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Characterization of *Pseudomonas fluorescens* polyhydroxyalkanoate produced from molasses as a carbon source

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KEYWORDS

Incubation period
Molasses
Polyhydroxyalkanoate
Polyhydroxybutyrate
Pseudomonas fluorescens

ABSTRACT

Polyhydroxyalkanoate (PHA) is a biopolymer produced by various types of bacteria under conditions of unbalanced growth. One of the bacteria generating PHA is *Pseudomonas fluorescens* with a carbon source in the form of molasses. This study aimed to determine the effect of molasses concentration and incubation period on biomass and PHA characteristics. This study utilised two factors, such as molasses concentration of 40, 60, 80 g/L, and incubation period for 3, 5, 7 days. The research result indicated the highest biomass yield was obtained from the treatment of 40 g/L molasses concentration and in 7-day incubation period, with dry cell weight values of 1,955 g/L, PHA weight of 0.756 g/L, dry cell yield of 2.036%, and PHA yield of 39.06%, respectively. The visual characteristics of PHA samples were indicated with brownish and granular state, while the melting point and functional groups were identical to pure polyhydroxybutyrate (PHB). The characteristics of the PHB structure obtained from Fourier Transform Infrared Spectroscopy (FTIR) analysis, depicting: C = O esters, CH_3, CH_2, -C-O-, -C-O-C Polymers, and C-C. The melting point of PHA samples, using Differential Scanning Calorimetry (DSC) analysis, was 166.4 ^oC and an enthalpy of -13.885 J/g with 9.5% degree of crystallisation.

Introduction

The development of the polymer industry has provided many benefits for human life. However, a greater negative impact presents if the product is unused and disposed of directly into the environment (Ashton et al., 2016). Decomposition of synthetic polymer products requires a noticeably long-time process by microbes to become simple molecules which potentially pollute the environment. An example is the use of conventional plastic derived from petroleum for everyday human needs. One commonly developed solution to overcome this problem is the use of natural polymers as a substitute for petrochemical-based plastics. Natural polymers is a renewable and perceivable natural resource, as well as have safe biodegradability attribute for the environment (Soroudi and Jakubowicz, 2013; Emadian et al., 2017; Aversa et al., 2019). One natural polymer material having the prospect of being further developed comes from the polyhydroxyalkanoate (PHA) group (Keshavarz and Roy, 2010; Albuquerque and Malafaia, 2018; Raza et al., 2018)

PHA is a biodegradable polymer produced by various types of bacteria as food and energy reserves in conditions of unbalanced growth (Sanhueza et al., 2019). PHA is extracted from bacterial cell granules and is utilized as a substitute for synthetic plastic in various applications such as plastic bags, film wraps, bottles, electrical insulation, pipes, packaging, latex paints, etc. The core characteristic of PHA is biodegradability, which can be degraded aerobically into carbon dioxide and water, or becoming methane under anaerobic conditions (Chanprateep, 2010). The number of bacteria that accumulates PHA consists of more than 250 types including gram-positive and gram-negative bacteria such as *Alcaligenes latus, Azotobacter, Bacillus, recombinant E. coli, Pseudomonas, R. eutropa, and Rhizobium* (Chen and Jiang, 2017). The PHA polyester group chiefly consists of poly-b-hydroxybutyrate (PHB), poly-b-hydroxyvalerate (PHV), poly-b-hydroxicatroat (PHC), poly-b-hydroxiheptanoate (PHH), poly-b-hydroxyoctanoate (PHO), poly-β-hydroxynonanoate (PHN), poly-b-hydroxydecanoate (PHD), poly-b-hydroxy-undekanoate (PHUD), and poly-b-hydroxi-

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dodecanoate (PHOD) (Zhila and Shishatskaya, 2018). PHA is divided into two large groups based on the number of chains of carbon atom in the monomer unit, which are short chain length containing of C3 - C5 atoms and medium chain length containing of C6 - C14 atoms (Dietrich et al., 2017).

