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**Comparative performance of medium-chain-length Polyhydroxyalkanoates production
between *Pseudomonas aeruginosa* and *Pseudomonas putida***

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Abstract

Non-degradable polymer waste enlarged over increasing of human population. This badly affected on environmental pollution and biosphere changing. Polyhydroxyalkanoates (PHAs), an eco-friendly biopolymer, were substituted for conventional- or petrochemical-derived polymer. They are reserve carbon energy produced from diversity of microorganisms in granular under limited nutrient for bacterial growth (e.g. nitrogen and phosphorus). *Pseudomonas aeruginosa* TISTR 1287 was used to catalyze emulsified palm oil to produce valuable PHAs. The highest yield of PHAs production (0.65 g/L, 38.0%) was obtained in MSM supplemented with 0.75% (v/w) emulsified palm oil after 72-hr cultivation, which was a few lower than that produced by *Pseudomonas putida* TISTR 1522 (0.95 g/L, 40.15%) cultivated in 1% (w/v) fatty acid salt for after 24-hr cultivation. The intracellular PHAs were detected by staining with Nile red. The characters of intracellular PHAs examined by Transmission Electron Microscope (TEM) exhibited that PHAs accumulate in white and roundish-shaped granules with 0.2-0.5 μ m diameter inside the cells, about 2-3 granules per rod-shaped bacterium cell. These optimizations were successfully demonstrated high content of intracellular PHAs accumulation in *P. aeruginosa* TISTR 1287 by utilization of emulsified palm oil.

Keywords Polyhydroxyalkanoates (PHAs), *Pseudomonas putida*, *Pseudomonas aeruginosa*, emulsified palm oil, fatty acid salt

Introduction

The overpopulation of human has led to the accumulation of enormous amounts of non-degradable petrochemical polymer waste causing environmental pollution and dramatically changing of biosphere [1-3]. To reduce these problems, an environment-friendly polymer was developed to substitute for petrochemical-derived polymers. Polyhydroxyalkanoates (PHAs) are biopolymer produced in granular by a variety of bacteria under unbalance nutrient condition as carbon or reserve material [1, 4-5]. They have attracted attention as alternative plastic to replace petrochemical polymers with favorable properties of their complete biodegradability and production from a low-cost, renewable feedstock such as palm oil [6-7]. The high carbon content and abundance of palm oil are promising of

feedstock that can be converted into valuable PHAs by bacteria. *Pseudomonas aeruginosa* and *Pseudomonas putida* are promising microorganisms that accumulate high PHAs content and capably utilize a number of feedstock including plant oil [7-10]. The fatty acids in plant oil were favorable carbon source for bacteria growth and catabolized via α -oxidation to produce PHAs [8, 10]. The PHAs have a broad range of advantageous applications, for instance, a nanoparticle for drug carrier, a part of bioplastic element for molded plastic or single-used polymer, and fertilizer coating film which was applied in agriculture [12].

This study reported the optimization of PHAs production by *Pseudomonas aeruginosa* TISTR 1287 from emulsified palm oil compared with our previous study by *Pseudomonas putida* TISTR 1522, lack of secretes lipase ability, from fatty acid salt [7]. The presence and the characters of intracellular PHAs accumulation were analyzed by Nile red staining, fluorescence microscope and Transmission Electron Microscope (TEM).

Materials and methods

Microorganisms and palm oil

Pseudomonas aeruginosa TISTR 1287 was obtained by Thailand Institute of Scientific and Technological Research. This pure culture was stored in 30% glycerol at -20 °C.

Palm oil (Morakhot olein palm oil), a plant oil, available in local market was used as a sole carbon source for PHAs production. The composition of fatty acid in palm oil was determined as described previously [8]. Gum arabic was discovered as an effective emulsifier for palm oil, with no effect on bacterial growth [10]. In this study, for PHAs production by *P. aeruginosa* TISTR 1287, bacteria secretes lipase, palm oil was emulsified using Gum arabic as emulsifying agent. Emulsified palm oil was sterilized, mixed with culture medium and adjusted pH to 6.90 [6, 10, 13]. In comparison to our pervious study, fatty acid salt obtained from saponified palm oil was used as a carbon source to produce PHAs by *P. putida* TISTR 1522, due to its lack of secreted lipase [11].

Culture medium and cultivation condition

Cells were grown in Nutrient Broth (NB) and induced for PHAs production in Mineral Salts Medium (MSM) [9]. The MSM was mixed with emulsified palm from previous experiment before using for PHAs production by *P. aeruginosa* TISTR 1287. Experiments were set up in an orbital shaker incubator (180 rpm, 30°C). Cells were harvested by centrifugation at 9,000 rpm for 20 min.

