

The effect of inoculum and glucose addition on polyhydroxyalkanoate production by *Brevibacterium* sp. B45

Pengaruh penambahan inoculum dan glukosa terhadap produksi polihidroksialkanoat oleh Brevibacterium sp. B45

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Abstrak

Plastik asal minyak bumi adalah penyebab utama pencemaran lingkungan karena plastik membutuhkan waktu bertahun-tahun untuk terdegradasi karena sulit lapuk oleh proses biologis. Kesulitan dalam penanganan limbah plastik berbasis minyak bumi telah memotivasi peneliti untuk menghasilkan bahan plastik ramah lingkungan yang dapat lapuk dengan proses biologis, salah satunya adalah polihidroksialkanoat (PHA). Polihidroksialkanoat adalah biopolimer alami yang dapat terurai secara alami dan diproduksi oleh bakteri sebagai karbon intraseluler dan penyimpanan energi. Senyawa ini merupakan sumber alternatif plastik yang memiliki sifat fisik mirip dengan plastik minyak bumi dan dapat dengan mudah terurai secara aerobik dan anaerobik. Penelitian ini menguji potensi salah satu isolat unggul penghasil PHA yaitu *Brevibacterium* sp. B45. *Brevibacterium* sp. B45 dibiakkan dalam medium minimal Ramsay dengan konsentrasi inoculum 1, 2 dan 3% (v/v) dan konsentrasi glukosa masing-masing 1, 3 dan 5% (w/v). Inkubasi *Brevibacterium* sp. B45 dilakukan dalam labu Erlenmeyer 500 mL pada mesin pengocok 150 rpm dan suhu 30 °C selama 72 jam. Produksi PHA diukur dengan metode ekstraksi kloroform dan dikarakterisasi dengan metode scanning electron microscopy (SEM), fourier transformed infrared (FTIR), dan differential scanning calorimetry (DSC). Rendemen biomassa kering hasil tertinggi (2,92%) diperoleh pada perlakuan kombinasi 3% inoculum dan 3% glukosa. Suhu leleh (T_m), entalpi (ΔH_f) dan kristalinitas (X_c) dari produk PHA berturut-turut adalah 172,1 °C, 61,04 J g⁻¹ dan 41,08%. Data SEM menunjukkan bahwa morfologi butir PHA murni dicirikan oleh permukaan berpori. Unit fungsional butiran PHA murni diidentifikasi sebagai C = O, CH₃, C-O, C-O-C, C-C, C-H, dan -OH. Spektrum yang muncul dalam butiran PHA

murni mirip dengan Poly-3-hydroxybutyrate (PHB) standar. Oleh karena itu, produksi PHA oleh *Brevibacterium* sp. B45 diidentifikasi sebagai PHB.

[Kata kunci: PHA, PHB, media minimal Ramsay, limbah plastik]

Abstract

Petroleum-based plastics are the major cause of environmental pollution because the plastics need years to be degraded. The difficulties in handling waste of petroleum-based plastic have motivated researchers to produce environmentally friendly plastic materials that are biologically degradable; one of them is polyhydroxyalkanoate (PHA). Polyhydroxyalkanoate is natural biodegradable biopolymers produced by bacteria as an intracellular carbon and energy storage. This polymer is an alternative source of plastics with similar physical properties to petroleum-based plastic. It can be easily biodegraded aerobically and anaerobically. This study examined the potential of one superior isolate as PHA producers, i.e., *Brevibacterium* sp. B45. *Brevibacterium* sp. B45 was cultivated in Ramsay's minimal medium with inoculum concentrations were 1, 2, and 3% (v/v) and glucose concentrations were 1, 3, and 5% (w/v). The cultivation of *Brevibacterium* sp. B45 was carried out in a 500 mL Erlenmeyer flask on a shaker incubator with 150 rpm and 30 °C for 72 hours. PHA recovery was carried out by chloroform extraction and characterized by scanning electron microscopy (SEM), Fourier transformed infrared (FTIR), and differential scanning calorimetric (DSC) methods. The highest yield of dried biomass (2.92%) was obtained using 3% inoculum and 3% glucose. The melting temperature (T_m), enthalpy (ΔH_f), and crystallinity (X_c) of the PHA product were 172.1 °C, 61.04 J g⁻¹, and 41.08%, respectively. Data of SEM show that a porous