One of the PHA producing bacteria is *Pseudomonas fluorescens* which is a gram-negative bacterium and is recognised to accumulate PHA as an energy reserve in the cell cytoplasm (Anjum et al., 2016). The production of PHA has some advantages due to its biodegradability and can replace plastics from petroleum derivatives. However, large-scale production is limited due to the high cost of the substrate as a carbon source. In terms of production costs, bacterial PHA is 5 to 10 times more expensive than petroleum derivative polymers such as polymers and polypropylene. Consequently, various methods are developed to produce high PHA with cheap substrates to reduce production costs (Koller et al., 2017; Sabapathy et al., 2019). One example of abundant renewable substrates as a source of carbon is molasses (Michailides et al., 2015; Sen et al., 2019), which is a by-product of the sugar factory in the form of thick-blackish-brown liquid with a distinctive odor, and sweet taste (Sarka et al., 2012). About 52% of molasses consists of total sugar (e.g. sucrose, glucose, and fructose), about 10% or more is inorganic salt, about 10-20% is water and the rest is non-sugar organic matter (Rashid et al., 2019). Molasses is a substrate with sufficient nutrient content for the growth of various types of microorganisms including bacteria and has a sufficient amount of glucose to be converted into PHA (Purama et al., 2018). In the study conducted by Liu et al. (2013), *Escherica coli* was utilised with molasses as carbon source.

Several research methods have been developed for the production of PHA. For example in studies conducted by Nair et al. (2014) and Xu et al. (2019), *Bacillus subtilis* bacteria is utilised with carbon molasses from sugarcane extraction by applying chloroform with hypochlorite method. In a study conducted by Jiang et al. (2008), *Pseudomonas fluorescens* A2a5 was employed with carbon sources of sugar cane liquor and extraction with chloroform solution. In the study of Snoei et al. (2015), *Pseudomonas fluorescens* SC4 was employed with a carbon source of fatty acids and extraction by utilizing chloroform performed with the soxhlet extraction method. In addition, the research conducted by Lopez et al. (2015) utilised *Pseudomonas fluorescens* with carbon sources in the form of sugarcane molasses and extraction by using chloroform. However, prior studies utilising *Pseudomonas fluorescens* with carbon molasses extracted with NaOH digestion method have never been conducted. Thus, it is necessary to determine the biomass and characteristics of PHA produced.

The PHA production process consists of two processes, include cultivation and isolation of PHA (Justyna and Kiewisz, 2016; Fabricio et al., 2017; Perez et al., 2018). Isolation in the PHA extraction process is conducted using NaOH digestion method due to its cheap cost, efficiency and safer for the environment. In addition, the amount of NaOH used in the PHA isolation process is less than other chemicals such as chloroform, methylene chloride, and other solvents, assisting the refining process and non-degradation of PHA (Yan et al., 2010). Lee et al. (1999) performed a recombinant solution of *Escherica coli* containing 69% PHB with 0.2 N NaOH for 1 hour at 30 ^{o}C obtaining PHB with 97% purity level. Therefore, this study aimed to characterize PHA from *Pseudomonas fluorescens* by utilising molasses as an alternative carbon source.

Research Methods

Materials

Material used in this study mainly consists of *Pseudomonas fluorescens* obtained from the Laboratory of Quality Testing and Food Safety, Faculty of Agricultural Technology, Universitas Brawijaya, molasses without any pre-treatment obtained from the sugar factory (Kebon Agung Ltd, Malang, Indonesia) and other ingredients, such as nutrient agar (NA) and nutrient broth (NB), aquades, 70% alcohol, and NaOH.

Experimental set-up

This study utilised two factors, including concentration of molasses at 40, 60 and 80 g/L and incubation period of 3, 5 and 7 days. As a result, 9 combinations of treatments were obtained. Each treatment was performed in triplicate, resulting 27 units of the experiment.

Culture preparation

Pure culture was firstly bred on nutrient media with as much as 1.4 g in 50 mL of distilled water and was added by 1 ose of pure bacteria, then was incubated for 24 hours. After 24 hours, one ose of *Pseudomonas fluorescens* from NA was withdrawn to grow at 15 mL NB for 24 hours. Cultures grown on NB were later utilised in PHA fermentation process and were stored for subsequent studies.

