Production of Medium Chain Length Polyhydroxyalkanoates by *Pseudomonas putida* ATCC47054 Using Glycerol and Sodium Octanoate as Substrates

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Abstract

In this paper, the production of medium chain length polyhydroxyalkanoates (mcl-PHAs) by *Pseudomonas putida* ATCC47054 using glycerol as a growth carbon source and sodium octanoate as mcl-PHAs was performed successfully both in batch and fed batch fermentation. The characterization of produced mcl-PHA by using nuclear magnetic resonance technique was found to be polyhydroxyoctanoate (PHO). The percentage of PHO per cell dry weight of *Pseudomonas putida* ATCC47054 in batch fermentation were found to be 47.37 and PHO yield was 11.61 mg/L. The preliminary study for production of PHO by using fed batch fermentation revealed high cell weight and PHO production which could be formed as a plastic sheet. This study suggested that glycerol and sodium octanoate were promising for using as a growth carbon source and PHO precursor and the production of PHO could be further scaled up by fed batch fermentation technique.

Keywords**:** *Polyhydroxyalkanoates, medium-chain-length PHA, biopolymers, sodium octanoate, Polyhydroxyoctanoate (PHO), fed batch fermentation*

1. Introduction

Petroleum-based plastic is cheap, capable of being made into any conceivable shape, strong and durable. It is believed that most of the plastic produced over the last 70 years has been thrown away, either into landfill sites or into the general environment. Some of the plastic are recycled. Recently, there has been the rising awareness of marine and terrestrial environments pollution by nondegradable petroleum based-plastics and the emerging of micro- or nano- plastic particles in marine that has affected various sea animal deaths. Moreover, the plastic which was degraded to pieces which small enough to pass through the stomach into fish and other animal can be accumulated in meat which human are eating. These reasons lead to a growing demand for decomposable biopolymers, such as PHAs (Ivar & Costa, 2014).

Polyhydroxyalkanoates (PHAs) are a group of natural biodegradable polymers showing properties quite similar to petroleum-based nondegradable plastics (Heinrich et al., 2016). They can be classified into three groups, depending on the number of carbons atoms in the monomeric units as the followings; short-chain-length (scl-PHAs), medium-chain-length and long-chain-length PHA. The scl-PHAs have 3–5 carbon atoms. Polyhydroxybutyrate (PHB), which is an scl-PHAs are found in many bacterial cells and shows limits in their mechanical properties (Hazer and Steinbuechel, 2007; Kim do et al., 2007; Samrot et al., 2011). The mcl-PHA consists of 6–14 carbon atoms, being amorphous or semi-crystalline and having elastomeric properties. The mcl-PHAs have promising potential applications in the medical field, such as medical devices, implants, drug delivery, and scaffolding for the regeneration of arteries and nerve axons (Chaudhry et al., 2011).

PHAs can be produced by many microorganisms as an intracellular energy storage material. There have been varying reports on their monomeric composition depending on the host and on the carbon source. The produced mcl-PHAs mainly consist of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) which shows much more elastomer and resistance. In contrast to PHB which can accumulate up to 90% of cell dry weight, mcl-PHAs are synthetized by only a few bacteria species and most of them are *Pseudomonas* sp. (Solaiman et al., 2000; Verlinden et al., 2007; Samrot et al., 2011; Kabilan et al., 2012). Among these bacteria, *Pseudomonas putida* KT2440 or ATCC47054 is able to synthesize large amounts of mcl-PHAs (Le Meur et al., 2012).

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According to the high price of a carbon source leads to the limitation of commercial production of PHAs (Ngozi & Adeniyi, 2012), as we aimed to study the possibility of using glycerol which is a by-product in the petrochemical process as a growth carbon source and using sodium octanoate as a precursor for mcl-PHA production by *Pseudomonas putida* ATCC47054.

2. Objectives

1. To study the possibility of glycerol as a growth carbon source and sodium octanoate as precursor for mcl-PHA production by *Pseudomonas putida* ATCC47054.
2. To identify the type of produced mcl-PHA by using nuclear magnetic resonance technique.
3. To conduct a preliminary study on production of mcl-PHAs by using fed batch fermentation.

