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Biosynthesis of medium chain length polyhydroxyalkanoates (mcl-PHAs) from palm oil

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ABSTRACT

Medium chain length polyhydroxyalkanoates (mcl-PHAs) are biopolyesters, derived from renewable biomass resources such as palm oil. These PHAs can be converted to added value products such as biopolymers, potentially increasing the economical worth of crude palm oil. Batch experiments were set up in an orbital shaker incubator at 30 °C to explore the feasibility of PHAs biosynthesized by *Pseudomonas aeruginosa* TISTR 1287 from palm oil. *P aeruginosa* was cultured under varied palm oil concentrations (0.50–2.00% w v^{-1}) and initial pH 7. The concentrations of palm oil and cultivation time influenced the growth of *P. aeruginosa* and intracellular accumulation of PHAs. Maximum cell dry weight of 2.33 g L^{-1} was obtained at 0.50% w v^{-1} palm oil after at 44-hr cultivation. The maximum PHAs concentration of 0.65 g L^{-1} and content was 38.02% at 0.75% w v^{-1} palm oil after 72-hr cultivation. Fourier Transform infrared (FTIR) and Gas Chromatography-Mass Spectrometry (GCMS) spectra indicated the biopolyesters are mcl-PHAs with heterogenous types of monomers. The findings showed the palm oil is a good economical, renewable feedstock of carbon for production of mcl-PHAs by *P. aeruginosa.*

1. Introduction

Palm oil has the lowest production cost among common vegetable oils, with 6–10 times greater yield per unit of land than other oil crops such as soy, rapeseed, sunflower, coconut and olive. Around 75% of crude palm oil (CPO) originating in Thailand is utilised domestically through production for food and biodiesel industry, with exports accounting for only a small fraction of total output. The growing cultivating areas surged CPO output by 45.6% to 2.6 million tonnes in 2017 and by another 5.8% to 2.8 million tonnes in 2018. The sharp rise in domestic CPO output and the phasing out of the EU on the use of biofuels derived from palm oil are the key influence of CPO price drop. Despite higher domestic consumption with the national policies to absorb excess supply of CPO in the system, the demand for CPO was insufficient to reduce CPO with prices dropping continuously since 2017 [1].

Petroleum-based plastics are generated from non-renewable resources and resistant to biodegradation in the environment. In response to this, waste plastic accumulation has become a global environmental issue and there has been considerable interest in the replacement of conventional plastics with degradable bioplastics. Polyhydroxyalkanoates (PHAs) are biopolyesters that accumulated in the cells of a wide range of bacteria, which have the potential of being used as renewable form of biomass. There are two groups of PHA according to the number of carbon atoms in the monomer units: short-chain length PHAs (scl-PHAs) with 3–5 carbon atoms in each monomer unit, and medium-chain length PHAs (mcl-PHAs) with 6–14 carbon atoms in each unit [2]. mcl-PHAs are attractive materials for biomedical applications, such as scaffolding for the regeneration of arteries and nerve axons, because of their natural origin, enhanced biocompatibility, biodegradability, and their ability to support cell growth and proliferation [3,4]. The most common examples of mcl-PHA are thermoplastic elastomers such as poly (3-hydroxyhexanoate) (P (3HHx)) and poly (3-hydroxyoctanoate) (P (3HO)) [5]. These materials are soft and

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flexible, having lower crystallinity and elastomeric nature, properties relating to side chain length (properties increases with increased side chain length). mcl-PHAs have not been widely used due to their high price of PHA production, which arises from the expensive carbon feedstocks, high recovery cost and relatively low PHA yield [6,7]. The present study focusd on the capability of *Pseudomonas aeruginosa* TISTR 1287, to biosynthesize mcl-PHAs from using a low-cost, renewable feedstock of variable palm oil concentrations using a lab-scale bioreactor. *P. aeruginosa* TISTR 1287, is a lipase producing bacterial strain locally isolated from shrimp pond in Thailand. Production of mcl-PHAs from the palm oil by this bacterial strain has never been reported by other research groups. The mcl-PHAs produced were structurally identified by FTIR and GCMS, while their thermal properties were determined by Differential Scanning Calorimetry (DSC).

2. Materials and methods

2.1. Microorganism

P. aeruginosa TISTR 1287 was obtained from Thailand institute of scientific and Technology Research (TISTR). *P. aeruginosa* was stored in 30% glycerol at −20 °C. It was cultured on nutrient broth (NB) medium to obtain cell mass for PHAs production. Cell growth was quantified by cell dry weight (CDW) and the optical density of the culture broth at 600 nm. CDW was analyzed according to APHA standard method [8]. The cell density was determined by the drop plate method [9]. This method was used to determine the numbers of initial microorganisms for PHAs production. The initial cell concentration of 10^{11} CFU mL^{-1} was used as inoculum in the PHAs production.

