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Biosynthesis of poly(3-hydroxybutyrate) (PHB) by *Cupriavidus necator* from various pretreated molasses as carbon source

Khok Yong Sen[a], M. Hazwan Hussin[b], Siti Baidurah[a,*]

[a] *School of Industrial Technology, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*
[b] *School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*

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ABSTRACT

The high production cost of polyhydroxybutyrate (PHB) particularly substrate cost limits its industrial applications. Reduced substrate cost by utilizing cheaper carbon source enables to improve its economic viability. Cane final molasses are considered potential feedstock due to its abundance, low cost, and high sugar content. However, sucrose in molasses required to convert to its monomers: fructose and glucose by pretreatments for the bacteria consumption during the fermentation process. In this study, the main components of molasses and sugar content in various pretreated molasses were analyzed. The PHB content and cell dry weight of *C. necator* biomass fermented utilizing glucose, untreated molasses and pretreated molasses as carbon sources are compared. The molasses consist of 34.49 ± 0.13% sucrose, 5.93 ± 0.07% glucose and 5.00 ± 0.04% fructose respectively. The hydrothermal acid pretreated molasses (oil bath) contains the highest fructose and glucose content which are 21.31 ± 0.04% and 20.32 ± 0.06% respectively. Although the hydrothermal acid pretreated molasses (oil bath) contains the highest fructose and glucose content, the cell dry weight and PHB produced by *C. necator* using hydrothermal acid pretreated molasses (oven) is the highest which are 2.86 ± 0.82 g and 27% respectively.

1. Introduction

The disposal of non-biodegradable plastic in large scale has become a major global problem not only to the environment and ecosystem but also human health (Cozar et al., 2014; Jambeck et al., 2015). In response to these issues, plastic derives from biological resources with biodegradability properties must be developed. Polyhydroxybutyrate (PHB) is one of the potential alternatives to replace conventional plastic and it fits into the above context perfectly.

PHB is a biodegradable thermoplastic polyester that belongs to polyhydroxyalkanoates (PHAs) family. In nature, PHB exists as a cytosolic inclusion body in various microorganisms such as bacteria and archaea (Reusch, 1992). PHB serves as carbon and energy storage for microorganism under stressed environmental condition (Castro-Sowinski et al., 2010). According to studies, the PHB production yield varies depends on bacterial species, nutrient and culture condition (Riedel et al., 2012; Batcha et al., 2014). *Cupriavidus necator* is among the bacteria widely studied for PHB production due to its ability to accumulate a remarkable amount of PHB which is up to 90% of its cell dry weight (CDW) (Obruca et al., 2014). Over the last few decades, PHB has garnered great interest in industrial applications and research fields due to its biodegradability, biocompatibility, thermoplastic processability as well as its physical property is similar to isotactic polypropylene (Barham et al., 1984). The biocompatibility and non-toxic nature of PHB can extend its industrial applications to medical, surgical and pharmacology fields as the biomedical implant material and drug carrier (Castro-Sowinski et al., 2010).

Although PHB offers many benefits over the conventional plastic, the production cost of PHB is expensive compared to the petrochemical-derived plastics. Currently, the production cost for PHB is 4–9times higher than the price of polyethylene (Roland-Holst et al., 2003). The high production cost of PHB partly due to the cost of raw material (50% of production cost) (Nonato et al., 2001). At present, pure fructose and glucose are the main carbon sources used for large-scale PHB production. Therefore, the choice of carbon source is one of the key factors to determine its economic feasibility in large-scale production. Utilization of abundant low-value materials as the substrate for fermentation, which is the most viable strategy to reduce the production cost (Koller et al., 2012). Many studies have been conducted on the PHB production by utilizing industrial by-products such as whey, molasses, glycerol, cellulosic material, waste lipid as an alternative carbon source (Adnadjevic and Jovanovic, 2012). Cane final molasses, the by-product

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[*] Corresponding author.
E-mail address: sitibaidurah@usm.my (S. Baidurah).

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from the sugarcane mill and sugar refinery used as the carbon source in this study due to its abundance, low cost and high amount of sugar which have the potential to reduce the PHB production cost. Moreover, molasses can be obtained from various parts of the world.