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surface characterized morphological of purified PHA grains. The functional units of purified PHA grains were C=O, CH₃, C-O, C-O-C, C-C, C-H, and -OH. The purified PHA grains show a similar spectrum to the standard Poly-3-hydroxybutyrate (PHB). Therefore, it could be assumed that PHA produced by *Brevibacterium* sp. B45 was most likely PHB.

[Keywords: PHA, PHB, Ramsay minimal media, plastic waste]

Introduction

Nowadays, the problem faced by almost every country, such as Indonesia, is the difficulty in managing and handling petroleum-based plastic waste. Difficulties in managing plastic waste encourage the researcher to develop and produce environmentally friendly plastics material that microorganisms can biologically degrade (bioplastic). The making of Polyhydroxyalkanoates (PHA) is one alternative to solve this problem (Kresnawaty *et al.*, 2014).

Polyhydroxyalkanoates (PHA) are natural polyesters synthesized as intracellular carbon and energy reserves by numerous bacteria and accumulated as granules in the cytoplasm of the cells in response to unbalanced growth (Laycock *et al.*, 2014; Godbole, 2016; Cardozo *et al.*, 2016; Aneesh *et al.*, 2017). Biodegradable plastics are biologically degradable by microorganisms into carbon dioxide and water (Naittam, 2017). Because of natural biodegradable, sustainable, and environment-friendly, PHA has received considerable attention for research and development as substitutes for conventional plastic (Anju *et al.*, 2016; Godbole, 2016). Some studies have reported more than 75 bacterial genera capable of accumulating the PHA as intracellular granules. However, only several bacteria are often studied included *Alcaligenes*, *Ralstonia*, *Bacillus*, *Azotobacter*, *Rhizobium*, and *Pseudomonas* (Babruwad *et al.*, 2015; Bhagowati *et al.*, 2015). Polyhydroxyalkanoates are natural, renewable, non-toxic, biodegradable, and biocompatible thermoplastic that can be biologically degraded (Mohapatra *et al.*, 2015; Lothar, 2016). These bioplastics can be used for food packaging, bottles, sanitary goods, containers, pharmacy, medicine, coating, active carrier in chemicals, packaging materials, bags, disposable item (such as disposable cups, bottles, containers), medical applications (such as surgical, bone replacement and plates, blood vessel replacement and stimulator of bone growth by piezoelectric properties) (Godbole, 2016; Shirani & Hivakumar, 2019).

Poly-3-hydroxybutyrate (PHB) is one of the famous and best-characterized bioplastics of the PHA group (Mahitha & Madhuri, 2016; Hertadi *et al.*, 2017). They have thermoplastic properties with good mechanical properties, similar to polypropylene (Atifah, 2006; Hamieh *et al.*, 2013). It is the most desirable plastic material, and

therefore, researchers have made many efforts to produce PHB. Rawa (2017) reported that PHB has potential as a promising alternative as a substitute for petroleum-based plastic and is expected to reduce pollution caused by increasing global demand for polymers (Mohapatra *et al.*, 2015). However, PHB as a plastic material is very fragile due to the high degree of crystallinity and the melting temperature (180 °C) near to thermal degradation temperature (200 °C) that makes the PHB molding process difficult (Lothar, 2016; Samanthary, 2020). Hence the crystallinity and thermal characteristics are the parameters for the quality of polymer purity (Wang *et al.*, 1997).