PHA fermentation

PHA fermentation process was performed by utilising NB under batch condition. In one fermentation process, a 150 mL NB solution is required. NB solution was obtained by using 2.4 g NB powder with the addition of 150 mL distilled water. The homogeneous NB solution was put into a 250 mL Erlenmeyer. Prior to the utilisation, NB solution was sterilised by using autoclave at 121 ^{o}C; then molasses was added according to the concentration of 40, 60 and 80 g/L respectively. After being homogenised, about 15 mL NB containing *Pseudomonas fluorescens* culture was included. Erlenmeyer was put into an incubator at 30 ^{o}C in various incubation periods of 3, 5 and 7 days. After the fermentation process was accomplished, a 50 mL of fermentation at each incubation period was taken and inserted into the centrifugation tube. Then the centrifugation process was carried out at a speed of 6000 rpm for 15 minutes to obtain supernatant and pellet in the form of biomass containing PHA. Pellet washing was performed by dissolving 10 mL of distilled water and was centrifuged at 6000 rpm for 15 minutes. Afterwards, it was processed with the drying process using an oven for 24 hours at a temperature of 70 ^{o}C to gain dry biomass.

PHA extraction

In PHA extraction process, the dry biomass was then dissolved in 0.05 M NaOH. The comparison used was 10: 1, where for every 10 mg of dry biomass would take about 1 mL of 0.05 M NaOH solution. The extraction process lasted for 1 hour at 30 ^{o}C. After the extraction process was accomplished, centrifugation with a speed of 6000 rpm was carried out for 15 minutes to separate the solvent from PHA. Then a further washing process was carried out by dissolving PHA in 10 mL distilled water and was then centrifuged to obtain a higher PHA purity level. The next process was continued with the drying process by using an oven with a temperature of 70 ^{o}C for 24 hours to remove the remaining distilled water in PHA.

Analysis

The analysis carried out included: dry cell weight, PHA weight, dry cell yield, PHA yield, functional group, melting point, and degree of crystallinity. PHA weight was analysed by measuring pellet weight after extraction with NaOH from dry cell weight. The dry cell yield value was calculated based on the ratio between dry cell weight divided by the weight of the solid material (Blanco et al., 2018). The solid weight consisted of molasses weight and NB (added during the fermentation process). Dry cell weight measurement is a method commonly used to determine the ability of bacterial strains to consume available organic compounds to be accumulated into PHA cells (Din et al., 2006). The value of PHA yield was calculated based on a comparison between weight values of PHA divided by dry cell weight (Figols et al., 2018). Analysis of functional groups was conducted by using fourier transform infrared (FTIR) spectroscopy (Shimadzu 8400S) to clarify the type of PHA produced from *Pseudomonas fluorescens* based on the band group (Kamnev et al., 2018). Melting analysis was carried out using differential scanning calorimetry (DSC) (Rigaku 8230, Japan) (Chan et al., 2019). Measurements of the degree of crystallinity were based on enthalpy values when measured with DSC. PHA with 100% crystallinity produced an enthalpy of 146 J/g. The degree of crystallinity was compared with the enthalpy value of the test sample with PHA having 100% crystallinity (Jost et al., 2017).

Results and Discussion

Biomass is the result of fermentation obtained as the main ingredient in the study. *Pseudomonas fluorescens* is produced by performing a batch fermentation method. Dry cell weight measurement is a method to determine the ability of bacteria to consume available organic compounds to be accumulated into PHA cells. Dry cell weight is obtained after centrifuging and drying the pellets (which were weighed to measure the final weight). Figure 1 presents the result of the dry cell weight graph of the study.

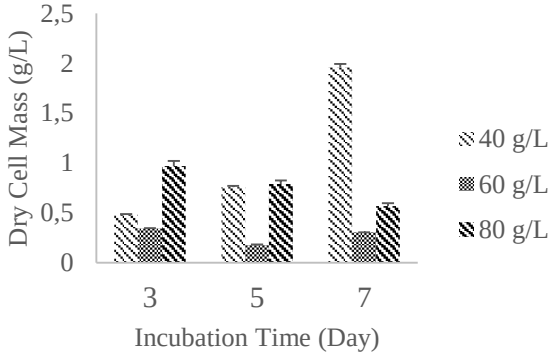

Figure 1. Graph of dry cell weight with incubation period

Figure 1 informs the average value of dry cell weight from all treatments were in the range of 0.176 to 1.955 g/L with a standard range deviation of 0.0007–0.0535. Based on statistical analysis, it is apparent that the factor of molasses concentration has a significant effect on dry cell weight. At a concentration of 40 g/L molasses, an increase in the

amount of dry cell weight occurs along with an increase in incubation period. An increase in heavy cell occurs due to bacteria growth phase in the presence of sufficient food sources and an appropriate environment to produce biomass. This result is consistent with the research conducted by Gumel et al. (2012) using carbon sources in the form of palm oil waste for biosynthesis of PHA mcl, where the dry cell weight was increased by increasing the concentration of carbon sources from 49.7% to 68.9%.

