHA at high temperature, would be very useful for practical application in industry.

INTRODUCTION

Polyhydroxyalkanoates (PHAs), which originate from renewable resources such as sugars and vegetable oils, have attracted great interest as alternatives to petrochemical-based plastics because of the similarity of their material properties to various thermoplastics and elastomers and their complete biodegradability (Sudesh et al. 2000; Chen 2009). PHAs comprise a large class of polyesters that are accumulated as carbon and energy storage material by a wide variety of bacteria. PHAs are commonly grouped into two major categories according to the number of carbon atoms in their monomeric units, which are short-chain-length (scl)-PHAs with C3 to C5 monomer units and medium-chain-length (mcl)-PHAs with C6 to C14 monomer units. The scl-PHAs are generally more crystalline than the mcl-PHAs and usually exhibit thermoplastic-like properties. In contrast, the mcl-PHAs exhibit elastomer- or adhesive-like properties depending on the specific side-chain length and the degree of unsaturation.

However, a major issue regarding industrial production of microbial PHAs is their much higher production cost compared with conventional petrochemical-based plastic materials. For industrial microbial production of PHAs, the cost of the fermentative substrate constitutes a significant proportion of the total costs (Lee and Choi 1998; Chen 2009). Production of biopolymers using inexpensive biomass and carbon sources available in by-products or waste streams potentially decreases the production cost and also helps to convert these materials into value-added products. Accordingly, research efforts abound to exploit inexpensive

fermentable raw materials as fermentative substrates for PHA production.

Another concern in the application of microbial PHAs to industrial production is the running cost of maintaining a temperature suitable for microbial growth (Fischer et al. 2008). In addition to the influence of external temperature increases, fermentation broths also heat up due to exothermic metabolic reactions. In many warm countries, summer temperatures are frequently >40°C and the fermentation vessel's temperature would be over 40°C in the absence of a cooling system. This overheating problem is partially overcome by spraying cold water on the fermentation vessel walls, but this increases the production cost. Moreover, an advantage of processing at higher temperatures is that it significantly reduces the chance of contamination. Thermophilic or thermotolerant microbial cells thus offer the advantage of enabling operation at elevated temperatures in industrial fermentation. It has been reported that the thermophilic bacterium *Thermus thermophilus* HB8 is a PHA producer (Pantazaki et al. 2003). This bacterium accumulated PHA composed of C4 to C12 monomer units at a cultivation temperature of 75°C. However, the optimal temperature range, 65−72°C, and the minimum temperature, 47°C (Oshima and Imahori 1974), are too high for industrial fermentation, because the need to maintain such high cultivation temperatures requires the expense of extra energy.

To overcome these issues, in this study we attempted to screen thermotolerant bacteria capable of producing PHAs from biodiesel fuel (BDF) by-products whose main component is glycerol (Yazdani and Gonzalez 2007; Sivakumar et al. 2010). BDF, an environmentally

friendly and sustainable alternative to fossil fuels, is derived from vegetable oils, fats and waste edible oils via a methyl-esterification process. BDF production on a large commercial scale has led to the accumulation of BDF by-product. Although BDF by-product was thought to be unfit for the growth and fermentation of some microorganisms, owing to its considerable amounts of certain impurities, such as soap, methanol and free fatty acids (Yazdani and Gonzalez 2007), we attempted to apply it to PHA production. As a result, we succeeded in isolating one microorganism, designated SG4502, which is thermotolerant and can produce mcl-PHA from BDF by-product as a sole carbon source at cultivation temperatures up to 55°C.

MATERIALS AND METHODS

Media

Mineral salt (MS) medium, containing 9.0 g of Na_2HPO_4 $12H_2O$, 1.5 g of KH_2PO_4, 0.1 g of NH_4Cl, 0.2 g of $MgSO_4$ $7H_2O$ and 1 ml of trace element solution per liter of distilled water (Kato et al. 1996), was used for the screening. Kato et al. also reported that higher C/N ratio promoted PHA accumulation in *Pseudomonas* sp. Therefore, NH_4Cl was decreased to 0.05 g l^{-1} for PHA production. Nutrient-rich (NR) medium containing 10 g of peptone, 2 g of yeast extract and 10 g of meat extract per liter of distilled water was used for the cultivation of bacteria. For growth on plates, NR medium with 1.5% (w/v) agar was used.

