Jurnal Teknologi

Full paper

Importance of Glucose and Pseudomonas in Producing Degradable Plastics

Farzaneh Sabbagh Mojaveryazdi[a*], Ida Idayu Muhamad[a], Shahabaldin Rezania[b], Hiro Benham[c]

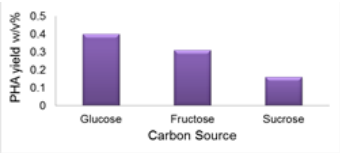
[a]*Faculty of Chemical Engineering Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*
[b]*Faculty of Civil Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*
[c]*Faculty of Bioscience, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*

*Corresponding author: farzaneh2464@yahoo.com.my

Article history

Received :5 March 2014
Received in revised form :
19 April 2014
Accepted :3 May 2014

Graphical abstract



Abstract

In this study, bacteria, *P. oleovorans*, was studied for its ability to produce PHA with glucose, fructose and sucrose as carbon sources that was grown at 25°C. The functional groups of the extracted PHA granules were identified as C=O group by Fourier transform Iinfrared (FTIR) spectroscopy analysis. The drastic absorption band at approximately 1720 cm^{-1} indicates the stretching vibration of the C=O groups in the PHA polyester. The optimized production of PHA was done by RSM (Response Surface Method) through various growth parameters. The best condition of productivity range for glucose is 93.4419 g/L. In addition, the highest PHA production after optimization is 2.28236 g/L with a desirability of 0.986 g/L, meanwhile the highest amount of PHA produced from *P. oleovorans* was 2.30 g/L.

Keywords: *Pseodomonas oleovorans*; carbon source; PHA; FTIR

Abstrak

Dalam kajian ini, kemampuan bakteria *P. oleovorans* untuk menghasilkan PHA dengan glukosa, fruktosa dan sukrosa sebagai sumber karbon ditumbuh pada 25°C telah dikaji. Kumpulan berfungsi daripada ekstrak granul PHA dikenal pasti sebagai kumpulan C=O dengan menggunakan analisis spektroskopi inframerah transformasi Fourier (FTIR). Jalur penyrerapan drastic lebih kurang pada 1720 cm^{-1} dan ini menunjukkan peregangan getarandaripada kumpulan C=O terkandung di dalam polyester PHA. Pengeluaran pengoptimuman PHA telah dilakukan dengan RSM (kaedah permukaan respons) melalui pelbagai parameter pertumbuhan. Setelah pengoptimuman dijalankan, keadaan produktiviti dalam julat yang terbaik diperolehi adalah glukosa dengan nilai 93.4419 g/L. Pengeluaran PHA yang paling banyak selepas pengoptimuman adalah 2.28236 g/L dengan kebolehinginan 0.986 g/L, sementara itu jumlah PHA yang paling banyak adalah dihasilkan oleh *P. oleovorans* dengan 2.30 g/L.

Kata kunci: *Pseodomonas oleovorans*; sumber karbon; PHA; FTIR

1.0 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polymers of hydroxyalkanoates. These polymers are often agglomerated insome bacteria as carbon and energy or to reduce storage materials in the bacteria [1]. PHAs are stored in the bacterial cytoplasm as inclusion bodies [2] and they are synthesized and accumulated intracellular as clear granules. These polymers are n [3] Cyctoplasm as inclusion bodies [2] and they are synthesized and accumulated intracellular as clear granules. These polymers are naturally synthesized biopolymers and decomposed by some microbial metabolisms, even though these biopolymers are able to be melted and molded similar to chemical and unnatural thermoplastics [3].

2.0 MATERIALS AND METHOD

Pseudomonas Sp. is one of the most versatile PHA accumulators. It is the bacteria strain used in all the current experiments. The organism was bought from United States Department of Agriculture (USDA). This genus has been strongly considered for its ability to synthesize Mcl-PHA [4]. The bacteria were grown in a 250 mL conical flask which contained 100 mL of MBM broth with different carbon sources such as sucrose, glucose and fructose, with concentration between 90–110 g/L. All flasks were incubated at 30°C on a shaker at 150 rpm for 48 hours. Based on the PHA yields, the best carbon source using sucrose was determined [5]. A central design (CCD) with a type response surface methodology (RSM) design was used.