However, the results are inversely proportional to the concentration of molasses (60 g/L and 80 g/L), indicating the decreasing pattern in the amount of dry cell weight. The decrease occurs due to high concentration of molasses inhibiting cell growth and substrate, limiting the number of biomass (Liu et al., 1998). The difference in the value of dry cell weight at each concentration produced is also due to the ability of bacteria to hydrolyze molasses into simple sugars (Jiang et al., 2008; Snoei et al., 2015). PHA-producing bacteria are efficient in utilizing glucose (99%), fructose (97%), and hydrolyzate sucrose (96%), and are less efficient in utilising sucrose (20%). In this study, the molasses consists of a total sugar of 25.13% and a reducing sugar of 15.43%. Reducing sugar is usually found in the monosaccharide group (i.e. glucose, fructose). In this study, the highest value of dry cell weight was 1.955 g/L at the molasses concentration of 40 g/L with an incubation period of 7 days.

The measurement of PHA weight is carried out after obtaining dry cell weight from biomass. After obtaining the dry cell weight, the extraction process with NaOH is performed to obtain PHA weight. The measurement of PHA weight is carried out after the centrifugation and drying process.

Figure 2 indicates the average weight of PHA ranging from 0.024-0.769 g/L with a standard deviation in the range of 0.0005-0.0266. At a concentration of 40 g/L molasses, PHA weight increases along with an increase in incubation period due to increase number of dry cells produced. In addition, longer time affects PHA weight produced because the bacteria continue to experience the growth phase with the number of substrates. The more dry cells produced, the more PHA is produced. Along with increasing fermentation time and concentration of carbon sources, PHA weight will continue to increase. Lopez et al. (2015) found that the maximum PHA weight produced by the *Pseudomonas fluorescens* bacteria was in the temperature range of 30 ^{o}C

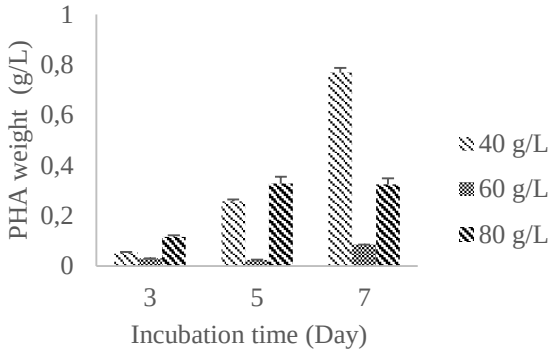

Figure 2. Graph of PHA weight and incubation periods. Error bars represent standard deviations of three measurements

However, the molasses concentrations of 60 and 80 g/L results are different and tend to decrease and increase PHA weight value due to mutual relationship between cell dry weight and PHA weight. The extraction process with NaOH removes impurities which are still present in dry cells. Although PHA weight shrinks, the obtained results still have high purity. In addition, a decrease in PHA weight appears due to the presence of fermented impurities found in dry cell weight such as protein, fat, and etc. Impurities are bound to the solvent during the extraction process with NaOH and then dissolved with water in the centrifugation process so that it changes the weight of the previous one.

The breakdown of non-PHA cell materials with NaOH (NaOH digestion) has several advantages, such as: (1) cheap and environmentally friendly; (2) PHB with high purity (> 98%); and (3) no degradation of PHB during the extraction process. In this study, the highest PHA weight is 0.769 g/L at 40 g/L molasses concentration with incubation period of 7 days. In this study, NB is utilised which is rich in meat and yeast extract to encourage more numerous cell growth.

Figure 3 presents a dry cell yield graph of the study, indicating the average value of dry cell yield ranging from 0.232 to 2.036% with a range of standard deviations between 0.0236 and 1.9133. At the concentration of 40 g/L molasses, the dry cell yield increases along with increasing incubation period due to the increase in dry cell weight. An increase in dry cell yield occurs due to ability of bacteria to adapt to growth media that are rich in food sources, especially carbon and other food sources (e.g. N, P, S, and O).