Although cane molasses contain high amount of sugar, the sugar composition is mainly comprised of sucrose (32%) and invert sugar (16%) (Hashizume and Yamagami, 1966). It is reported that *C. necator* unable to assimilate sucrose (Arikawa et al., 2017). Therefore, pretreatment of molasses is required to hydrolyze the sucrose to its monomer glucose and fructose for bacteria consumption. The previous study reported sucrose hydrolysis can be carried out using chemical (acid, alkali) or the enzyme (sucrase, invertase) (Tomatoni and Vitolo, 2010). Tomatoni and Vitolo (2010) reported that the acid catalyzed sucrose hydrolysis pretreatment has advantages due to its potential to reduce the production cost in contrast to utilization of enzyme. However, environmental pollution related to the toxic by-product such as hydroxymethylfurfural (HMF) formation should take into consideration during performing the acid catalyzed sucrose hydrolysis pretreatment (Khajavi et al., 2005; Bower et al., 2008; Tomatoni and Vitolo, 2010). Therefore, microwave heating and hydrothermal pretreatment were also applied to assist the acid catalyzed sucrose hydrolysis.

Microwave heating is defined as a rapid form of dielectric heating, whereby the heat is generated in polar molecule during exposure to high frequency electric field (Jacob et al., 1995). The process of heat transfer in microwave heating involves energetic coupling at the molecular level. Moreover, microwave heating reduces the activation energy and accelerates the rate of chemical reactions and physio-chemical processes, which in turn gives higher yields and reduce toxic by-product formation (Adnadjevic and Jovanovic, 2012). For these reasons, the use of microwave in pretreatment has gained a lot of attention in recent years (Nomanbhay et al., 2013). The reducing sugar produced from oil palm empty fruit bunch fiber (EFB) that undergo microwave assisted alkaline pretreatment coupled with microwave assisted enzymatic saccharification is 5.8-fold higher than EFB from non-microwave alkaline pretreatment and enzymatic saccharification (Nomanbhay et al., 2013).

Contrarily, hydrothermal pretreatment is referred to the process whereby moist biomass subjected to elevated temperature (above water boiling point) with hot water or steam under the high pressure. Hydrothermal pretreatment provides advantages not only to reduce the inhibitory byproduct formation, but also to reduce the chemical usage (Yang et al., 2018). Furthermore, hydrothermal pretreatment offers moderate reaction temperature and a low-cost operational method, which indirectly reduce the overall production cost (Zha and Yu, 2016). The reducing sugar produced from hydrothermal pretreated potato peel residues (PPR) (57.8 ± 2.1) is 3.12-fold higher than PPR without pretreatment (18.5 ± 0.5) (Ben Taher et al., 2017).

To the best of our knowledge, there was no research conducted to determine the effect of various pretreatments on the glucose and fructose production from cane final molasses for PHB production. This is the first study to report the impact of various pretreatment methods on the glucose and fructose production as well as the effect of molasses pretreatment on bacterial biomass and PHB production by *C. necator*.

2. Materials and methods

2.1. Cane final molasses and its nutrient composition

Cane final molasses were obtained without any costs from Malayan Sugar Manufacturing (MSM) Prai Berhad, Malaysia. Cane final molasses is defined as the final effluent from sugar refining by repeated evaporation, crystallization and affination of raw sugar. The main nutrient compositions of molasses were analyzed, which includes total solids, total sugar, total nitrogen, sucrose, fructose, glucose, water and inorganic content.

2.1.1. Total solids and water determination of cane final molasses

The water content in cane molasses was analyzed using sand pan technique from AOAC International (2008) and modified based on Gafta (2003). The sand pan technique was used to prevent surface crust formation and promote efficient moisture evaporation (Nielsen, 2010). The sand functions as an extender for evaporation of water in molasses. The sand was screened through 40 mesh sieve, pretreated with 0.1 M HCl, washed with distilled water thrice, dried in an oven model Binder ED 115 (Binder GmbH, Germany) at 110 °C for 2 h and finally ignited in muffle furnace model Carbolite ELF 11/6 (Carbolite Gero, United Kingdom) at 800 °C for 2 h. Prior conducting analysis, 30 g of clean, acid pretreated sand was weighed in a glass petri dish (90 mm diameter, 19 mm depth) and placed in the hot air oven with lid and mixing rod at 110 °C for 2 h. After drying, the sand was placed in the desiccator to cool down to ambient temperature and reweighted. Twenty grams of molasses were diluted with 10 ml of distilled water and stirred until the mixture was uniform. Two grams of the molasses solution were added into the glass petri dish with sand and mixed thoroughly with glass rod ensuring no loss of material occurred. The petri dish with its lid was placed in the vacuum oven model Binder VD 53 (Binder GmbH, Germany) for drying at 65 °C, 18 h under vacuum condition. Then, the sample was transferred to a desiccator for cooling. The water and total solid contents of cane molasses were calculated based on the following equation:

$$\%\text{water}\left(\frac{\text{wt}}{\text{wt}}\right) = \frac{\text{weight of water in molasses}}{\text{weight of raw molasses}}\text{ X 100 }\% \tag{1}$$

$$\%\text{solid}\left(\frac{\text{wt}}{\text{wt}}\right) = \frac{\text{weight of dry matter in molasses}}{\text{weight of raw molasses}}\text{ X 100 }\% \tag{2}$$

2.1.2. Total nitrogen determination by Kjeldahl method

The Kjeldahl protocol is based on Horwitz and Latimer (2005) and the digestion catalyst formula was modified according to Silva et al. (2016). The Kjedahl method started with approximately 1.0 g of molasses sample was weighed into the micro Kjeldahl tubes. A blank was prepared and 2 g of catalyst which consists of sodium sulfate and copper (II) sulfate in the ratio 20:1 was added. The catalyst and 20 ml of concentrated sulfuric acid were added in the sample and the mixture was heated until it appeared clear and colourless. The digestion was continued for another 2 h, followed by cooling down at room temperature. After the digestion stage, the condenser water was turned on and 60 ml of boric acid (H_3BO_3) solution with indicator was added to graduated 500 ml Erlenmeyer titration flask. The methyl red and methylene blue were used as indicators. The flask contains H_3BO_3 solution was placed under the condenser tip while the tube contains sample was placed into the distillation unit model BÜCHI K-350 (BÜCHI Labortechnik AG, Sweden). Sodium hydroxide (NaOH) solution 40% was added into the tube until the indicator level reach 160 ml and the solution was distilled into H_3BO_3 solution in the receiving flask. The receiving flask was lowered, and the liquid was drained from the condenser tip. H_3BO_3 receiving solution was titrated with standard 0.1 M HCl solution to first trace of pink. The volume (ml) of HCl was recorded to at least nearest 0.05 ml. The total nitrogen content was calculated based on the following equation:

$$\% \quad nitrogen = \frac{(ml\ standard\ acid - ml\ blank)\text{x}N of\ acid\text{x}1.4007}{weight\ of\ sample\ ingrams} \tag{3}$$

2.1.3. Inorganic content determination of raw molasses by sulphated ash method

The analysis method of inorganic content is based on sulphated ash method, AOAC International (1995). Molasses weighing five grams were transferred into the silica crucible. The molasses sample was moistened using 0.5 ml of concentrated sulfuric acid and heated gently until the sample was carbonized. The carbonized sample was placed in the muffle furnace model Carbolite ELF 11/6 (Carbolite Gero, United Kingdom) at 550 °C for 2 h. The sample was removed, cooled, re-moistened with two drops of concentrated sulfuric acid and placed in the furnace at 800 °C for 1 h and finally cooled in the desiccator to room temperature. The inorganic content was calculated based on the following equation:

$$\%\text{Inorganic content} = \frac{\text{Weight of sample after ignition}}{\text{Weight of molasses}} \text{ x } 100\ \% \tag{4}$$

2.2. Molasses solution preparation and clarification

The preparation of the untreated molasses solution was based on Ashraf et al. (2015). Fifteen grams of molasses were weighed in 250 ml beaker and then diluted with 100 ml distilled water. The clarification process begins with centrifuging the molasses solution at 3500 × *g* for 15 min and filtered using Whatman filter paper no. 1 (25 × 180 mm). The obtained solution was later used for further analysis, such as high-performance liquid chromatography (HPLC) and various pretreatment method.