3. Materials and Methods

3.1 Bacteria and culture condition

A laboratory mutant strain of *Pseudomonas putida* ATCC47054 was used in this study. The strain was adapted in a glycerol and sodium octanoate - rich environment and effectively forms PHO. It was maintained in 45% glycerol kept in -80 ^{o}C. An inoculum of *Pseudomonas putida* ATCC47054 was prepared by aseptically transferring the cells from -80 ^{o}C into 5 mL of nutrient broth medium in a test tube and incubated for 24-48 h at 30 ^{o}C and then subcultured on nutrient agar for a single colony. The cells taken from a single colony were further cultivated in a 500-mL flask containing 200 mL of a mineral solution supplemented with 20 g/L glycerol and 1 g/L sodium octanoate and shaken at 30 ^{o}C and 200 rpm for 36-40 h on a rotary shaker. The mineral solution contained the following (per liter): 1.2 g of NaH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 2 g of $(NH_4)_2SO_4$, 7.34 g/L K_2HPO_4, and 1 mL of trace element solution which contained 700 μM $Fe(NH_4)SO_4$, 17 μM $ZnSO_4{\cdot}7H_2O$, 25 μM $MnCl_2{\cdot}4H_2O$, 8 μM $CuSO_4{\cdot}5H_2O$, 7.2 μM $NaB_4O_2{\cdot}10H_2O$, and 8.3 μM $NaMoO_4{\cdot}2H_2O$. The flask culture was repeated two times to obtain highly active cells as the inoculum for fermentation in a bench-top bioreactor.

Batch fermentations were performed in a 5 L bioreactor (MDFT-N-5L/B.E.Marubishi/Thailand) containing 2.5 L mineral solution as described above. The temperature, pH, and dissolved oxygen probes were used to monitor and control the fermentations. Culture conditions were maintained at 30 ◦C and pH 6.8. Agitation speeds were 200 rpm or above to control the dissolved oxygen at 10% of air saturation. Cultures were inoculated with a 200 mL seed flask culture mentioned above. Aliquots of 10–15 mL culture medium were collected for analysis of OD600 and cell dry weight. At 60 h incubation period, the cells were harvested, centrifuged, lyophilized and used for determination and characterization of mcl-PHAs.

3.2 Extraction and quantitation of PHO by gas chromatography

About 50 milligrams of lyophilized cells were added with 2 mL chloroform and 2 mL of 3% H_2SO_4 (v/v) in methanol supplemented with 10 mg/mL benzoic acid as the internal standard and then heated at 100 °C for 4 hours with mixing at 30 minutes intervals. After that, the tube of mixture was placed at room temperature and let it cool down overnight. Then, 2 mL of distilled water was added, mixed vigorously by vortex for 4 minutes and let the tube stand overnight. Chloroform layer was used for determination of PHO in milligram by reading from sodium octanoate standard curve. For gas chromatography, it was carried out on gas chromatography instrument (Agilent technologies 7890A) percentage of PHO per cell dry weight (% w/w) and PHO yield (milligram/L) were calculated.

3.3 Extraction and characterization of mcl-PHAs

The lyophilized cells were added with chloroform at the ratio of 15 milliliter chloroform per 1 gram of the lyophilized cells. Mixed the cells and chloroform together by using magnetic stirrer and then placed the suspension on heat box at 60 ^{o}C overnight, and repeating this step twice. After that the suspension was filtered through filter paper (Whatman number 1), the filtrate was dropped into a beaker containing 60 milliliters of methanol. Then, methanol was evaporated by using an evaporator at 55 ^{o}C. The

polymer appeared as white matter and was further characterized by using nuclear magnetic resonance at Scientific and Technological Research Equipment Centre Chulalongkorn University.