2.1 Determination of Bacteria Density and Cell Dry Weight

It was found that the PHA accumulation was in proportion to the bacterial density and cell weight when the bacterial culture was incubated for four days. The most amount of accumulation was observed on the second and third days of bacteria growth, respectively [6]. Cell concentration was determined by measuring the cell's dry weight (CDW). The bacterial culture was centrifuged at 6000 rpm for 7 minutes and washed with distilled water to obtain the pellet form of the cell and dried to estimate the dry cell weight in units of g/L. The precipitated cells by centrifugation were transferred to a 15 mL universal bottle. Bottles containing cells were dried in an oven at 55°C and cooled to room temperature before any measurements were taken. Cell's dry weight values were also recorded. Residual biomass was estimated by the difference in weight between dry cell and extracted PHA. The percentage of intracellular PHA accumulation is estimated by the composition percentage of PHA present in the dry cell weight.

$$\text{Residual biomass (g/L)} = \text{CDW (g/L)} - \text{Dry weight of extracted PHA}$$

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L)} \times 100\ \%}{\text{CDW (g/L)}}$$

2.2 Extraction of PHA

The PHA was directly extracted using chloroform as the solvent. The cells were incubated at 95°C for 1 h and filtered before the sediment granules were incubated in an oven at 65ºC till dry. Chloroform was added to the dried granules and incubated at 95°C for 10 min. After cooling, the mixture was moderately mixed overnight. Then the solution was filtered to get the debris. Eventually, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water and then was washed with acetone and dried [6]. Sudan black-B is a procedure to confirm the presence of PHA as intracellular granules by staining the cells [7]. After the complete production of PHA under appropriate growth conditions, by using a clean glass slide, a thin smear of strains was made and was heat-fixed. This smear was air dried and fixed by immersing in 2% acetic acid for 5 min. This glass slide containing the sample was immersed in a filtered solution of 0.3 % (w/v) Sudan black-B (0.3 g of Sudan black in 100 mL of ethanol) and kept at room temperature for 15-20 min. The excess stain was drained off. Eventually, the microscopic slide was counter-stained for 10 s with (0.5 % w/v) aqueous safranin as the second dye. After that, the slide was rinsed with distilled water and examined under a microscope [6].

2.3 PHA Identification by UV Spectrometer

Briefly, 3 mL of bacterial culture grown in N-free medium was transferred to plastic centrifuge tubes. These tubes were washed with acetone and methanol to remove plasticizers and centrifuged at 5000 rpm for 10 min. The cell pellet was suspended in 10 mL of standard sodium hypochlorite solution and incubated at 37ºC for 2 h to complete the digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was scrapped. The sediment was washed twice with 10 mL of distilled water and was centrifuged. The PHA granules in the sediment were washed twice with three ingredients; acetone, methanol and diethyl ether. The polymer was dissolved in boiling chloroform and the chloroform was then evaporated. Eventually, the polymer was mixed with 10 mL of concentrated sulfuric acid and the capped tube was heated for 10 min at 100°C in a water bath. PHA's concentration was determined from an established standard graph in which the absorbance was plotted against the concentration of crotonic acid as the standard (235 nm). PHA granules which were extracted from the boiling chloroform method were used for measurement at a UV spectrum between 200 nm and 400 nm. To confirm the presence of PHA, indicated by the presence of a peak, should be obtained between 230-240 nm [6].

2.4 Estimation of Phase by Crotonic Acid Essay

The amount of PHA in the sample can be determined by the spectrophotometric assay. PHA polymer was extracted by chloroform and was exposed to evaporate the chloroform from the tube. Then 10 mL of sulfuric acid was added to the polymer and was heated at 100ºC on a water bath for 10 min. By adding sulfuric acid (H_2SO_4) to the polymer, it converts to crotonic acid and the color inside the glass tube changes to brown. Crotonic acid standard solution was prepared with different concentrations (10–40 µg). Before the determination of PHA, the solution was diluted by sterile distilled water. This solution was diluted with the dilution factor of 10. As the initial solution contains concentrated sulfuric acid and cannot be read by UV-spectrophotometer, dilution of the solution is needed. PHA in the extracted sample was confirmed by FTIR spectroscopy. The absorbance of the UV-spectrophotometer was set to 235 nm and the sample was transferred to a silica cuvette and the absorbance reading of the sample was recorded [8].