2.3. Sucrose, fructose and glucose analysis using HPLC method

The sucrose, fructose and glucose content of raw molasses and pretreated molasses were determined using HPLC method. The standard solutions of sucrose, fructose and glucose were prepared in five various concentrations ranging from 0.3 to 5.0 mg/ml. The HPLC analyses were carried out using a Waters 515 HPLC pump and Waters 2414 differential refractive index detector (Waters Corporation, USA), operated at 90 °C and nitrogen pressure set at 655 Psi. Standard and sample solution (20 µl) were directly injected into Sugar-Pak 1 column (10 µm, 6.5 mm x 300 mm). HPLC analysis was carried out by isocratic elution for 13 min using the 0.001 mol Calcium EDTA solution as mobile phase at a flow rate of 0.5 ml/min. The mobile phase was filtered using 0.22 µm nylon filter and degassed by ultrasonic bath model Elma TI-H 10 (Elma Schmidbauer GmbH, Germany) for 15 min prior use. All sample solutions were filtered through Bioflow 0.22 µm PES membrane cartridge and Sep-Pak classic Alumina A cartridges before analysis. The chromatograms were acquired and then handled using the CSW32-chromatography station software.

2.4. Molasses pretreatment

In this research, ten types of molasses pretreatments were conducted. The thermal pretreated molasses solution was prepared by incubating 100 ml of untreated molasses solution in 90 °C water bath for 1 h. After incubation and cooling, clarification process was conducted.

Sulfuric acid pretreated molasses solution was prepared by adding 5 ml of 1 N or 1.5 N of sulfuric acid into the 100 ml untreated molasses solution and was incubated at 90 °C in water bath for 1 h. After incubation and cooling, the molasses solution was adjusted to pH 7 using 1 M NaOH solution and followed by clarification process (Waisbren et al., 1951; Baei et al., 2009). Upon clarification process, a clear black supernatant and the red brown pellet was formed. The supernatant was kept for subsequent analysis.

Microwave assisted sulfuric acid pretreated molasses solution was prepared by adding 5 ml of 1 N or 1.5 N of sulfuric acid into the 100 ml untreated molasses solution and was placed in the 800 W microwave oven model Sharp R202ZS (Sharp Corp, Japan) for 5 min.

Alkaline pretreated molasses solution was prepared by adding 5 ml of 1 N potassium hydroxide solution into the 100 ml untreated molasses solution and was incubated at 90 °C in water bath for 1 h.

Hydrothermal pretreated molasses solution was prepared by adding 10 ml of untreated molasses solution into the 15 ml stainless steel hydrothermal synthesis autoclave reactor and submerge into the 110 °C pre-heated paraffin oil bath for 30 min, followed by incubation for 1 h.

Hydrothermal oven pretreated molasses solution was prepared by adding 200 ml of untreated molasses solution into the 600 ml stainless steel hydrothermal synthesis autoclave reactor and transferred into the 105 °C hot air oven model Venticell LSIS-B2V/VC55 (MMM Medcenter Einrichtungen GmbH, Germany) preheated for 1 h and followed by incubation for 1 h.

Hydrothermal acid pretreated molasses (oil bath) solution was prepared by adding 10 ml of 1.5 N sulfuric acid pretreated molasses solution into the 15 ml stainless steel hydrothermal synthesis autoclave reactor and submerge into the 110 °C paraffin oil bath for 1 h.

Hydrothermal acid pretreated molasses (oven) solution was prepared by adding 200 ml of 1.5 N sulfuric acid pretreated molasses solution into the 600 ml stainless steel hydrothermal synthesis autoclave reactor and transferred into the 105 °C hot air oven pre-heat for 1 h and followed by incubation for 1 h.

For all pretreatment process, after the incubation process, the pretreated molasses were cooled at room temperature for 30 min, adjusted the pH to 7 with 1 M NaOH and followed by the clarification process. Subsequently, 1 ml of pretreated sample was kept for sugar determination by HPLC method. The untreated molasses solution and three pretreated molasses solution with highest glucose and fructose content were subjected to further fermentation process.