To obtain cell mass for PHAs production, *P. aeruginosa* TISTR 1287 was grown overnight in 50 mL NB (first-subculture) before transferring to 500 mL NB (second subculture). During late-exponential growth, bacterial cells were harvested by centrifugation and washed with MSM. The standard curve between Colony Forming Unit (CFU) and cell dry weight (CDW) content was used to calculate the amount of initial bacterial cells (10^{11} CFU mL⁻¹) for PHAs production in MSM supplemented with emulsified palm oil [10]. For *P. putida* TISTR 1522, cell mass was obtained as described above.

Optimization for PHAs production

The influence of various parameters on PHAs production by *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522 was studied which are concentration of emulsified palm oil, pH values, CDW content and cultivation time. To optimize concentration of carbon source for PHAs production, bacteria was grown in 300 mL MSM supplemented with various concentration of emulsified palm oil (0.50, 0.75, 1.00 and 1.50% (w/v)) for *P. aeruginosa* TISTR 1287, and various concentration of fatty acid salt (0.50, 0.75, 1.00 and 1.50% (w/v)) for *P. putida* TISTR 1522. The pH value of culture medium was measured every 8 hr by pH meter (Mettler Toledo LE409). To determine CDW content, 2 mL of culture medium was harvested by centrifugation, washed and dried at 105 °C for 2 hr [14]. The percentage of emulsified palm oil and fatty acid salt that gave the highest CDW content was selected for the following optimization cultivation time.

PHAs determination

The accumulation of intracellular PHAs was monitored by fluorescence microscope and Transmission Electron Microscope (TEM). In brief, the aliquot of PHAs-accumulating cell was

harvested by centrifugation, smeared on glass slide, fixed by heat, stained with 0.05 mM Nile red for 30 min in darkness and analyzed by Fluorescence Microscope [15]. To study the character of intracellular PHAs, TEM was performed. In brief, PHAs-accumulating cell was collected, washed and stored in 2.5% glutaraldehyde solution overnight. The resuspension was dehydrated and embedded in BEEM capsule. The polymerized plastic was trimmed, stained with lead citrate followed by 1-2% uranyl acetate. The sample was washed, dried and analyzed by TEM (TECNAI 20 TWIN) [7].

PHAs extraction

PHAs extraction was performed as described previously [7]. The PHAs-accumulating cells were harvested, washed with 0.85% (w/v) NaCl solution and dried at 80 °C for 5 hr. The dried cells (0.4 g) were soaked in 95% ethanol, placed in Cellulose Extraction Thimble and extracted by dichloromethane for 5 hr using Soxhlet extractor. The dried weight product (PHAs) was determined after incubation of pellet at 70 °C for 2 hr.

Results and Discussion

Monitoring of bacterial growth

In the growth curve of *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522, late-exponential growth of bacterial cells exhibited between 10-24 hr and 5-11 hr, respectively (Fig 1a). Moreover, the first- and second-subculture showed both late-exponential growths after 24 hr cultivation for *P. aeruginosa* TISTR 1287, and after 18 and 30 hr cultivation for *P. putida* TISTR 1522 (Fig 1b, c). The initial amount of bacterial cells that calculated from standard curve between CFU and CDW content of *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522 (Fig 1d, e) was grown in MSM supplemented of different concentration of emulsified palm oil or fatty acid salt for PHAs production.

Fig. 1 The growth comparison between *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522 from (a) purified stock culture; (b) first-subculture; (c) second-subculture in Nutrient Broth (NB); The linear correlation graph between CFU and CDW content of (d) *P. aeruginosa* TISTR 1287 and (e) *P. aeruginosa* TISTR 1287 which were used to calculate the amount of initial bacterial cells for PHAs production.