2.5 Fourier Transform-Infrared Spectroscopy Analysis (FTIR Analysis)

The PHA extracted from the *P.oleovorans* was analyzed by FTIR spectroscopy. To confirm the functional groups of the extracted polymer, the spectrum was recorded in it was used in 400-4000 cm^{-1} spectral range. The presence of absorption bands at 1722cm^{-1} and 1279 cm^{-1} are attributed to the carbonyl bands C=O and C–O stretching ester in polymer. The bands at 1183 and 1134 cm^{-1} are characteristic of the asymmetric and the symmetric stretching of the C–O–C group, respectively [6].

3.0 RESULTS AND DISCUSSION

3.1 Effect of Different Carbon Sources on Production of PHA

The effect of different carbon source for PHA composition has been studied. The production of PHA accumulated by *P. oleovorans* grown on different carbon sources at 30°C for 48 hours is presented in Figure 1. Among the three sources of carbon, it was found that glucose was the best source of carbon which gave PHA yield of 0.400 w/v%. Other sugars tested, such as fructose and sucrose, yielded less PHA, which are 0.310 and 0.160 w/v%, respectively. The glucose molecule is made up of six carbon atoms, 12 hydrogen atoms and six oxygen atoms. As most of the carbon atoms are in a reduced state, the molecule contains lots of energy. By reacting the carbon in such a way that it is oxidized rather than reduced, allowing the bacteria to liberate energy from glucose and use it for cellular processes.

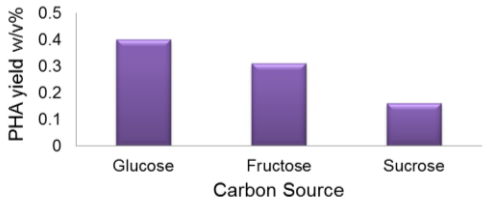


Figure 1 Effect of different carbon source on PHA production (w/v%)

3.2 Isolation and Characterization of PHA

The UV absorption spectra in Figure 2 show a distinct absorption peak around 230 nm. The sample containing PHA was digested in concentrated sulfuric acid and consequently diluted with 0.014 N of H_2SO_4 (0.3 to 12 µg of sample per mL of final concentration). UV analysis of the product from sulfuric acid digestion of sample confirmed the presence of a single peak whose retention time was identical to that of crotonic acid. The UV spectrum of this product was identical to that of crotonic acid. PHA granules which were extracted by boiling chloroform method were used for UV measurement between 200 nm and 400 nm. PHA's concentration was determined from an established standard graph in which the absorbance was plotted against the concentration of crotonic acid as the standard (235 nm). To confirm the presence of PHA, the presence of a peak should be obtained between 230–240 nm.

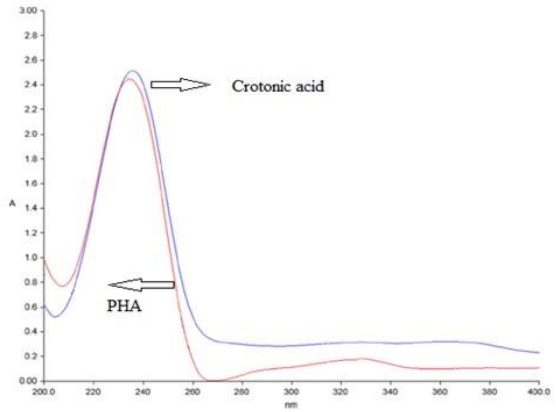


Figure 2 UV Spectrophotometer of PHA (red line) and crotonic acid (blue line)

3.3 FTIR Analysis

The PHA extracted from the *P.oleovorans* was analyzed by FTIR spectroscopy. To confirm the functional groups of the extracted polymer, it was recorded in the range of 400–4000 cm^{-1}. The spectrum shows the presence of different conformations bands in the extracted PHA from mixed culture. The presence of absorption bands at 1722 cm^{-1} and 1279 cm^{-1} demonstrated the carbonyl bands C=O and C–O stretching ester in polymer, respectively. The bands at 1183 and 1134 cm^{-1} are characteristic of the asymmetric and the symmetric stretching of the C–O–C group, respectively [6]. As shown in Figure 3, the drastic absorption band located at approximately 1720 cm^{-1} indicates the stretching vibration of the C=O groups in the PHA polyester. Accompanying bands of the C-O-C groups emerged in the spectral region of1150 to 1300 cm^{-1}. One more absorption region is from 2800 to 3100 cm^{-1}, which corresponds to the stretching vibration of C-H bonds [6]. The absorption band at 2955.76 cm^{-1} is assigned to an asymmetric methyl group. Asymmetric CH_2 of the lateral monomeric chains were assigned to the stretching vibration at 2925.98 cm^{-1}. Absorption at 1378.83 cm^{-1} is assigned to terminal CH_3 groups. Series of absorption bands at 1166.87 cm^{-1} to 619.39 cm^{-1} were assigned to C–O and C–C stretching vibration in the amorphous phase [9].