2.5. Organism and culture media

The bacteria strain *C. necator* was used in this study which is a PHB producing soil bacterium. The inoculum culture medium was nutrient broth, which comprised of beef extract 10 g/L, peptone 10 g/L and sodium chloride 5 g/L (Waisbren et al., 1951). The seed culture medium was adjusted the pH to 7 using 1 M NaOH solution and autoclaved at 121 °C, 15 psi for 15 min. The culture medium used in this study for the seed culture was mineral salt medium (MSM). The MSM is based on Kim et al. (1994). This culture medium is made up of carbon source: glucose or molasses solution 10 g/L; nitrogen source and macronutrients $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 1.5 g/L, $Na_2HPO_4{\cdot}2H_2O$ 4.47 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L and 1 ml of trace element solution. Trace element solution containing: $FeSO_4{\bullet}7H2O$ 10 g/L, $ZnSO_4{\cdot}7H_2O$ 2.25 g/L, $CuSO_4{\cdot}5H_2O$ 1 g/L, $MnSO_4{\cdot}5H_2O$ 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 2 g/L, $Na_2B_4O_7{\cdot}10H_2O$ 0.23 g/L, $(NH_4)_6Mo_7O_{24}$ 0.1 g/L, and 35% HCl 10 ml/L. The culture medium was adjusted to pH 7 and autoclaved at 121 ∘C, 15 psi for 15 min. The carbon source, nitrogen source, magnesium sulfate, trace element solution and calcium chloride solution were prepared and autoclaved separately to avoid Millard reaction and precipitation (Lund and Ray, 2017).

2.6. Bacterial cultivation

The inoculum was prepared in 250 ml flask and incubated in the reciprocal shaker model Infors Ecotron (INFORS HT, Switzerland) at 30 ± 1 °C, 250 ± 10 rpm for 24 h. Forty milliliters inoculum was transferred to 500 ml conical flask which consist of total 400 ml culture medium. The culture medium contains different carbon sources were conducted in triplicate. Temperature and agitation speed were maintained at 30 ± 1 °C, 250 ± 10 rpm. The culture samples of the culture were taken for determining the OD at the time interval of 2, 4, 6, 12, 18, 24, 30, 36, 42, 48, 60, 66, 72, 78, 84 h. At every 12 h, 40 ml sample of the culture was taken for cell dry weight determination by taking account on the weight loss upon drying. Whereas PHB and residual sugar content were analyzed by means of gas chromatography (GC) and HPLC, respectively.

2.7. Cell biomass and PHB determination

Samples of 3 ml were taken from the bacterial culture flask for OD determination at 620 nm (Baei et al., 2009). For CDW determination, 40 ml of the aliquots from culture flask were sampled in pre-weighted 15 ml centrifugal tubes. The samples were centrifuged at $3500 \times g$, 4 °C for 15 min. Ten ml of the supernatant was kept at − 20 °C. The cell pellets were washed with distilled water to remove residual culture medium. The washed cell pellets were resuspended in 2 ml of cold distilled water, frozen at − 80 °C, lyophilized using Labconco Freezone 12 L freeze dryer (Labconco Corporation, USA) followed by the determination of CDW. The PHB content and composition accumulated in the cells were determined using methanolysis followed by GC analysis. The methanolysis protocol was conducted according to Braunegg et al. (1978) and modified according to Alves et al. (2017). During the methanolysis procedure, 8–10 mg of the lyophilized cell was weighted in a borosilicate tube with screw cap. Two ml of chloroform and 2 ml of methanol supplemented with 15% sulfuric acid was added into the borosilicate tube which contain dried cell and tightly screwed. The mixture was incubated in 100 °C digital dry bath for 3.5 h. After cooling, 1 ml of distilled water was added to the mixture and vigorously vortexed for 1 min. The upper aqueous layer of the mixture was removed and retained the lower organic layer which contain methyl ester of 3-hydroxybutyrate which is subjected to the GC analysis. Sodium sulfate was added to the lower organic layer to remove the remaining trace water. The standard sample of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] in the monomer ratio of 78:22 were used to prepared standard calibration curve. Analysis was performed using a Shimadzu model GC-2010 Plus (Shimadzu Corp., Japan) equipped with the Merck SPB-1 capillary GC column (30 m × 0.25 mm I. D, 0.25 µm film thickness). Helium was used as the carrier gas with a flow rate 0.91 ml/min. The injector was set at 270 °C, a pressure of 84.2 kPa, flow rate 13.0 ml/min and the flame ionization detector at 280 °C. The column temperature was programmed from 70 °C to 280 °C at a rate of 5 °C/min. The total measurement time for each sample is 42 min.