Influence of carbon source concentration on pH value and CDW content

The culture medium of *P. aeruginosa* TISTR 1287 in MSM supplemented with different concentration of emulsified palm oil was taken at interval time to measure the changes of pH value and CDW content. The result exhibited that pH value of culture medium of *P. aeruginosa* TISTR 1287 rapidly decreased in the first 12 hr cultivation for all emulsified palm oil concentration and then remained or slightly decreased. On the other hand, previous study reported that the pH value of culture medium of *P. putida* TISTR 1522 extremely decreased in the first 16 hr cultivation for all carbon source concentration and then slightly increased over cultivation time [7], which showed the pH values in the ranging of 6.34-6.40 for *P. aeruginosa* TISTR 1287 and 6.22-6.47 for *P. putida* TISTR 1522 (Fig 2). The pH value of culture media decreased in consequence of triglyceride catabolism in palm oil by secreted lipase from *P. aeruginosa* TISTR 1287, which breaks ester bond of triglycerides into fatty acids and glycerol resulting in lower pH value in culture medium [16]. Moreover, the variation of pH value in culture medium depends on types and concentration of carbon sources [17].

The result of CDW content (Fig 3) exhibited rapid bacterial growth in the first 12 hr cultivation for *P. aeruginosa* TISTR 1287 (this study) and 24 hr cultivation for *P. putida* TISTR 1522 [7] which correspond to rapidly decreasing of pH value of culture medium. The highest CDW content was 2.33 g/L in 0.75% (w/v) emulsified palm oil after 44 hr cultivation of *P. aeruginosa* TISTR 1287 and 3.00 g/L in 1.00% (w/v) fatty acid salt after 24 hr cultivation of *P. putida* TISTR 1522. Afterwards, bacterial growth rate decreased result from continuously decreasing of pH value which was unsuitable for bacterial growth. This result led to unbalance of hydrogen and hydroxide ions which impact on nutrients feeding of bacterial cell [18]. The observation could be established the correlation between the variation of pH value of culture medium and CDW content, and carbon source limitation for bacterial growth and PHAs accumulation which was 0.75% (w/v) emulsified palm oil for *P. aeruginosa* TISTR 1287 (this study) and 1.00% (w/v) fatty acid salt for *P. putida* TISTR 1522 [7]. Therefore, this emulsified palm oil concentration was selected for further optimization for PHAs production.

Fig. 2 pH variation of *P. aeruginosa* TISTR 1287 during 72-hr cultivation in MSM supplemented with various concentration of emulsified palm oil.

Fig. 3 CDW variation of *P. aeruginosa* TISTR 1287 during 72-hr cultivation in MSM supplemented with various concentration of emulsified palm oil.

Profile of PHAs production

The PHAs-accumulating cells were taken at interval time, extracted to measure CDW content and PHAs concentration. The result revealed that PHAs concentration increased over cultivation time up to maximum value at 0.65 g/L with CDW content of 1.71 g/L and 38.0% PHAs content after 72 hr cultivation for *P. aeruginosa* TISTR 1287 (Table 1). This observation was noticeable that PHAs accumulation and CDW content increased when the pH value decreased (Fig 4), consistent with previous result that PHAs production by *P. putida* TISTR 1522 in MSM supplemented with 1% fatty acid salt which obtained maximum value at 1.23 g/L with CDW content of 3.23 g/L after 60 hr cultivation [7]. Accordingly, *P. putida* KT2442 and *P. aeruginosa* ATCC 9027 produce PHAs during exponential growth [19-20], and PHAs production relied on the nutrient depletion on medium [6, 9, 11, 13-14]. These result show that emulsified palm oil and fatty acid salt can be a good carbon source for PHAs production using *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522. Comparison of our PHAs production yield with that from other reports reveals that our PHAs production from *P. aeruginosa* TISTR 1287 was higher than previously reported data using other *P. aeruginosa* strains, and comparable or higher than previously reported data using other *P. putida* strains (Table 2). The FTIR result of purified PHAs that produced from *P. aeruginosa* TISTR 1287 cultivated in 0.75% (w/v) emulsified palm oil, and NMR result of purified PHAs obtained from *P. putida* TISTR 1522 cultivated in 1.0 % (w/v) fatty acid salt were characterized as medium-chain length PHAs [7, 27]. These previous results were consistent with previous report that there are 2 major monomer units (medium-chain length) of PHAs production from palm oil which are 3-hydroxyoctanoate (C8) and 3-hydroxydecanoate (C10) [8].

Table 1

Fig. 4 The correlation between PHAs content, CDW content and PHAs concentration of *P. aeruginosa* TISTR 1287 during 60-hr cultivation in MSM supplemented with 0.75% emulsified palm oil.