2.2. Culture media and palm oil

The culture media used in the PHAs production was mineral salt medium (MSM) [10]. Palm oil was used as a carbon source and gum arabic (GA) was used as an emulsifier. GA was mixed with palm oil at 0.30:1.00 mass ratio using homogenizer at 16,000 rpm for 1 min [11] before 300 mL MSM was added and sterile by autoclaving.

2.3. PHAs production and extraction

Batch experiments were conducted to determine the influence of palm oil concentration, 0.50–2.00% (w v^{-1}) range, on PHAs production. All batch experiments were performed in duplicate of 1000 mL volumetric flask and conducted at 30 °C in the orbital shaker incubator with 180 rpm for 72 hrs (Fig. 1). Liquid samples were collected for pH analysis and cell dry weight (CDW) measurement every 8 hrs. Cell suspension was collected for microscopic analyses; and PHAs extraction and structural analyses. The cell suspension was centrifuged to harvest the cells, washed with 0.85% NaCl solution and acetone solution to remove unused oil from cell surface. After twice washes, the cells were dried in an oven at 80 °C for 5 hrs. before extracted by dichloromethane [12].

2.4. Microscopic analyses of PHAs accumulation

Cell suspension collected after 24- and 44-hr cultivation was centrifuged and the pellet was resuspended in 1 mL of distilled water. Subsequently, 40 μL of Nile red (80 μg mL^{-1} dissolved in dimethyl sulfoxide [DMSO]) was added to the suspension to give a final concentration of 3.1 μg Nile red per mL suspension prior being observed under a fluorescence microscope at excitation and emission wavelength 535 and 605 nm [13].

The cell suspension after 72-hr cultivation was centrifuged and washed with 0.1 M PBS buffer solution (pH 7.4) and embedded in resin according to Tian et al. (2005) [14] prior to Transmission Electron Microscopy (TEM) imaging.

2.5. PHAs characterization

The samples were analyzed for the major functional groups on the purified PHAs biopolymer using PerkinElmer Frontier, FTIR spectrometer