3. Results and discussion

3.1. Molasses main constituents

In this study, cane final molasses were utilized as the main carbon source for *C. necator* fermentation. As an agro-industrial by-product, there are difference in the composition of each batch cane molasses. The variation in chemical composition of molasses was contributed by soil factor, climatic factor (temperature, moisture, season of production), sugarcane variety, maturity, processing and storage condition (Binkley and Wolform, 1953). Molasses contains low amount of carbohydrate, nitrogenous material, fat, nucleotides, pigmented material, odorant, phenolic compounds, acids, and vitamins (Binkley and Wolform, 1953; Hashizume and Yamagami, 1966). While the sugar content and composition of molasses are the main focus of this study. The chemical composition of molasses before pretreatment is shown in Table 1.

Table 1
Chemical constituents of untreated molasses.

Molasses constituents	This study	Hashizume and Yamagami (1966)	Bayley et al. (1983)	Bortolussi and O'Neill (2006)
Source of molasses	Malaysia	Okinawa	Cuba	Eastern Australia
Sugar:				
Sucrose	34.49 ± 0.13	31.76	27.10	45.20 ± 0.12
Glucose	5.93 ± 0.07	15.44	6.10	18.70 ± 0.17
Fructose	5.00 ± 0.04	(invert sugar)	17.50	(reducing sugar)
Total Nitrogen	0.51 ± 0.04	0.90	0.34	–
Water Content	15.80 ± 0.64	18.85	–	23.40 ± 0.09
Total Dry Matter	84.20 ± 0.64	81.15	82.50	76.60 ± 0.09
Ash	10.47 ± 0.17	13.82	9.10	17.50 ± 0.10

Referring to the Table 1, sugar content constitutes a large portion in molasses which is 45.43%. The high sugar content in molasses can be utilized as a potential carbon source in PHB production. However, the sugar content in molasses obtained in this study is slightly lower than previous studies (47.20–63.80%) (Hashizume and Yamagami, 1966; Bayley et al., 1983; Bortolussi and O'Neill, 2006). The sugar composition in molasses mainly consists of sucrose (34.5 ± 0.13%), glucose (5.93 ± 0.07%) and fructose (5.0 ± 0.04%). Other type of sugars and carbohydrates such as raffinose, psicose, pectin, pentosan, and uronic acid may also present in low amount (Chen and Chou, 1993; Atiyeh and Duvnjak, 2003; Bortolussi and O'Neill, 2006;). As presented in Table 1, the molasses contains very low total nitrogen (0.51 ± 0.04%). The low total nitrogen content in molasses also reported in other studies which are ranging from 0.34% to 0.90% (Hashizume and Yamagami, 1966; Bayley et al., 1983; Bortolussi and O'Neill, 2006). The nitrogenous materials in molasses are made up of amides, albuminoids, amino acids and other simple nitrogenous compounds (Binkley and Wolform, 1953). In order to promote the accumulation of PHB, it is desirable to have a culture medium with limited nitrogen sources and high amount of carbon sources (Baidurah et al., 2016). The water content obtained in this study is 15.8%, which is lower than the water content range of molasses reported from previous studies (Hashizume and Yamagami, 1966; Bayley et al., 1983; Bortolussi and O'Neill, 2006). The reason possibly due to the sugar refining process technology employed vary in each country.

3.2. Molasses pretreatment

As discussed in the previous section, the major sugar components in molasses are sucrose which accounts for 34.49%. Whereas the glucose and fructose content of untreated molasses are very low which contains only 5.93% and 5.00%. The fructose content of untreated molasses is lower than glucose due to the composition of cane juice and fructose reacts with amino acid faster than glucose in Milliard reaction during the sugar refining process (Lund, 2017). Sucrose hydrolysis is required to improve bacteria consumption of simple sugar in molasses. Therefore, pretreatment is required to hydrolyze sucrose to its monomers: β-D-fructose and α-D-glucose prior bacterial fermentation. Upon hydrolysis, each sucrose molecule will yield equivalent of one glucose molecule and fructose molecule. Based on previous studies, sucrose hydrolysis can be accomplished via acid, alkali, invertase, subcritical water and cation exchange resin pretreatments (Khajavi et al., 2005). The rate of acid or alkali catalyzed sucrose hydrolysis were influenced by several factors such as concentration of hydronium and hydroxide ion, temperature, pH, sucrose and monosaccharides concentration, and lastly nature and pressure of gas supplied. In this study, ten pretreatments were conducted on molasses to increase its fructose and glucose content (Baidurah et al., 2016).