Although it has broad-spectrum applications, the use of PHA is still limited due to its high price. The high cost of biodegradable plastic is due to the high cost of production, namely the use of substrates as a carbon source. The high cost of PHA production can be five times higher than that of conventional plastic production. More than 70-80% of the cost of raw materials containing carbon used as a substrate for both microorganism's growth and biopolymer production (Aneesh *et al.*, 2016). Various studies have been conducted so that PHB prices are more competitive, among others are utilizing inexpensive substrate sources, bacterial strains, more efficient and effective fermentation processes that can reduce the production cost (Godbole, 2014). Some researchers have been explored new strains and recombinant bacteria which can synthesize the PHB (Scheper *et al.*, 2001; Girdhar *et al.*, 2014; Peters, 2017; Joyline & Aruna, 2019). Several bacterias such as *Bacillus subtilis* (Babruwad *et al.*, 2015), *Bacillus megaterium* OUAT (Wang *et al.*, 1997), *Bacillus cereus* PW3A (Ahmad *et al.*, 2017), *Azohydromonas australica* (Ramsay *et al.*, 1990), *Bacillus* sp PSA10 (Yanti *et al.*, 2010), are reported successfully produced PHB by synthetic media. This study examined the potential of one superior isolate, *Brevibacterium* sp. B45 as PHA producer.

Materials and Methods

Brevibacterium sp. B45 subculture preparation

The bacteria cultures were grown on nutrient agar (NA), incubated at 30 °C for 24 hours. Then the culture was grown in the nutrient broth (NB) media and incubated on a shaker incubator at 150 rpm and 30 °C for 24 hours. After 24 hours, the inoculum of *Brevibacterium* sp. B45 was harvested and used for PHB production.

PHA production

The production of PHA was carried out according to Kresnawaty *et al.* (2014) with modification. Each 75 mL of Ramsay minimal media containing different concentrations of glucose (1%, 3%, and 5%) in 500 mL Erlenmeyer flask was inoculated with 1%, 2%, and 3% inoculum of *Brevibacterium* sp. B45 aseptically and

then incubated in a shaker incubator agitated at 150 rpm and 30 °C for 72 hours.

Biomass production

After 72 hours of incubation, the culture containing PHA was centrifuged at 7000 rpm for 15 minutes. The supernatant was removed and the pellet was suspended in 3.5 mL of distilled water, and shaken to be homogeneous. After that, it was dried using oven at 70 °C for 24 hours. The dried biomass yield was determined by using the following formula:

$$\text{Yield (\%)} = \frac{\text{dry cell weight (g)}}{\text{volume culture bacteria (v)}} \times 100\% \quad (1)$$

PHA purification

The purification of PHA was carried out using the Hahn method (Hahn *et al.*, 1994). The crude of PHA was extracted with a mixture of 0.125 mL distilled water, 1 mL 5% sodium hypochlorite and 1.5 mL chloroform. The extraction was carried out at room temperature, 180 rpm for 72 hours. The solution was then centrifuged at 3000 rpm for 15 minutes, and resulted three-layer solutions. The bottom layer (chloroform containing PHA) was separated and collected for the following step: the chloroform phase formed was dropped into cold methanol in a ratio of 5:12 (v/v), washed with 3 mL of acetone and centrifuged again to precipitate the PHA. The precipitated PHA was dried in the oven at 40 °C for 24 hours. The dried product was used for further characterization using SEM, FTIR and DSC.

Scanning Electron Microscopy (SEM)

The surface morphology of purified PHA product was analyzed by a scanning electron microscopy technique (SEM; JSM IT300, JEOL, Japan at 20 kV) technique according to the method described by Saraswaty *et al.* (2019). The sample was placed on double sided conducting adhesive carbon tapes and coated with a gold layer for 2 min by D11-29030SCTR Smart Coater. Representative SEM images were taken for each samples at 2000x magnification.

The functional group of PHA and PHB product

The functional group of PHA products was analyzed by Fourier transform infrared (FTIR) spectroscopy according to Bhagowati *et al.* (2015). The presence of functional groups in the purified PHB grains were analyzed by an FTIR spectrophotometer (Perkin-Elmer, RX 1). The scanning range was from 400 to 4000 cm⁻¹ at ambient temperature.