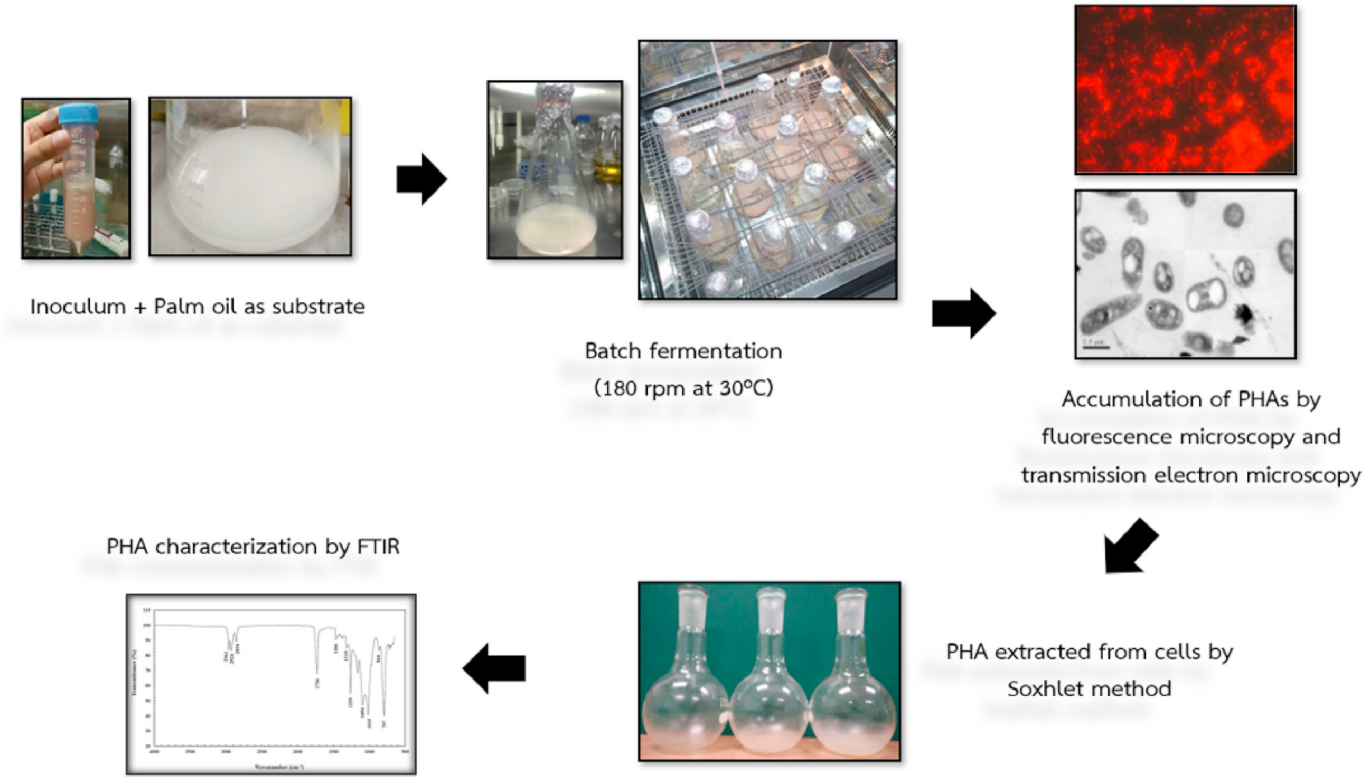

Fig. 1. Schematic of the PHAs production experiment.

operation in attenuated total reflectance (ATR) mode (45 mg of *P. aeruginosa* TISTR 1287). A thin PHA film was prepared and deposited on the ATR crystal and scanned between the ranges of 650–4000 cm^{-1} wavenumber with an accumulated frequency scan of 12.

In addition to FTIR characterisation, complementary thermal analysis was carried out using a PerkinElmer simultaneous thermal analysis STA600, hyphenated to a PerkinElmer Clarus 680 gas chromatograph (GC) and 600 T single quadrupole mass spectrometer (MS). The material was heated from 30 to 700 °C with a heating rate of 10 °C min^{-1} under a constant nitrogen flow of 30 mL min^{-1}. The GCMS was triggered to fill the sample loop of evolved gases at 300 °C. The sampled evolved gases were then separated on the GC using non-polar column with a 5% diphenyl dimethyl polysiloxane phase was purchased from Restek Ltd (Worthing, UK) using column dimensions of 250 μm for the internal diameter and a film thickness of 0.25 μm. The column flow was established at 1.2 mL minute (ultra-pure helium) and GC oven ramp of 50–300 °C at 10 °C min^{-1}. The inlet conditions were set at 250 °C with the split vent open at ratio of 20:1. Detection of separated bands using MS 600 was carried out using standard hot EI source at 70 eV and 250 °C with acquisition parameters operated in full scan mode between 50 and 400 *mz*.

3. Results and discussion

3.1. PHA production

P. aeruginosa TISTR 1287 was able to synthesize PHA under different concentrations of the palm oil. The result clearly indicated palm oil can be a good carbon source for the PHA production. The increase of palm oil concentration in the range of 0.50–2.00% significantly increased the cell mass with the maximum 2.93 g CDW L^{-1} at 0.50% palm oil after 60-hr culturation (Fig. 2). *P. aeruginosa* grew rapidly during 12-hr cultivation which corresponds to rapid decreasing pH from 7.00 to the range of 6.34–6.40 in all conditions (Fig. 2). When the bacteria catabolizes triglycerides in the palm oil, the lipase breaks the ester bonds of triglycerides and becomes fatty acids and glycerol, resulting higher acidity in the culture medium [15]. The microbial cells growth rate slightly increased after 12-hr culturation due to the continuously decreasing pH causing unsuitable condition for cell growth and a lack of balance between the amount of hydrogen and hydroxide ion affecting the uptake of substrates and nutrients into the cells [16].

3.2. PHA accumulation

The culture time of *P. aeruginosa* plays an important role on the PHA accumulation. Maximum CDW was obtained at 0.50% palm oil with maximum CDW after 44-hr cultivation (1.99 g CDW L^{-1}) (Fig. 3) while the maximum PHA concentration and content was obtained after 72-hr cultivation of 0.65 g L^{-1} and 38.02%, respectively. The percentage of mcl-PHAs accumulation in the bacterial cells can be varied between 0.94 and 86.00% dependent on the bacterial strains, types of substrates, and reactors' modes [17].