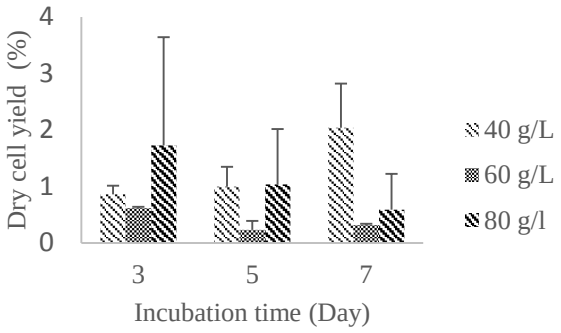

Figure 3. Graph of dry cell yield and incubation periods. Error bars represent standard deviations of three measurements

However, at 60 and 80 g/L molasses the results are different and tend to decrease in dry cell yield value. This decrease is thought to occur due to the large number of bonds in sugar groups (in molasses), hindering bacteria to break down the existing sugar bonds. This result is consistent with the previous research of Liu et al. (1998) using *Eschericha coli*, at a concentration of 60% (g/L) molasses, indicating that the concentration of PHB produced was greater than the concentration of molasses at 40 and 20%. The high concentration of molasses inhibits cell growth because molasses is inhibitory to the substrate but can increase the synthesis of PHB by *Eschericha coli*. In addition, a decrease in the amount of dry cell yield occurs due to the centrifugation process to separate between filtrate and dry cell weight. During the centrifugation process of solids in the form of NB and molasses which have been mixed with distilled water, the fermentation process of the bacteria is incorporated into the filtrate. Therefore, lots of solids are also wasted along with the filtrate decreasing the values. Based on the research, it was found that the highest dry cell yield was 2.036% at 40 g/L molasses concentration with 7 days incubation period. Meanwhile, the lowest dry cell yield value was 0.232% at the concentration of molasses 60 g/L with an incubation period of 5 days.

Figure 4 presents PHA yield in the study indicating the average value of PHA yield ranging from 8.80 to 39.06% with a standard deviation range between 3.6135 and 29.7057. At the concentration of molasses (40, 60, 80 g/L), an increase in PHA yield occurs along with increasing incubation period. This increase is expected to occur because the bacteria have been in an exponential phase because in the first 3 days the bacteria are still in the environmental adjustment stage. After passing through the adjustment stage, the bacteria will automatically induce the growth phase and carry out cell division to continuously grow on day 5. Afterwards, the bacteria enter the stationary phase where the bacteria remain in growth but enter the deceleration phase where the substrate needed for growth begins to run out and decreases in growth rate. In addition, the increase in PHA yield occurs because bacteria are able to adapt to growth media that are rich in food sources, especially carbon and other limited food sources (N, P, S, O). The environment in which bacteria grow which is rich in carbon, phosphate and nitrogen sources will trigger bacteria to produce carbon stocks in the form of PHA. In this study, the highest value of PHA yield is 39.06% at molasses concentration of 40 g/L with incubation period of 7 days. The yield value produced is much smaller than the study conducted by Jiang et al. (2008) using *Pseudomonas fluorescens* A2a5 with a PHB concentration of 22 g/L and a PHB content of up to 70% (of dry cell weight). In addition, in a study conducted by Snoei et al. (2015) using the ability of *Pseudomonas fluorescens* SC4 to produce PHA using different fatty acids i.e. linoleic acid, oleic acid, and sunflower oil, with a concentration of 1.5% (v/v), linoleic acid accumulates the highest PHA (44.2%) in pumpkin fermentation, and (45.2%) with a cell dry weight of 6.75 g/L after 48 hours in the bioreactor. The study conducted by Nair et al. (2014) obtained a dry cell weight of 2.09 g/L, PHA weight of 1.5 g/L, and PHA yield of 75.5%.