Table 2 shows the sucrose, fructose and glucose content of untreated molasses and various pretreated molasses. Among all the pretreatment method, the hydrothermal acid pretreated molasses (oil bath) produced the highest fructose and glucose content which are 21.31 ± 0.04% and 20.22 ± 0.06% respectively. The hydrothermal acid pretreatment on molasses using oil bath is capable to achieve 76.05% of sucrose conversion from 34.43% to 8.26%. The fructose content of molasses increases 3.2-fold to 16.31% whereas the glucose content of molasses increases 2.4-fold to 14.29% of total mass of molasses respectively. The high sucrose conversion by hydrothermal acid pretreatment using oil bath was due to the combined effect of high pressure, high temperature (110 °C), low pH and subcritical water as catalyst which increase the rate of acid-catalyzed sucrose hydrolysis. Under high pressure and high temperature (above 100 °C), the ionization products of water increase, which make the subcritical water act as a source of hydronium and hydroxide ion and a catalyst (Khajavi et al., 2005).

Moreover, the molasses with hydrothermal acid pretreatment either using oven or oil bath as heating source and hydrothermal pretreatment

Table 2
The sucrose, fructose and glucose content of untreated molasses and various type of pretreated molasses.

Types of pretreatment on molasses	Types of sugar (%)		
	Sucrose	Fructose	Glucose
No pretreatment	34.49 ± 0.13	5.00 ± 0.04	5.93 ± 0.07
Thermal pretreatment	37.82 ± 0.05	6.02 ± 0.69	6.73 ± 0.19
Alkaline pretreatment (1.0 N KOH)	36.52 ± 0.04	4.82 ± 0.08	5.90 ± 0.07
Acid pretreatment (WB) (1.0 N H_2SO_4)	29.38 ± 0.06	7.46 ± 0.03	8.05 ± 0.13
Acid pretreatment (WB) (1.5 N H_2SO_4)	26.98 ± 0.05	11.81 ± 0.07	12.10 ± 0.18
Acid pretreatment (MW) (1.0 N H_2SO_4)	37.58 ± 0.05	6.28 ± 0.06	7.32 ± 0.12
Acid pretreatment (MW) (1.5 N H_2SO_4)	36.27 ± 0.63	7.69 ± 0.11	8.24 ± 0.14
Hydrothermal pretreatment (oven)	34.46 ± 0.11	5.07 ± 0.55	5.59 ± 0.16
Hydrothermal acid pretreatment (oven)	24.25 ± 0.19	12.38 ± 0.22	11.43 ± 0.38
Hydrothermal pretreatment (oil bath)	33.90 ± 0.13	6.54 ± 0.12	6.38 ± 0.15
Hydrothermal acid pretreatment (oil bath)	8.26 ± 0.05	21.31 ± 0.04	20.22 ± 0.06

WB - water bath.
MW - microwave assisted.

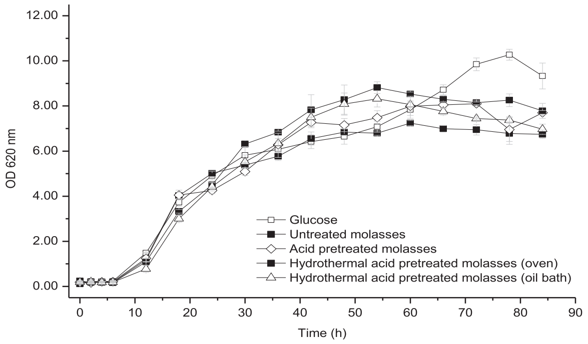

Fig. 1. Comparison of growth curve of *C. necator* utilizing glucose, untreated molasses and various pretreated molasses as carbon sources for fermentation.

(oil bath) contain higher fructose content than glucose content which differs from other pretreatments used in this study. This phenomenon occurs due to high pressure and high temperature in hydrothermal pretreatment promote the isomerization of glucose to fructose. Increases in temperature and pressure shift the chemical equilibrium of isomerization of aldose (glucose) to ketose (fructose) (Usuki et al., 2007). Furthermore, the obtained result in Table 2 shows that the rate of sucrose hydrolysis and invert sugar production were increased correlation with the increased in the concentration of sulfuric acid used in the molasses pretreatment. The similar observation was reported by Khajavi et al. (2005).