The BDF by-product used in this study was obtained from Hokusei Kigyou Co. Ltd.

(Sapporo, Japan). The by-product contained 41.5% glycerol, 12.4% methanol and 21.4% ether-soluble materials such as fatty acid methyl ester. This was neutralized by H_2SO_4 before used.

Isolation of bacteria accumulating PHA

Samples, which were collected from sewage sedimentations and surface of an apparatus for producing BDF, were used as sources for the isolation of thermotolerant PHA producing bacteria. Each sample was put into 10 ml of sterilized water and mixed. After centrifugation at 1000 × g for 3 min, each of the supernatants (0.2 ml) was added into 50 ml of MS medium containing 1% (w/v) BDF by-product and incubated for 3 d at 45°C with shaking at 160 rpm. After repeating subcultures, each culture was streaked on NR agar medium and incubated at 45°C for 3 d. The isolated strains were tested for PHA synthesis activity as the following: the cells were cultured in 5 ml of NR medium at 45°C for 16 h. They were inoculated in 100 ml of nitrogen-limited MS medium containing 1% (w/v) BDF by-product in 500-ml Erlenmeyer flasks with baffles and incubated at 45°C for 48 h with shaking at 160 rpm. The collected cells were washed with distilled water, followed by ethanol and distilled water again and then lyophilized. The dried microbial cells were subjected to gas chromatography (GC) analysis as described below.

PHA production

SG4502 was cultured in 60 ml of NR medium at 45°C for 16 h. The cultures were inoculated into 1.2 l of nitrogen-limited MS medium containing a carbon source in 3-l Erlenmeyer flasks with baffles, and incubated at 45 or 55°C with shaking at 130 rpm for 48 h. To this medium, BDF by-product was fed twice at the concentrations of 1% (w/v) at 0 and 24 h, respectively, during the course of cultivation. Glycerol or acetate was fed four times at the concentrations of 1, 2, 3 and 4% (w/v) at 0, 12, 24 and 36 h, respectively, during the course of cultivation. Octanoate, dodecanoate or soybean oil was added four times at the concentrations of 0.1% (w/v) at 0, 12, 24 and 36 h, respectively, during the course of cultivation. The collected cells were washed with distilled water followed by ethanol and distilled water again and then lyophilized. Polymer was extracted from each dry microbial cell body by a Soxhlet extractor with chloroform as a solvent and then dried under reduced pressure after the solvent was removed by an evaporator.

Analyses

The sequence of 16S rDNA was analyzed with MicroSeq Full Gene 16S rDNA PCR and Sequencing kits and by the ABI PRISM 3100 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

Polymer composition and accumulation were analyzed by the GC-2010 capillary gas chromatography system (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a Zebron ZB-1 GC capillary column (0.25 mm I.D. × 30 m; Phenomenex Inc.,

CA, USA). A sample was prepared as follows: approximately 10 mg of polymer or 50 mg of dried microbial cell was treated with a solution containing 1.7 ml of methanol, 0.3 ml of sulfuric acid and 2.0 ml of chloroform at 100°C for 140 min to convert the constituents to their methyl esters. One milliliter of water was added to the reaction mixture, and then the lower chloroform layer was used for GC analysis.

The ^{1}H-NMR spectra of the polymers were obtained in deuterated chloroform ($CDCl_3$) using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4 ms, 3,000 Hz spectral width and a 4 s repetition rate. The chemical shifts were reported in ppm with tetramethylsilane as the internal reference.

The molecular weights of the obtained polymers were determined by using a gel-permeation chromatography (GPC) system (Shimadzu) equipped with tandem TSKgel Super HZM-H columns (6.0 mm I.D. × 150 mm; Tosoh Co., Tokyo, Japan) at 40°C. Chloroform was used as an eluate, and the calibration was performed using polystyrene samples as standards.

RESULTS

Phenotypic and phylogenetic characterization

To obtain thermotolerant bacteria capable of accumulating PHA in a chemically defined medium containing a BDF by-product as a sole carbon source, we first carried out enrichment culture with MS medium containing a BDF by-product at a cultivation temperature of 45°C.