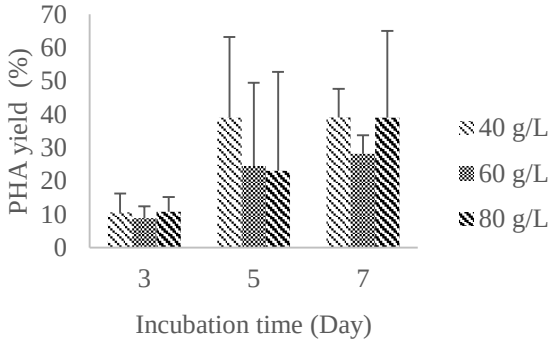

Figure 4. Graph of PHA results and incubation periods. Error bars represent standard deviations of three measurements

FTIR spectroscopic result from PHA samples are depicted in Figure 5 indicating the results of analysis from PHA sample functional groups in this study (having 27 peaks, of which 15 peaks are equal to the standard PHB spectrum using Sigma-Aldrich brand). It is assumed that the PHA samples tested are PHB types to match the characteristics of PHA, methyl free (CH_3) and single methylene (CH_2) groups which also are in accordance with the PHB structure. A distinctive feature of PHA molecules is

the presence of carbonyl esters (C = O), –C-O bonds and –C-O-C-polymeric bonds. Other bonds also have C-H bonds that can be detected at waves of 2900 cm^{-1} and C-C at waves of 977 cm^{-1}. In addition, the main bonds in PHB molecules are carbonyl esters (C = O) which are read in waves of 1738-1728 cm^{-1}, methyl (CH_3) deformation, methylene (CH_2), and CO bonds at wave numbers of 1450-1000 cm^{-1} (Kansiz et al., 2000). The infra-red spectrum of a material indicates the chemical composition of the material due to producing a typical absorbance spectrum. The difference in each spectrum is determined by the chemical structure and the level of contribution of each component to the spectrum. The Interpretation of FTIR spectra of PHA samples is depicted in Table 1.