Differential Scanning Calorimetri (DSC)

The melting point of PHA product was analyzed by using 2920 DSC technique according to Nair *et al.* (2014). Thermal analysis is under nitrogen gas purge (50 mL min⁻¹). The

calorimeter had one sample cell and one reference cell. Samples (less than 10 mg) were exposed to a temperature profile over 20 °C to 200 °C, at a heating rate of 10 °C min. The heat flow evolved during the isothermal crystallization was recorded as a function of time. The melting temperature (T_m) and melting enthalpy (ΔH_f) were determined from DSC endothermal peaks. The crystallinity (X_c) of PHB in blend was calculated as per the equation given below.

$$X_c = \frac{\Delta H_f \times 100}{\Delta h^p} \quad (2)$$

X_c = crystallinity (%), ΔH_f = melting enthalpy of the sample, Δh^p = the melting enthalpy of 100% crystalline PHB which is assumed to be 146 J g⁻¹ (Bhagowati *et al.*, 2015).

Results and Discussion

Figure 1 presents the effect of inoculum and glucose concentrations on the yield of dried biomass of *Brevibacterium* sp B45. The highest yield of dried biomass (2.92%) was obtained by the addition of 3% inoculum and 3% glucose. The use of carbon sources in PHA production has been studied including molasses, whey hydrolysates, lignocelluloses and others. Xilose and glucose have been used as carbon surces for PHA production by *E. coli* PTS mutant which obtained the PHA was 0.476 g L⁻¹ and cell density 2.3 g L⁻¹ (Poonam *et al.*, 2015).

Scanning Electron Microscopy (SEM) analysis

Figure 2 shows SEM micrographs of purified PHA grains from *Brevibacterium* sp. B45 cultivated by using 3% inoculum and 3% glucose. Pure PHB grains indicate that there are pores throughout the PHB grains. This is due biopolymers have very low inter molecular density as biopolymers synthesized by microorganisms are very easy to be degraded (Asranudin & Surya, 2014).

Fourier Transform-Infrared (FTIR) spectroscopy analysis

FTIR spectrum (Figure 3) obtained for extracted polymer shows prominent peaks at 1722.70 and 1279.54 cm⁻¹. The peak at 1722.70 cm⁻¹ corresponds to C=O stretching of thioester group. The peak at 1279.54 cm⁻¹ corresponds to C-O aliphatic stretching of carbonyl group. Wicaksono (2005) reported that FTIR spectrum of standard PHB shows peaks at 1726.2 and 1278.7 cm⁻¹ and also Bahgowati *et al.* (2015), the prominent peaks at 1725 and 1288 cm⁻¹. Absorption bands occurring at 1456.13 cm⁻¹, indicate the presence of aliphatic -CH₂. And there are addition the band detected at 2957.39 cm⁻¹ corresponded to the (C-H) carbon hydrogen stretching. The results of the extracted PHA from *Brevibacterium* sp B45 closely the result of the standard PHB molecule thus confirmed PHA structure was most likely PHB.

Thermal analysis

The results of DSC analysis for melting point (T_m) are shown from the results of PHA heating, where glucose was used as carbon source in growth media causes of changes in values (T_m) from PHA obtained. The DSC spectrum (Figure 4) shows three peaks obtained. The first peak at 141.2 °C is the peak that shows the presence of impurities that are bound to PHA, the peak is organic materials such as carbonated compounds and proteins. The second peak at 172.1°C representing melting point of purified PHA from *Brevibacterium sp.* B45.

Whereas the third peak at 149.8 °C is crythallization temperature. The enthalpy of fusion was 61.04 J g⁻¹. The crystallinity degree (X_c) was calculated based on the melting enthalpy of 146 J g⁻¹ of 100% crystalline PHB (Bhagowati *et al.*, 2015). The enthalpy of melting is 32.56 J g⁻¹ for standard PHB, while for purified PHB, it was 41.08 J g⁻¹. Based on the statement of Lafferty *et al.* (1998) the PHB melting point varies between 157-188 °C. Refer to Laferty & Korsatko (1998), the purified biopolymer analyzed was classified as PHB.