3.4 Preliminary study of PHO production by *Pseudomonas putida* ATCC47054 using fed batch fermentation

The fed batch fermentations were performed as the same as batch fermentation as described above except the fed-batch fermentation began with 2 L of the mineral solution with 20 g/L of glycerol and 7.5 g/L $(NH_4)_2SO_4$ with an initial C/N ratio at 5, and 200 mL of *Pseudomonas putida* ATCC47054 starter inoculum. After the cell density reached the desired level (about 5 g/L) or during 20-40 h of incubation, a glycerol solution (500 g/L) was added via an automatic pump to keep the residual glycerol concentration above 10 g/L. For the initial pH control and nitrogen feeding, a solution of 15% ammonia water was added to the culture to maintain an ammonia–nitrogen concentration at 3 g N/L and a C/N ratio of 5. When cell density reached the desired level (OD600 about 80) at 40 h of incubation and no additional nitrogen was needed, the ammonia nitrogen solution and the glycerol solution were replaced with a NaOH/KOH (5M/5M) solution and glycerol: sodium octanoate solution (7: 3 ratio) to initiate the nitrogen limitation condition for mcl-PHAs accumulation. Aliquots of 25–35 mL culture medium were dispensed into pre-weighed polypropylene centrifuge tubes. Microbial growth was monitored with a UV/Vis spectrophotometer (Shimadzu UV-1700, Japan) by measuring the optical density of the culture medium at 600 nm. Fermentation was stopped when the OD600 began to decrease or 60 h of incubation. The samples of the culture medium were centrifuged at 5000 g for 10 min to separate the supernatant (culture solution) from wet pellets (PHAs-containing cells). The wet pellets were subsequently washed with distilled water and lyophilized to determine the dry cell mass and PHAs content.

4. Results and Discussion

4.1 Biosynthesis of natural mcl-PHAs polyester by batch fermentation

Batch fermentations were performed in a 5 L bioreactor containing 2.5 L mineral solution supplemented with glycerol as a carbon source and sodium octanoate as mcl-precursor. During 60 h of incubation the cells were harvested and OD600 and cell dry weight were measured as shown in Figure 1. The lag phase, log phase and stationary phase were at 0 to 20 h, 20-40 h and 40-60 h, respectively. This result suggested that at 40 h of incubation the limitation of nitrogen source should be set to lead the accumulation of PHAs in the cells (Tanadchangsaeng & Yu, 2013). The cells at 60 h of incubation period were harvested, lyophilized and were further extracted and characterized for chemical structure by nuclear magnetic resonance (NMR). The mcl-PHAs polymers that accumulated in the cells were extracted with chloroform (10 mL chloroform/1 g dry cells) as described above and the wet extract was white sticky matter (Figure 2a). After allowing the solvent to evaporate at 55 ^{o}C, the white dry PHAs (figure 2b) was further characterized by nuclear magnetic resonance.

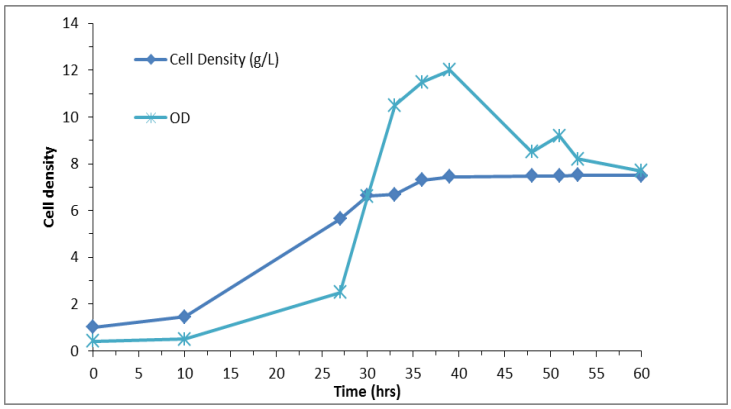


Figure 1 Showing the cell density (g/L) and optical density at 600 nm (OD) of *Pseudomonas putida* ATCC47054 cultured by batch fermentation

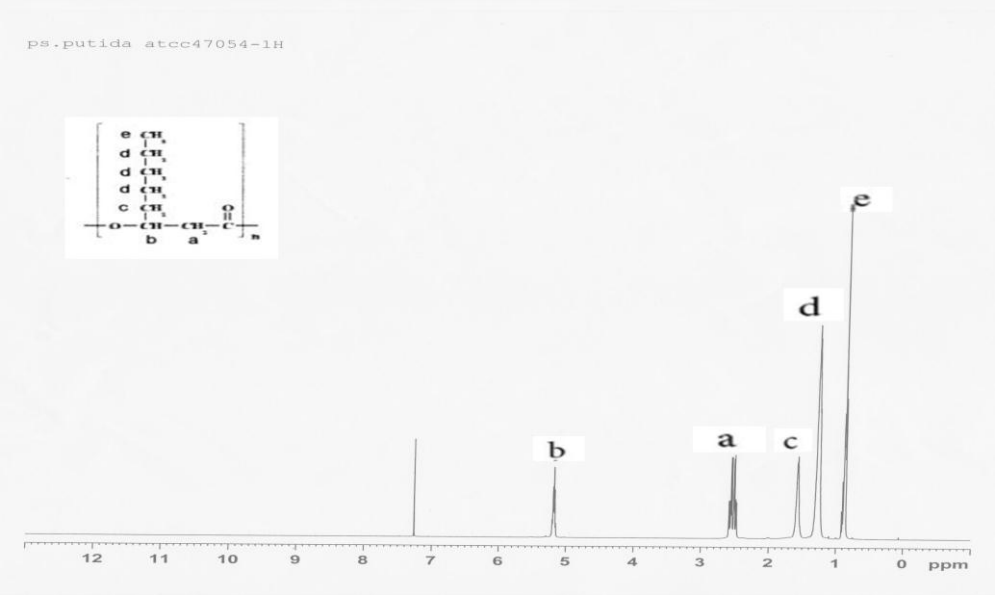
(2a) (2b)