The tens of colonies isolated on NR agar medium were evaluated for their ability to produce PHA from a BDF by-product as a carbon source. The nitrogen source, NH_4Cl, in MS medium was decreased to promote PHA accumulation. The PHA accumulated in the cells was determined by GC analysis. We successfully found a strain that could produce mcl-PHA at a cultivation temperature of 45°C. The strain was designated SG4502.

The microbiological properties of strain SG4502 were analyzed. SG4502 is an aerobic, Gram-negative, polar flagellate bacillus. The strain was able to grow up to a cultivation temperature of 55˚C, and not at 60˚C (Figure 1). We found the strain positive for catalase, oxidase and arginine dihydrolase activities and negative for urease activity. Nitrate was not reduced. The strain grew at pH 6−10. Growth was observed at 37−50˚C in the presence of 4% NaCl. Anaerobic growth did not occur in the presence of nitrate. Tweens 20 and 80 were hydrolyzed, but aesculin, gelatin, starch and skimmed milk were not. Furthermore, strain SG4502 assimilated citrate, pyruvate, succinate, malate, acetate, propionate, octanoate, decanoate, betain, L-lysine, L-asparagine, L-serin, L-glutamine, L-alanine, L-isoleucine, L-proline, dodecane, tridecane, hexadecane, nonadecane, eicosane, palmitic acid, octanal, 1-octanol, 1-dodecanol, and benzoate, but not fructose, D-mannose, D-trehalose, D-cellobiose, L-arabinose, D-glucose, L-sorbose, D-sucrose, D-xylose, D-maltose, D-mannitol, gluconate, phenylacetate, adipate, glycerol, glycine, L-cystein, L-phenylalanine, L-histidine, L-arginine, N-acetylglucosamine, decane, SDS, salicylate, naphthalene, anthracene or phenanthrene.

The sequence of the 16S rRNA-encoding gene of strain SG4502 was determined and

deposited in DNA Data Bank of Japan (DDBJ) sequence database under accession number AB616678. The 16S rRNA gene sequence analysis using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that strain SG4502 belongs to the genus *Pseudomonas* and that the closest phylogenetic neighbors of the strain are *P. thermotolerans* (99% identity; accession number AJ311980) (Manaia and Moore 2002). Phylogenetic analyses using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007, Figure 2) show that strain SG4502 and *P. thermotolerans* are clustered and are relatively distant from the most closely related other *Pseudomonas* species.

PHA productivity

To investigate the PHA productivity of strain SG4502 at a cultivation temperature of 45°C, the strain was cultivated under nitrogen-limiting conditions in MS medium supplemented with several types of carbon sources. Polymers accumulated in the cells were extracted from the dried cells with chloroform and then analyzed. BDF by-product was first tested as a sole carbon source. As shown in Table 1, strain SG4502 reached a total biomass (cell components plus PHA) of 1.50 g l^{-1} for 48 h and accumulated 0.61 g l^{-1} of PHA (40.6 % [w/w] of total biomass). The number-average molecular weight (Mn) and polydispersity (Mw/Mn) of the product were determined to be 36×10^3 and 1.95, respectively (Table 2). The ^{1}H NMR analysis clearly indicated that the product was PHA (Figure 3). Based on GC analysis, PHA was calculated to be composed of 0.8 mol% 3-hydroxybutyrate (3HB, C4), 5.7

mol% 3-hydroxyhexanoate (3HHx, C6), 25.1 mol% 3-hydroxyoctanoate (3HO, C8) 59.8 mol% 3-hydroxydecanoate (3HD, C10) and 8.6 mol% 3- hydroxydodecanoate (3HDD, C12) units.

We also used glycerol, a main component in BDF by-product, as a sole carbon source, although the assimilation test for glycerol was negative as described above. Under the polymer production conditions employed, however, the strain showed weak growth and accumulated trace amounts of polymer composed of a 3HD unit (Table 1).