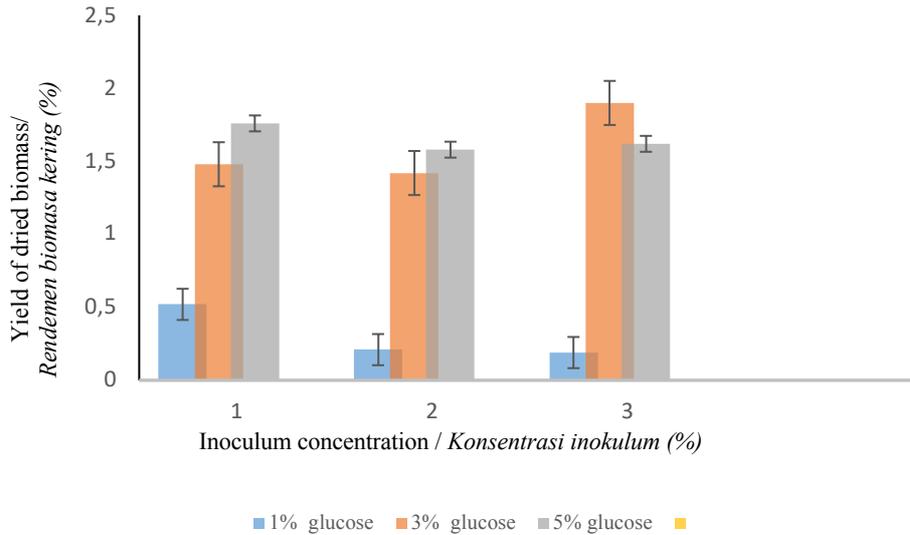


Figure 1. Effect of inoculum and glucose concentration on yield of biomass of *Brevibacterium sp.* B45
 Gambar 1. Pengaruh konsentrasi inokulum dan glukosa terhadap rendemen biomassa kering *Brevibacterium sp.* B45

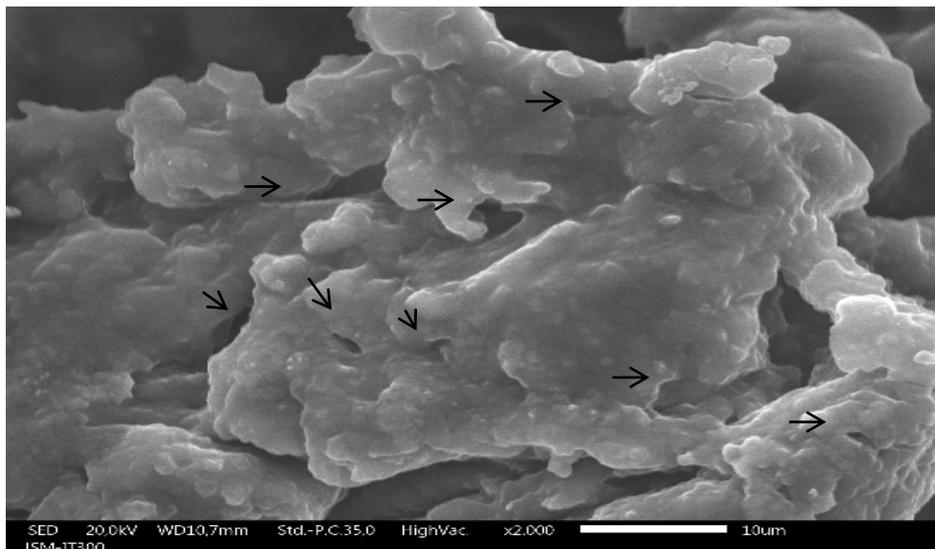


Figure 2. SEM photomicrograph of purified PHB from *Brevibacterium sp.* B45 at 20 kV (Mag. 2000x)
 Gambar 2. Fotomikrograf SEM hasil pemurnian PHB dari *Brevibacterium sp.* B45 pada 20 kV (perbesaran 2000x)