Figure 2 The PHA–chloroform extract from *Pseudomonas putida* ATCC47054 lyophilized cells in wet form (2a) and dried form (2b)

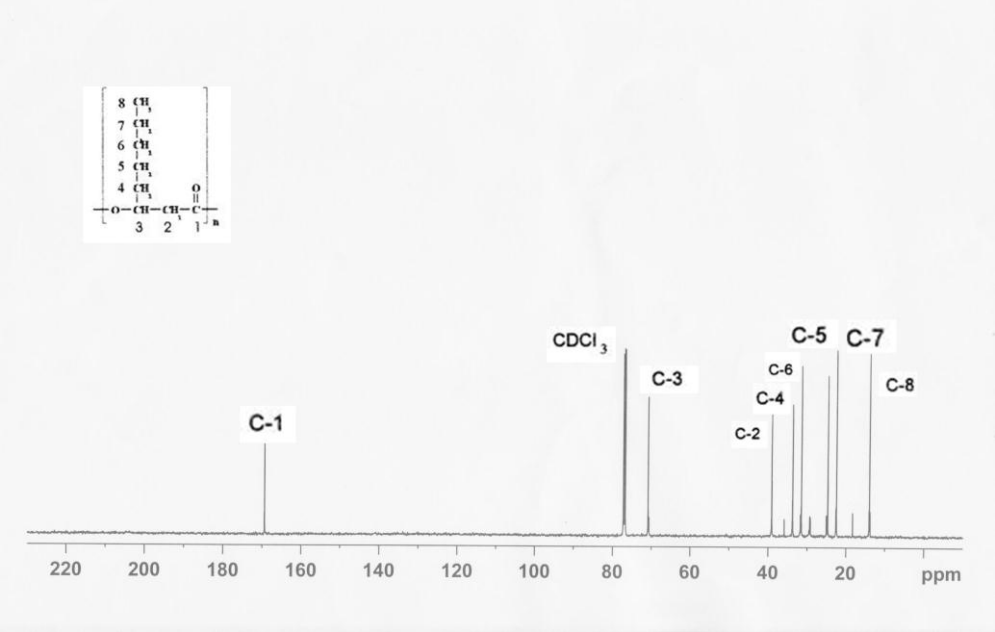
4.2 Nuclear magnetic resonance study of mcl-PHAs produced by *Pseudomonas putida* ATCC47054

In bacteria, mcl-PHAs are synthesized by using activity of PHA synthetase (pha C) which polymerized (R)-3-hydroxyacyl-CoA, the intermediates from catabolism of sugar or beta-oxidation of alkanoic acids or fatty acids. The monomer composition of the mcl-PHA produced is directly influenced by the carbon source added to the growth media (Steinbüchel, 2001). In this study, the carbon sources were glycerol and sodium octanoate which consist of 8 carbon atom and the results of proton and the number of carbons from NMR study showed that chemical structure of mcl-PHAs produced from *Pseudomonas putida* ATCC47054 composed of hydroxy octanoates (Figure 3a) and consisted of 8 carbons (Figure 3b), therefore the mcl-PHAs produced by *Pseudomonas putida* ATCC47054 was mcl-PHAs with 8 carbons and named as polyhydroxyoctanoates or PHO. Then, the amount of PHO in the cells harvested at 60 h was determined by gas chromatography and it was found that the percentage of PHO per cell dry weight was 47.37 and PHO yield was 11.61 g/L. These values were quite low when compared to the study of PHO production by recombinant *Pseudomonas putida* KT2440 grown on a defined medium supplemented with xylose as the growth substrate and octanoic acid as the precursor for mcl-PHA production which increased to 20% w w^{-1} of mcl-PHA was obtained. A yield of 0.37 g mcl-PHA per g octanoic acid was achieved under the employed conditions (Le Meur et al., 2012). In this work, the attempt was done by using sodium octanoate

which is an organic sodium salt comprising equal numbers of sodium and octanoate ions, high solubility in water instead of octanoic acid which is a colorless to light yellow liquid with a mild odor, burns and is corrosive to metals and tissue.