Vegetable oils such as soybean oil, which have been used as feedstocks for PHA production (Kahar et al. 2004), were also examined as possible sole carbon sources. As shown in Table 1, however, strain SG4502 was unable to assimilate soybean oil under the cultivation conditions, suggesting that the strain would have no lipases to be secreted into the medium, because fatty acids were assimilated as described below.

We next compared properties of the PHA produced by strain SG4502 with those produced by *P. putida* and *P. oleovolance* (Gross et al. 1989; Huisman et al. 1989), both of which are typical mcl-PHA producers, with acetate (C2), octanoate (C8), and dodecanoate (C12) as sole carbon sources. Strain SG4502 produced PHA having 3HD units as a major component (84.4 mol%) with acetate as a sole carbon source. In this case, the substrates for polymer syntheses would be supplied from *de novo* fatty acid biosynthesis pathway (Rehm et al. 1998). When octanoate was added to MS medium as a sole carbon source, the total biomass of strain SG4502 was highest among all cultivation conditions and the 3HO (C8)

content in PHA was drastically increased (82.5 mol%) (Table 1). When dodecanoate (C12) was used as the sole carbon source, almost equal amounts (40 mol%) of 3HD (C10) and 3HO (C8), which would be formed respectively by one and two cycles of β-oxidation of dodecanoate, were incorporated. These results suggested that fatty acids fed in the culture broth would be efficiently incorporated into PHA. In all cases with acetate, octanoate or dodecanoate as the sole carbon source, the Mn and Mw/Mn were between 7 to 11×10^4 and 2.0 to 2.5, respectively. All these properties were in good agreement with those of *P. putida* and *P. oleovolance*.

The effects of cultivation temperature on cell growth, PHA productivity, and monomer composition were examined with dodecanoate as a sole carbon source at 55˚C, since strain SG4502 was able to grow at 55˚C in NR medium and the solubility of dodecanoate increased as temperature increased (McBain and Sierichs 1948). Under the cultivation condition, the strain grew and accumulated a polymer. As for the monomer composition in the PHA, it was almost the same as that produced at 45˚C. We thus demonstrated that strain SG4502 had the ability to produce mcl-PHA at a cultivation temperature of 55˚C.

DISCUSSION

We successfully isolated a thermotolerant bacterium, strain SG4502, capable to accumulate PHA from a BDF by-product as a carbon source at temperatures up to 55˚C. Taxonomic studies and 16S rRNA gene sequence analysis revealed that strain SG4502 was

phylogenetically affiliated with species of the genus *Pseudomonas*. The closest species is *P. thermotolerans* (Manaia and Moore 2002), whose PHA productivity has not been reported. Moreover, the results of assimilation tests for citrate, pyruvate, succinate, L-lysine, L-arginine, SDS and benzoate were different from each other. As a result, strain SG4502 was concluded to be a *Pseudomonas* sp.

The BDF by-product used in this study contained glycerol as the major component and fatty acid methyl ester as the minor component. Based on the results of cell growth and the properties of the produced PHA with BDF by-product, glycerol and fatty acids, strain SG4502 preferentially assimilated minor components rather than glycerol in the BDF by-product. However, strain SG4502 produced a trace amount of homopolymer composed of 3HD when glycerol was used as a sole carbon source, suggesting that the strain was able to utilize glycerol for PHA accumulation perhaps via *de novo* fatty acid biosynthesis (Rehm et al. 1998).

The PHA produced from BDF by-product has a lower molecular weight than PHAs produced from acetate, octanoate and dodecanoate under the same cultivation conditions (Table 2). Previously, the same phenomenon was reported: *Ralstonia eutropha* produced PHA with a lower molecular weight when cultured with glycerol as a carbon source (Madden et al. 1999). In this study, it was shown that glycerol functions as a termination reagent during a chain transfer/elongation in PHA biosynthesis, resulting in decreased molecular weight of the polymer produced. This was also the case with strain SG4502.