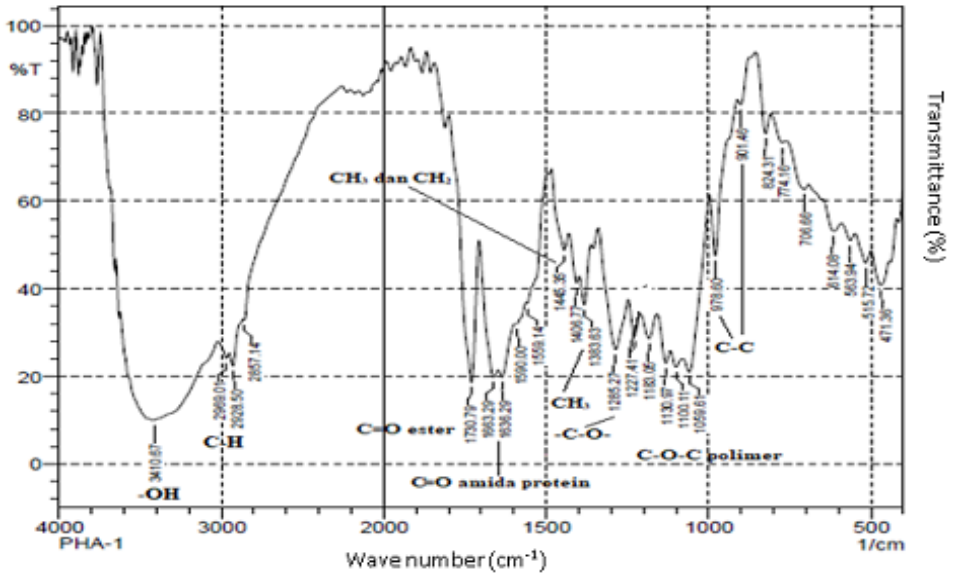

Figure 5. Results of functional group analysis on PHA

Table 1. Interpretation Results of FTIR spectrum PHA

No	PHA from molasses			Pure PHB (Atifah et al., 2007)	
	Wave number (cm^{-1})	Identification	Intensity	Wave number (cm^{-1})	Identification
1.	471.36, 515.72, 563.94, 614.08, 706.66, 774.16, 824.31	Not Identified	Moderate	515.0, 597.9, 827.4 827.4	Not Identified
2.	901.46*	C-C	Moderate	979.8*, 956.6*, 935.4	C-C
3.	978.6*	C-C	Strong		
4.	1059.61*, 1100.11*,1130.97*, 1183.05*, 1227.41 *	C-O-C Polymer	Moderate	1058.8*,1101.3*, 1132.1*,1186.1*	C-O-C Polymer
5.	1285.27*	-C-O-	Strong	1278.7*	-C-O-
6.	1383.63*, 1406.77*	CH_3	Moderate	1380.9*,	CH_3
7.	1445.35*	CH_3 and CH_2	Moderate	1454.2*	CH_3 and CH_2
8.	1559.14, 1590	N-H Amide Protein	Poor		
9.	1636.29, 1663.29	C=O Amide Protein	Moderate	1658.7	C=O amide protein
10.	1730.79 *	C=O Ester	Strong	1726.2*	C=O Ester
11.	2857.14	C-H Aldehyde	Poor		
12.	2928.5*, 2969.01*	C-H	Moderate	2933.8*	C-H
13.	3410.67*	-OH	Strong	<3500*	-OH

** Spectrum of PHA Samples at Standard PHB*

Based on Table 1, other groups such as C = O, and N-H appear in wave numbers of 1636.29, 1663.29, 1559.14, and 1590 which are protein amide groups. Protein residues in PHA samples are

considered to be the residual molasses or cell fragments in the fermentation process; thus, other impurities have not been separated during the extraction process. In addition, the presence of protein residues is believed to be the membrane lining the PHA. The result of this study presents a strong intensity of wave (3410.67 cm^{-1}) which is the -OH spectrum. This compound is assumed to be a residue from NaOH carried out in the process of extraction which has not undergone evaporation during the drying process. In addition, it is also suspected that the sample contains glucose compounds into derivative acids or intermediate compounds which contain several OH groups. Gumel et al. (2012) conducted a study utilising carbon sources in the form of palm oil waste for biosynthesis of mcl-PHA with *Pseudomonas putida* Bet001 bacteria, indicating several peaks due to the typical characteristics of PHB, such as: –OH (3420.20 cm^{-1}), metil (2955.76 cm^{-1}), C=O ester (1741.44 cm^{-1}), CH_3 (1378.83 cm^{-1}), and C-O-C (1259.89 cm^{-1} and 1166.87 cm^{-1}).

The measurement of PHA fusion melting and enthalpy points in this study is illustrated in Figure 6 demonstrating a peak of 166.4 ^oC which is the melting point of PHA with an enthalpy value of -13.885 J/g. Peak on the graph in a sharp downward direction indicates a dominant component in the form of PHA. The peak leading down indicates that the sample absorbs energy to change enthalpy value (endotherm). The energy absorbed by the sample causes melting in the sample; thus, the temperature at the peak of absorption of heat energy is called the melting temperature (Tm). The results of the comparison of melting and enthalpy points with standard PHB are presented in Table 2.

Based on Table 2, it is apparent that the melting point values in the study are lower than the standard PHB melting point values of 170.15 ^oC. This result is in contrast with Atifah et al. (2007) applying Sigma-Aldrich natural origin and PHA from starch. sago resulting a melting point of 163.96 ^oC with an enthalpy value of 12.23 J/g. PHA samples are in the temperature range that is in accordance with the PHB of the biocycle brand industry with 165-170 ^oC (Bugnicourt et al., 2014). Therefore, based on the existing melting point, it is suspected that the PHA sample is a type of PHB having property close to propylene.

Table 2. Comparison of melting points of samples with standard PHB

No	Material	Melting Point (^oC)	Enthalpy (J/g)
1.	PHA Sample in the Research	166.9	13.885
2.	Commercial PHB Sigma Aldrich (Atifah et al., 2007)	170.5	32.35
2.	PHB Industrial Biocycle (Bugnicourt et al., 2014)	165-170	-
3.	PHBV5% Metabolix Inc (Thellen et al., 2008)	143	-