(3a)



(3b)

Figure 3 Nuclear magnetic resonance of PHAs produced by *Pseudomonas putida* ATCC47054 (3a) showing the proton of PHAs and revealed as hydroxy octanoate and (3b) showing the number of carbons found in the PHAs polymers which consisted of 8 carbons

4.3 Biosynthesis of natural mcl-PHAs polyester by fed batch fermentation

Glycerol and sodium octanoate were used as substrate and mcl-precursor, respectively by *Pseudomonas putida* ATCC47054 to synthesize mcl-PHAs biopolyesters. Unless specified otherwise, a fed-batch culture of mutated *Pseudomonas putida* ATCC47054 grown on glycerol and sodium octanoate was conducted in a 5-L bench top fermenter. The culture consisted of an initial cell growth phase on glycerol under nutrient-rich conditions, followed by mcl-PHAs formation from glycerol and sodium octanoate under nutrient limitation. Figure 4 shows time-dependent changes in dry cell density. At 40 h incubation, when the cell mass reached the desired level, the ammonia solution was replaced with a base solution to apply nitrogen limitation. Early nitrogen limitation can result in a low cell density (Tanadchangsaeng & Yu, 2013). During 40-60 h of incubation, the mcl-PHAs were formed. Then, the cells at 60 h were harvested and lyophilized. The mcl-PHAs polymers that accumulated in cells were extracted with chloroform (10 mL chloroform/1 g dry cells) for at least 48 h at 60 ^{o}C and purified by two rounds of precipitation with cold methanol. Polymer films were prepared by casting the PHA–chloroform solution onto a Petri dish and allowing the solvent to evaporate at room temperature for 1 week. The films were colorless, quite transparent, soft and possessed elasticity (Figure 5).

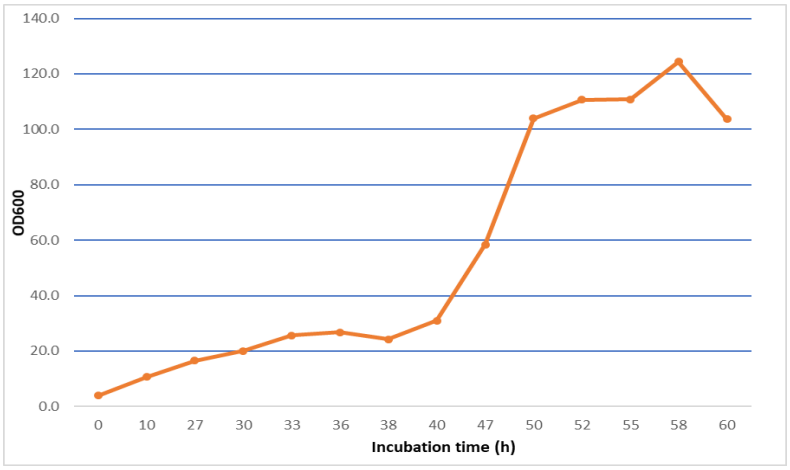


Figure 4 The optical density at 600 nm of *Pseudomonas putida* ATCC47054 at various incubation time of fed batch fermentation

Figure 5 The mcl-PHAs biopolymers film prepared by casting the PHA–chloroform solution onto a Petri dish and allowing the solvent to evaporate at room temperature for 1 week

5. Conclusion

In this work, the production of polyhydroxyoctanoates, a medium chain length polyhydroxyalkanoates (mcl-PHAs) by *Pseudomonas putida* ATCC47054 using glycerol as a growth carbon source and sodium octanoate as mcl-PHAs was performed successfully both in batch and fed batch fermentation. In batch fermentation, the percentage of PHO per cell dry weight and yield were quite low (47.37% and 11.61 mg/L, respectively), however the preliminary study for production of PHO by using fed batch fermentation revealed high cell weight and PHO production. It is worthy to note that the optimization and scaling up of PHO production by fed batch fermentation should be further studied.

6. Acknowledgements

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7. References

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