To date, *T. thermophilus* HB8 is the only heat-tolerant bacterium producing PHA (Oshima and Imahori 1974; Pantazaki et al. 2003). However, *T. thermophilus* requires a cultivation temperature above 47^{o}C, which in turn requires more energy to maintain. As shown in this study, we have succeeded in isolating a PHA-producing strain capable of producing mcl-PHA at cultivation temperatures up to 55˚C. Although strain SG4502 would be more useful for practical application in industry, the strain did not efficiently assimilate BDF by-products, including glycerol. However, this could be overcome by the construction of a genetically engineered strain to reinforce the glycerol assimilation pathway. Previously, Solaiman et al. succeeded in conferring the assimilation capacity of oils to *P. putida* and *P. oleovorans*, which were impossible to grow on vegetable oils and fats, by introducing a gene encoding lipase; these strains produced PHA from oils (Solaiman et al. 2001). Application of the same strategy to strain SG4502 would enable the construction of an engineered strain with a high ability to assimilate BDF by-product.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Eiji Yamada, Hokkaido University, for his technical assistance with NMR measurements. We also thank Mr. Yusuke Soeda, Ms. Reiko Yamato and Mr. Tetsuya Toriyabe, Agri Bio Industry Inc., for their technical support. This work was supported by the Global COE Program (Project No. B01: Catalysis as the Basis for Innovation in Materials Science) from the Ministry of Education, Culture, Sports, Science,

and Technology, Japan; and by the Northern Advancement Center for Science & Technology (NOASTEC).

REFERENCES

Chen, G.Q. (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem Soc Rev* **38**, 2434−2446.

Fischer, C.R., Klein-Marcuschamer, D. and Stephanopoulos, G. (2008) Selection and optimization of microbial hosts for biofuels production. *Metab Eng* **10**, 295−304.

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.

Huisman, G.W., de Leeuw, O., Eggink, G. and Witholt, B. (1989) Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent Pseudomonads. *Appl Environ Microbiol* **55**, 1949−1954.

Kahar, P., Tsuge, T., Taguchi, K. and Doi, Y. (2004) High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. *Polym Degrad Stab* **83**, 79−86.

Kato, M., Bao, H.J., Kang, C.K., Fukui, T. and Doi, Y. (1996) Production of a novel copolyester of 3-hydroxybutyric acid and medium chain length 3-hydroxyalkanaic acids by *Pseudomonas* sp. 61-3 from sugars. *Appl Microbiol Biotechnol* **45**, 363−370.

Lee, S.Y. and Choi, J.I. (1998) Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by *Alcaligenes latus*. *Polym Degrad Stab* **59**, 387−393.

Madden, L.A., Anderson, A.J., Shah, D.T. and Asrar, J. (1999) Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxy-compounds as chain transfer agents. *Int J Biol Macromol* **25**, 43−53.

Manaia, C.M. and Moore, E.R. (2002) *Pseudomonas thermotolerans* sp. nov., a thermotolerant species of the genus *Pseudomonas sensu stricto*. *Int J Syst Evol Microbiol* **52**, 2203−2209.

McBain, J.W. and Sierichs, W.C. (1948) The solubility of sodium and potassium soaps and the phase diagrams of aqueous potassium soaps. *J Am Oil Chem Soc* **25**, 221−225.

Oshima, T. and Imahori, K. (1974) Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. *Int J Syst Bacteriol* **24**, 102−112.

Pantazaki, A.A., Tambaka, M.G., Langlois, V., Guerin, P. and Kyriakidis, D.A. (2003) Polyhydroxyalkanoate (PHA) biosynthesis in *Thermus thermophilus*: purification and biochemical properties of PHA synthase. *Mol Cell Biochem* **254**, 173−183.

Rehm, B.H.A., Krüger, N. and Steinbüchel, A. (1998) A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. The PhaG gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme A

transferase. *J Biol Chem* **273**, 24044−24051.

Sivakumar, G., Vail, D.R., Xu, J.F., Burner, D.M., Lay, J.O., Ge, X.M. and Weathers, P.J. (2010) Bioethanol and biodiesel: Alternative liquid fuels for future generations. *Eng Life Sci* **10**, 8−18.

Solaiman, D.K., Ashby, R.D. and Foglia, T.A. (2001) Production of polyhydroxyalkanoates from intact triacylglycerols by genetically engineered Pseudomonas. *Appl Microbiol Biotechnol* **56**, 664−669.

Sudesh, K., Abe, H. and Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci* **25**, 1503−1555.