Table 2

PHAs accumulation analysis by fluorescence microscope and Transmission Electron Microscope (TEM)

The present of intracellular PHAs was monitored by staining of Nile red on PHAs-accumulating cells and visualizing by fluorescence microscope. An increasing of fluorescence intensity was observed inside bacterial cells which clearly demonstrate *P. aeruginosa* TISTR 1287 accumulated intracellular PHAs (Fig 5). However, no significantly different in fluorescence intensity during 24 and 44 hr cultivation was observed. Therefore, this technique can be used for only physical analysis to monitor the PHAs production. Similarly, PHAs accumulation in *P. putida* TISTR 1522 was noticed by Nile red staining [7].

Accumulated PHAs inside the *P. aeruginosa* TISTR 1287 from TEM image are white, roundish-granules with approximately 0.2-0.5 μ m diameter (Fig 6). About 2-3 granules of intracellular PHAs were observed inside a single rod-shaped bacterium, consistent with granule character from previous studies of *Pseudomonas* sp. [7, 28-29].

Fig. 5 Nile red staining of PHAs accumulation by *P. aeruginosa* TISTR 1287 in MSM supplemented with 0.75% (w/v) emulsified palm oil (a) 24-hr (b) 44-hr cultivation visualized by fluorescence microscope with 400x magnification.

Fig. 6 The PHAs character accumulating inside *P. aeruginosa* TISTR 1287 in MSM supplemented with 0.75% (w/v) emulsified palm oil during 72-hr cultivation under TEM with (a) 2,500x and (b) 5,000x magnification.

CONCLUSION

Substitution of petrochemical-derived polymer by environmentally degradable biopolymer such as PHAs was developed to reduce environmental pollution. One of the major limitations of the PHAs production in industry is mainly depends on cost of carbon source for bacterial growth. This study demonstrated that a low-cost and abundance palm oil can be a good carbon source for production of valuable PHAs as it can induce the high content of PHAs accumulation using *P. aeruginosa* TISTR 1287. We also concluded that the culture medium supplemented with 0.75% (v/w) emulsified palm oil is the most suitable condition for intracellular PHAs production by *P. aeruginosa* TISTR 1287 in lab scale. However, it is unlikely to apply in industry owing to cost of gum arabic. Thus development of utilization of low-cost materials could lead to economical and sustainable PHAs manufacturing in the future.

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Ethical Approval

Our work did not involve human subjects and/or animals.

Consent to Participate

'Not applicable'

Consent to Publish

'Not applicable'

Authors Contributions

Conceptualization: Nipon Pisutpaisal and Pinanong Tanikkul; Methodology: Siriorn Boonyawanich, Nipon Pisutpaisal, and Pinanong Tanikkul; Formal analysis and investigation: Pornpanna Thenchartanan, Nipon Pisutpaisal and Pinanong Tanikkul; Writing - original draft preparation: Pornpanna Thenchartanan, Nipon Pisutpaisal and Pinanong Tanikkul; Writing - review and editing: Nipon Pisutpaisal; Funding acquisition: Nipon Pisutpaisal; Resources: Nipon Pisutpaisal; Supervision: Nipon Pisutpaisal.

We confirm that the manuscript has been read and approved by all named authors.

We confirm that the order of authors listed in the manuscript has been approved by all named authors.

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Competing Interests

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.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Figures

Figure 1

The growth comparison between *P. aeruginosa* TISTR 1287 and *P. putida* TISTR 1522 from (a) purified stock culture; (b) first-subculture; (c) second-subculture in Nutrient Broth (NB); The linear correlation graph between CFU and CDW content of (d) *P. aeruginosa* TISTR 1287 and (e) *P. aeruginosa* TISTR 1287 which were used to calculate the amount of initial bacterial cells for PHAs production.

Figure 2

pH variation of *P. aeruginosa* TISTR 1287 during 72-hr cultivation in MSM supplemented with various concentration of emulsified palm oil.

Figure 3

CDW variation of *P. aeruginosa* TISTR 1287 during 72-hr cultivation in MSM supplemented with various concentration of emulsified palm oil.

Figure 4

The correlation between PHAs content, CDW content and PHAs concentration of *P. aeruginosa* TISTR 1287 during 60-hr cultivation in MSM supplemented with 0.75% emulsified palm oil.

Figure 5

Nile red staining of PHAs accumulation by *P. aeruginosa* TISTR 1287 in MSM supplemented with 0.75% (w/v) emulsified palm oil (a) 24-hr (b) 44-hr cultivation visualized by fluorescence microscope with 400x magnification.

Figure 6

The PHAs character accumulating inside *P. aeruginosa* TISTR 1287 in MSM supplemented with 0.75% (w/v) emulsified palm oil during 72-hr cultivation under TEM with (a) 2,500x and (b) 5,000x magnification.

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