Fluorescence image (Fig. 3a and b) illustrated the area where intracellular lipids stained with Nile Red is fluoresced at the emission wavelength of 610 nm, indicating the synthesis and accumulation of PHAs inside the bacterial cells, while TEM image displayed round, insoluble inclusions of PHA located in the cytoplasm (white area) with approximately 0.2–0.5 μm granule size (Fig. 3c). Both microscopic images qualitatively confirmed the presence of PHA accumulation in the bacterial cells [18].

3.3. PHA characterization

FTIR absorption spectra were scanned in the range of 4000–650 cm^{-1}. The presence of methyl (CH3) and methylene (CH2) spectra in the region from 2961 to 2854 cm^{-1} and of ester carbonyl group (C═O) spectra at 1726 cm^{-1} (Fig. 4) strongly indicated mcl-PHAs [16]. The intermittence band between 1466 and 1000 cm^{-1} are attributed to the stretching vibration of the C–O groups. The strong vibration band at 1259 and 1094 cm^{-1} were observed of C–C and C–O stretching, respectively. Other characteristic bands present for PHA at 1015, 864 and 797 cm^{-1} were assigned to C–C group [15] (see Fig. 5).

GC-MS chromatogram confirmed the purified PHAs contained four mixed types of monomers including 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxy-2-octenoate (3H2O), and 3-hydroxydecenoate (3H2D) with the molar percentages of 5.21, 2.63, 88.85, and 3.31, respectively.

The DSC scan of the purified PHA showed that the decomposition of the polymer began at 260.95 °C and lost its 90% of mass at 315.29 °C (Fig. 3).

Based on the types of monomers in mcl-PHAs produced in this work, we suggest that in the palm oil, which consists of approximately 50% saturated fatty acids, with 44% palmitic acid (C16:0), 5% stearic acid (C18:0), and myristic acid (C14:0) could be the precursors of 3HHx, 3HO, and 3HD. The unsaturated fatty acids are approximately 40% oleic acid (C18:1) and 10% polyunsaturated linoleic acid (C18:2) and linolenic acid (C18:3) could be the precursors of 3H2O and 3H2D. Glycerol in the palm oil may be converted to acetyl-CoA and used to generate either biomass or intermediates for the synthesis of mcl-PHAs.

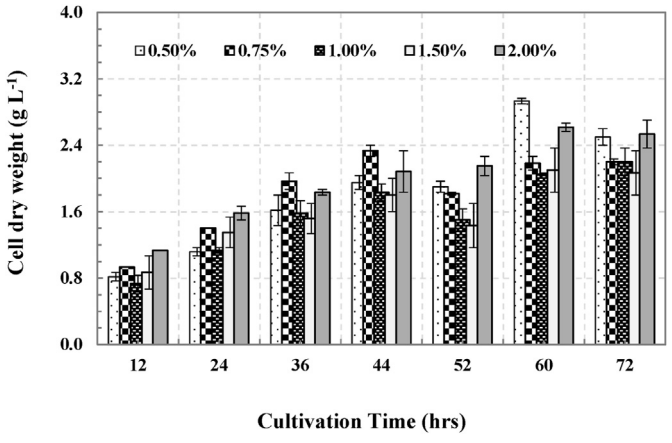

Fig. 2. Net CDW (g L^{-1}) at varying concentrations of palm oil concentration during 72-hr cultivation. Histogram bar represents average value of duplicate experiments (n = 2).

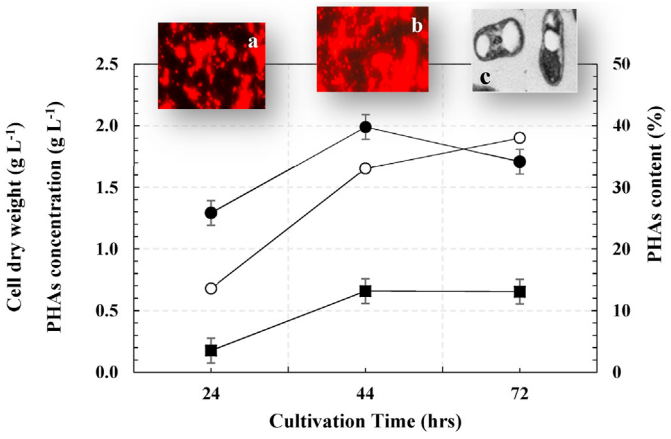

Fig. 3. CDW (g L^{-1}) (●) PHAs concentration (g L^{-1}) (■) and PHAs content (%) (○); and fluorescence and TEM images (magnification c:5,000x) of intracellular PHA granules after 72-hr cultivation of *P. aeruginosa* in 0.50% palm oil. Histogram bar represents average value of duplicate experiments (n = 2).