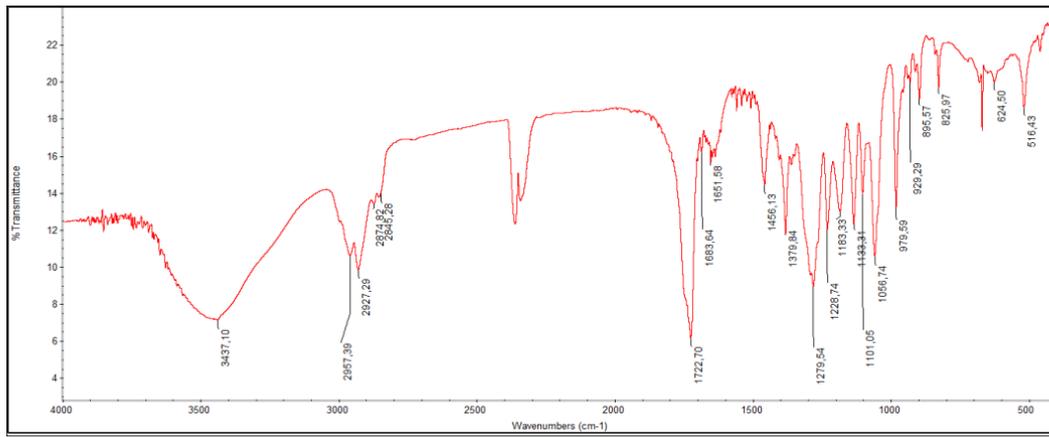


Figure 3. FTIR spectrum of purified PHB from *Brevibacterium* sp. B45
 Figure 3. Spektrum FTIR hasil pemurnian PHB dari *Brevibacterium* sp. B45

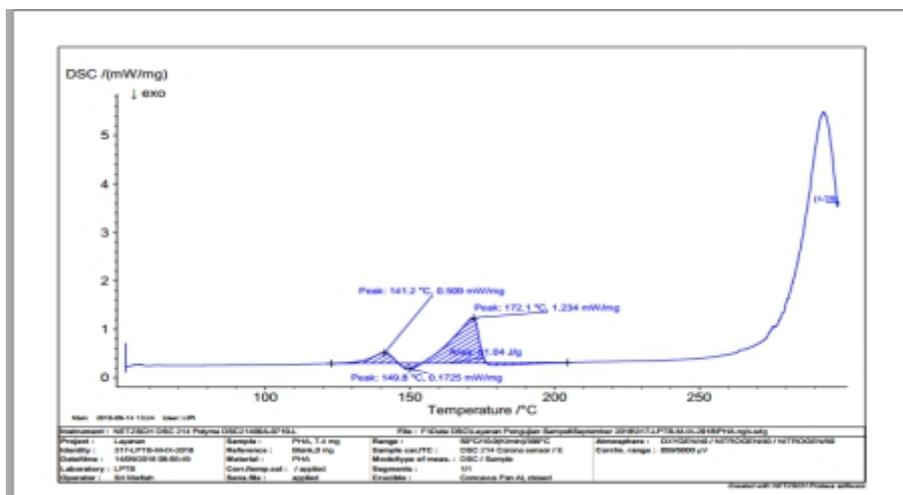


Figure 4. DSC analysis of purified PHB from *Brevibacterium* sp B45
 Gambar 4. Analisis DSC hasil pemurnian PHB dari *Brevibacterium* sp B45

Table 1. DSC result of the purified PHB sample in comparison to the standard PHB.

Tabel 1. Hasil DSC dari pemurnian sampel PHB dibandingkan dengan PHB standar.

Sample/ Sampel	Tm (°C)	ΔHf (J g ⁻¹)	Xc (%)
Standard PHB/PHB standar ^{*)}	174.5	32.56	22.30
Purified PHB /PHB murni	146	61.04	41.08

^{*)}Bhagowati et al. (2015)

Notes: Tm= the melting temperature, ΔHf= melting enthalpy, Xc = the crystallinity

Keterangan: Tm= suhu leleh, ΔHf= entalpi lebur, Xc= kristalinitas

Conclusion

It has been shown that PHA can be successfully produced by using *Brevibacterium* sp. B45 in Ramsay minimal medium reaching the highest dried biomass (2.92%) with the addition of 3% inoculum and 3% glucose. Polyhydroxyalkanoate produced by *Brevibacterium* sp. B45 was identified as PHB groups.

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