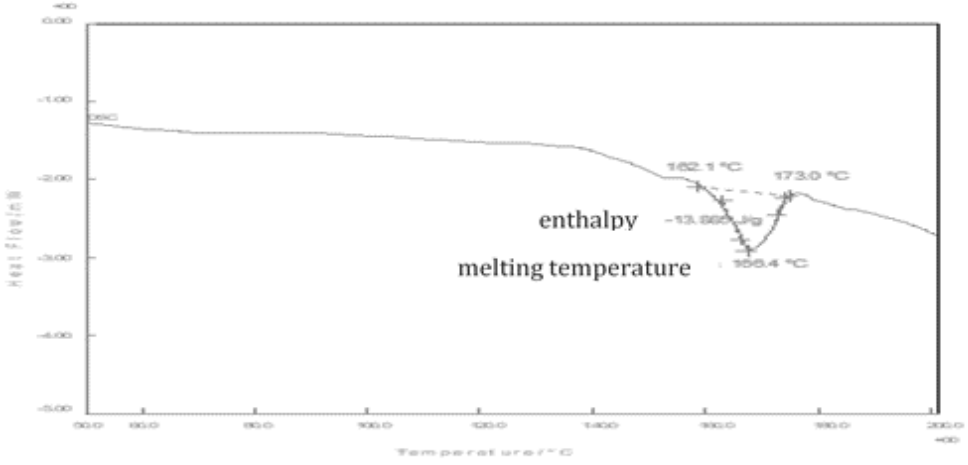

Figure 6. Analysis of melting temperature at PHA

In addition, this allegation is strengthened in the functional group test of PHA molasses samples, indicating that the types of bonds in the PHA sample are in accordance with the PHB structure. Melting temperature (Tm) of PHB varies between 157-188 ^oC and the glass transition temperature (Tg) of 5-20 ^oC depends on the composition and thermal history of the material (Saratale et al., 2019; Moura et al.,

2019). In this study, the temperature of Tg glass transition in the sample and standard PHB is not detected due to the limited ability of the device to be operated at temperatures below 50 ^{o}C. Gumel et al. (2012) conducted a study by utilising carbon sources in the form of palm oil waste for biosynthesis of PHA mcl, obtaining a melting point of 43 (± 0.2) ^{o}C with an enthalpy value of 100.9 (± 0.1) J/g. This value is slightly different in the study, where the carbon source used is different with addition of oleic acid and octanoic acid. Differences in carbon sources and types of bacteria produce different types of PHA.

Based on enthalpy values generated from DSC measurements in Figure 6, the value of PHA crystallinity is estimated. In theory, samples with 100% crystallinity have fusion enthalpy of 146 J/g (Hahn et al., 1995). The degree of crystallinity is the percentage of PHA enthalpy distribution tested with 100% crystallinity. Previous research found that enthalphy value of 13.885 J/g and standard PHB of 32.35 J/g (Atifah et al., 2007) utilising the Sigma-Aldrich brand. The results of the approximate degree of crystallinity of the PHA and PHB standards are around 9.5% and 22%. Based on the estimations, there are samples of PHA and standard PHB dominant which are in the amorphous form with a low degree of crystallinity.

This study is in accordance with the research of Hahn et al. (1995) utilising *Ralstonia eutropha* that most PHB granules are in amorphous form. The melting point value in this study is not much different from the research conducted by Atifah et al. (2007) which produced PHA from sago starch with a melting point at 163.96 ^{o}C with an enthalpy value of 12.23 J/g. Thus, the sample is considered to be a type of PHB. This value is different from the study conducted by Gumel et al. (2012) using carbon sources in the form of palm oil waste for biosynthesis of mcl-PHA with the addition of oleic acid (the melting point was 43 (± 0.2) ^{o}C with an enthalpy value of 100.9 (± 0.1) J/g. The degree value of crystallinity obtained is 69.11% and tends to be crystalline. This difference in the degree of crystallinity occurs due to the different sources of carbon used and the type of bacteria producing different types of PHA. In addition, thermoplastic polymers with crystallinity tend to highly melt at high temperatures than in amorphous polymers. As a result, the analysis of the degree of crystallinity is based more on the sharpness of the peak formed at the melting temperature.

Conclusions

Based on the results, it was found that the treatment of molasses concentration significantly affected dry cell weight but did not significantly affect the PHA weight and PHA yield. The incubation period had a significant effect on the yield of PHA, but it did not significantly affect PHA weight, dry cell weight, and PHA yield. The highest biomass results were obtained in a combination of 40 g/L molasses treatment with a 7-day incubation period, with the value of dry cell weight of 1.955 g/L, PHA weight of 0.756 g/L, dry cell yield of 2.036% and PHA yield of 39.06%.

Conflict of interest

The authors declare that there is no conflict of interest in this publication.

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