Tamura, K, Dudley, J, Nei, M. and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596−1599

Yazdani, S.S. and Gonzalez, R. (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Curr Opin Biotechnol* **18**, 213−219.

Table 1 Monomer compositions of PHAs produced by strain SG4502

Carbon Sources	Temp. (˚C)	Total Biomass[a] ($g\ l^{-1}$)	PHA yield ($g\ l^{-1}$)	PHA content (% [w/w])	Monomer composition (mol%)[b]				
					3HB	3HHx	3HO	3HD	3HDD
BDF by-product	45	1·50	0·61	40·6	0·8	5·7	25·1	59·8	8·6
Glycerol	45	0·053	trace[c]	-	0	0	0	100	0
Acetate	45	0·57	0·06	10·1	1·0	1·4	10·4	84·4	2·9
Octanoate	45	1·80	0·50	28·0	0·9	10·7	82·5	5·7	0·1
Dodecanoate	45	0·30	0·11	36·1	0·7	6·3	40·2	37·8	15·0
	55	0·41	0·10	24·0	0	6·0	57·0	37·0	0
Soybean oil	45	trace[c]	-	-	-	-	-	-	-

[a] Total biomass was defined as cell components plus PHA.

[b] 3HB, 3-hydroxybutyrate (C4); 3HHx, 3-hydroxyhexanoate (C6); 3HO, 3-hydroxyoctanoate (C8); 3HD, 3-hydroxydecanoate (C10); 3HDD, 3-hydroxydodecanoate (C12).

[c] Trace < 0.02 g/L

Table 2 Molecular weights of PHAs produced by strain SG4502

Carbon source	Temp. (˚C)	Mn×10^{-4} (g mol^{-1})	Mw/Mn
BDF by-product	45	3·57	1·95
Acetate	45	7·34	2·16
Octanoate	45	10·7	2·43
Dodecanoate	45	7·67	2·21
	55	3·61	3·59

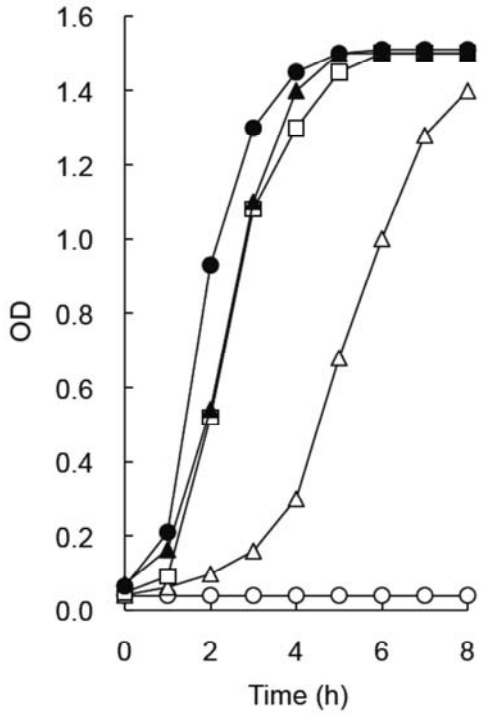


Figure 1 Growth curve of strain SG4502. The strain was cultured in NR medium at the indicated temperatures. Closed triangle, 37°C; closed circle, 45°C; open square, 50°C; open triangle, 55°C; open circle, 60°C.