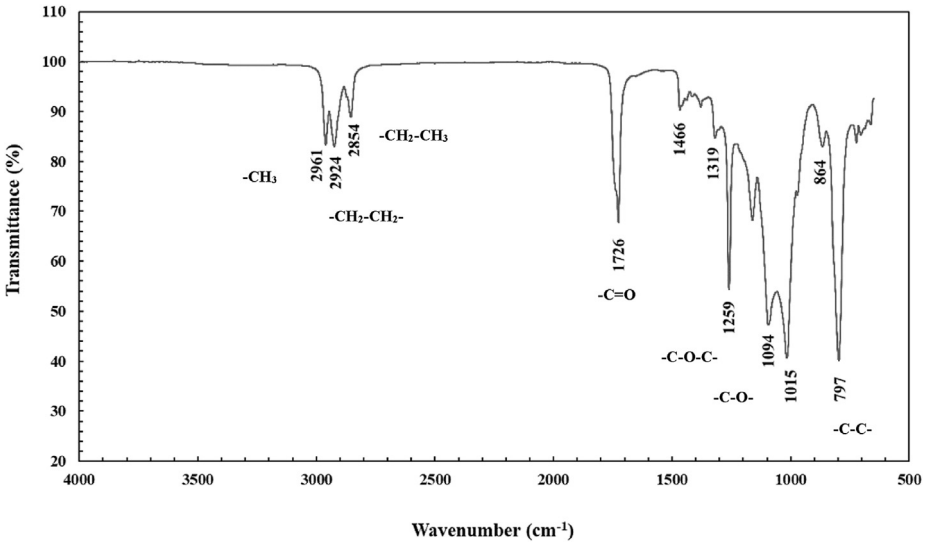

Fig. 4. FTIR spectra of purified PHA polyester of *P. aeruginosa*. The spectra were recorded ranging from 4000 to 650 cm^{-1} wavenumber.

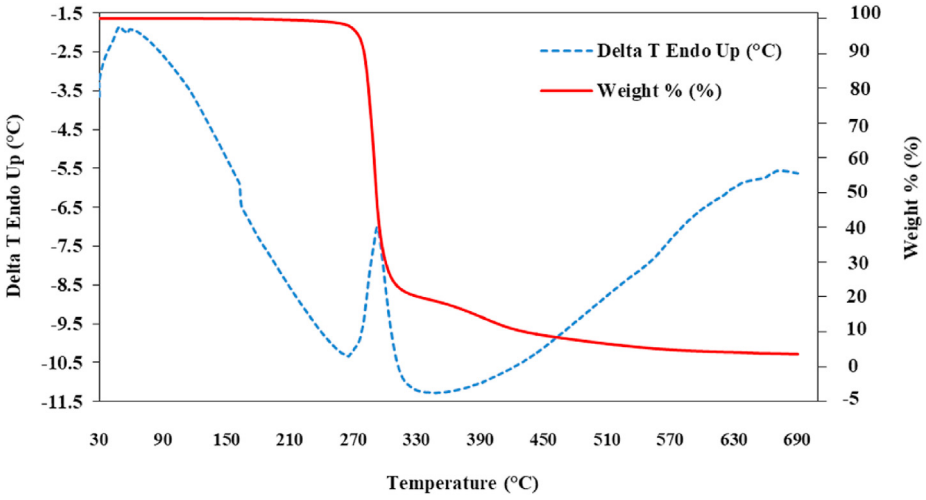

Fig. 5. Thermo-Gravimetric Analysis (TGA) thermogram of the purified mcl-PHA biopolyester starting to decompose at 260.95 °C and lost its 90% of mass at 315.29 °C (blue line) and DSC scanning result of the purified mcl-PHA (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

Pseudomonas aeruginosa TISTR 1287 is capable of synthesizing mcl-PHA with heterogenous types of monomers from palm oil. The maximum cell dry weight of 2.33 g L^{-1} was obtained after 60-hr cultivation at 0.50% palm oil. The palm oil concentrations and cultivation time influenced the bacterial growth and mcl-PHA synthesis. The maximum mcl-PHA concentration was 0.65 g L^{-1} (13.56%) after 44-hr cultivation and the highest mcl-PHA content was 38.02% after 72-hr cultivation. Palm oil is apparently renewable feedstock for mcl-PHAs production, yet further investigation on pilot-scale production and downstream PHAs extraction is required in order to elucidate the economic feasibility of the industrial applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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