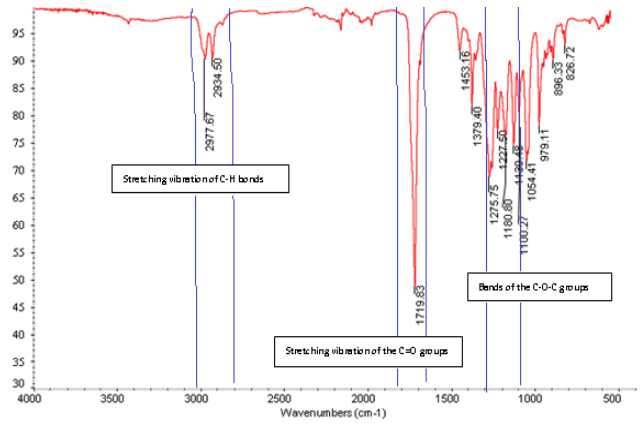


Figure 3 FTIR spectrum of PHA produced by Pseudomonas oleovorans

4.0 CONCLUSION

Bacteria, *P. oleovorans*, is able to produce PHA by carbon sources at 25 °C as proven by the presence of C=O functional groups from PHA in the FTIR spectrum. The optimized production of PHA was carried out by Response Surface Method through various growth parameters. It was found that glucose was the best source of carbon at 30 °C for 48 h. Thus, bacteria can liberate energy from glucose and use it for cellular processes.

Acknowledgement

The authors would like to thank Universiti Teknologi Malaysia (UTM) for providing necessary facilities to carry out this research.

References

[1] H. Salehizadeh, M.C. Van Loosdrecht. 2004. Production of Polyhydroxyalkanoates by Mixed Culture: Recent Trends and Biotechnological Importance. Kluyver Laboratory for Biotechnology.
[2] S.Y. Lee. 1996. Bacterial Polyhydroxyalkanoates. *Biotechnol Bioeng.* 49(1): 1–14.
[3] M. Koller, R. Bona, G. Braunegg, C. Hermann, P. Horvat, M. Kroutil, J. Martinz, J. Neto, P. Varila, L. Pereira. 2005a. Production of Polyhydroxyalkanoates from Agricultural Waste and Surplus Materials. *Biomacromolecules*. 6: 561–565.
[4] M.R. Zakaria, S. Abdul.-Aziz, H. Ariffin, N. A. Abdul Rahman, P.L. Yee, M. A Hassan. 2008. Comamonas sp. EB172 Isolated from Digester Treating Palm Oil Mill Effluent as Potential Polyhydroxyalkanoate (PHA) producer. *Afr. J. Biotechnol.* 7: 4118–4121.
[5] P. S. Chandrashekharaiah. 2005. Isolation, Screening and Selection of Efficient Poly-B-hydroxybutyrate (Phb) Synthesizing Bacteria. Master Thesis, Dharwad University Of Agricultural Sciences.
[6] A. Santhanam, S. Sasidharan. 2010. Microbial Production Of Polyhydroxyalkanotes (PHA) from Alcaligens spp. and Pseudomonas Oleovorans Using Different Carbon Sources. *Afr. J. Biotechnol.* 9: 3144–3150.
[7] S. S. Amutha Santhanam. 2010. Microbial Production of Polyhydroxy alkanotes (PHA) from Alcaligens spp. and *Pseudomonas oleovorans* using different carbon sources. *Afr. J. Biotechnol.* 9(21): 3144–3150.
[8] K. Selvakumar, G. Srinivasan, V. Baskar, R. Madhan. 2011. Production and Isolation of Polyhydroxyalkanoates from Haloarcula Marismortui

MTCC 1596 Using Cost Effective Osmotic Lysis Methodology. *Eur. J. Exp. Biol.* 1–3: 180–187.

[9] A. Gumel, M. Annuar, T. Heidelberg. 2012. Biosynthesis and Characterization of Polyhydroxyalkanoates Copolymers Produced by *Pseudomonas Putida* Bet001 Isolated from Palm Oil Millеffluent. 7(9): 45214.