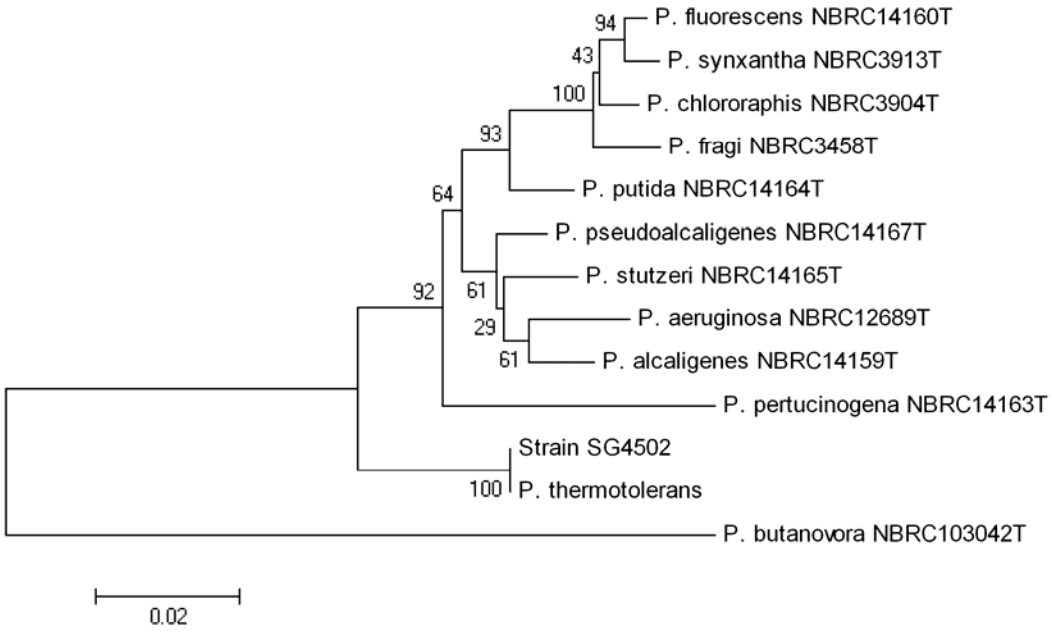


Figure 2 Phylogenetic tree of strain SG4502 and its related microorganisms. The 16S rRNA gene sequence of strain SG4502 was aligned with those of 11 type strains in *Pseudomonas* species collected at NITE Biological Resource Center (NBRC) as well as *P. thermotolerans* by using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007). A phylogenetic tree was constructed using the neighbor-joining method. The numbers on the tree indicate the percentages of bootstrap sampling, derived from 1000 replications.

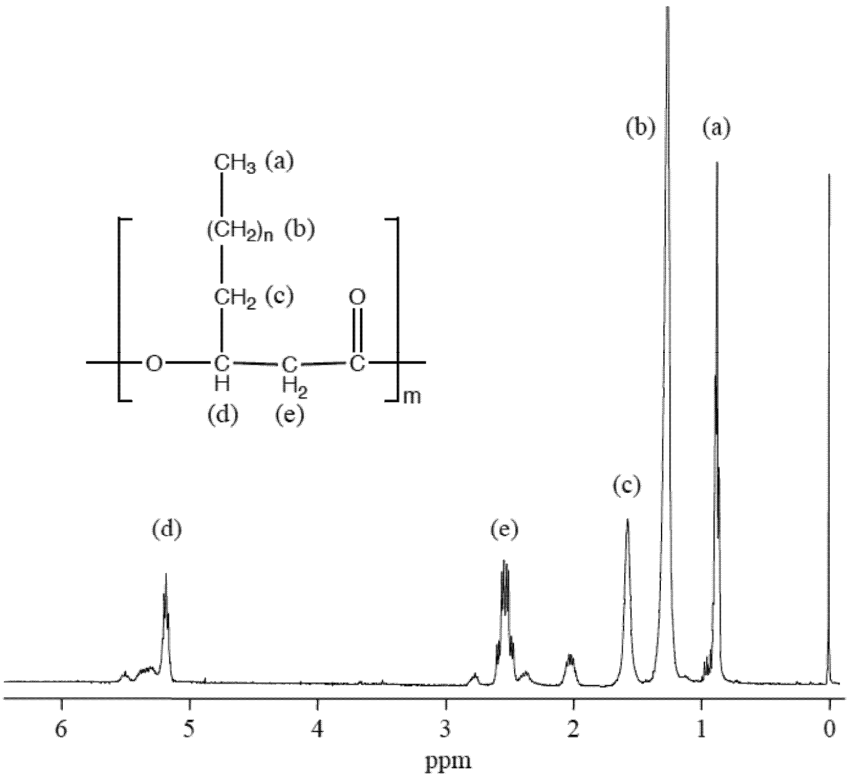


Figure 3 ^{1}H NMR spectrum of the polymer produced from BDF by-product.