Contrarily, the alkaline pretreated molasses contains the lowest fructose and glucose contents among the pretreatments which are 4.82 ± 0.08% and 5.90 ± 0.07%, respectively. The fructose and glucose content in alkaline pretreated molasses is lower than untreated molasses may due to Millard reaction and alkaline degradation of fructose and glucose to a mixture of hydroxyl acid (Yang and Montgomery, 2007).

There are previous studies reported that microwave assisted acid pretreatment can reduce the reaction time and promote the reduction of toxic compounds (Chen et al., 2011; Adnadjevic and Jovonovic, 2012; Nomanbhay et al., 2013). Adnadjevic and Jovanovic (2012) reported that the isothermal acid-catalyzed hydrolysis using microwave heating is 4–7 times higher than the conventional heating method while the activation energy of microwave heating acid catalyzed sucrose hydrolysis is 1.6 times lower than conventional heating. It is interesting to note that, the glucose and fructose content from both microwave assisted acid pretreated molasses only increase slightly (8.24 ± 0.14%, 7.69 ± 0.11%). While the sucrose content from both microwave assisted acid pretreated molasses (1.0 N and 1.5 N sulfuric acid) increase from 34.49 ± 0.13% to 37.58 ± 0.05% and 36.27 ± 0.63% respectively. The increased in sucrose content of microwave assisted acid pretreated molasses indicates the hydrolysis of other complex sugar (raffinose, 1-kestose) present in the molasses (Yang and Montgomery, 2007).

3.3. *Effect of glucose, untreated molasses and various pretreated molasses as carbon source on cell dry weight and PHB production*

The effect of various pretreated molasses on the production of cell biomass and PHB yield by the *C. necator* were studied. Untreated molasses and three pretreated molasses with the highest fructose and glucose content which are acid pretreated molasses (WB) (1.5 N sulfuric acid), hydrothermal acid pretreated molasses (oven) and hydrothermal acid pretreated molasses (oil bath) were utilized as a carbon source for bacterial fermentation. Glucose is chosen as standard carbon source due to its wide application as carbon source in current large-scale PHB production. The carbon source concentration used in this study is 10 g/L. The phrase “acid pretreated molasses (WB) (1.5 N sulfuric acid)” is simplified as acid pretreated molasses in subsequent explanation.

Fig. 1 shows the growth curve for *C. necator* fermentation utilizing glucose, untreated molasses and various types of pretreated molasses as carbon source. Referring to Fig. 1, the first 6 h of *C. necator* fermentation shows low cell growth where the cell is entering lag phase, regardless of any type carbon sources supplied. However, the cell at this stage is metabolically active and adapting to the new environment. The bacterial cells at lag phase undergoes the repair of macromolecular damage or synthesis of the necessary cellular component for later growth (Rolfe et al., 2012). After 6 h of fermentation, the cells enters the exponential growth phase. For untreated molasses and all pretreated molasses as carbon source, the *C. necator* enter the stationary phase at fermentation time 54 or 60 h, whereas the *C. necator* using the glucose as carbon source enter the stationary phase at fermentation time 78 h. *C. necator* utilizing glucose as carbon source require a longer time to reach stationary phase due to the high glucose concentration in the culture medium. The *C. necator* using hydrothermal acid pretreated molasses (oven) as carbon source shows the highest growth with $OD_{620\,nm}$ 8.82 at time 54 h among the molasses whereas the untreated molasses has the lowest growth with $OD_{620\,nm}$ 7.24 at time 60 h. The *C. necator* using glucose as carbon source achieve the highest growth compared to the other molasses with $OD_{620\,nm}$ 10.27.

Figs. 2–6 shows the CDW (g/L), PHB (g/L), PHB (% of CDW) in *C. necator* biomass and residual sugar from the medium utilizing glucose, untreated molasses and various pretreated molasses as carbon source for different time interval. Referring to Fig. 2, the highest cell dry weight and the PHB yield of *C. necator* for glucose as carbon source are obtained at time 72 h with 2.81 ± 0.10 g/L and 1.61 ± 0.71 g/L, although the glucose depleted at time 60 h. This observation may due to

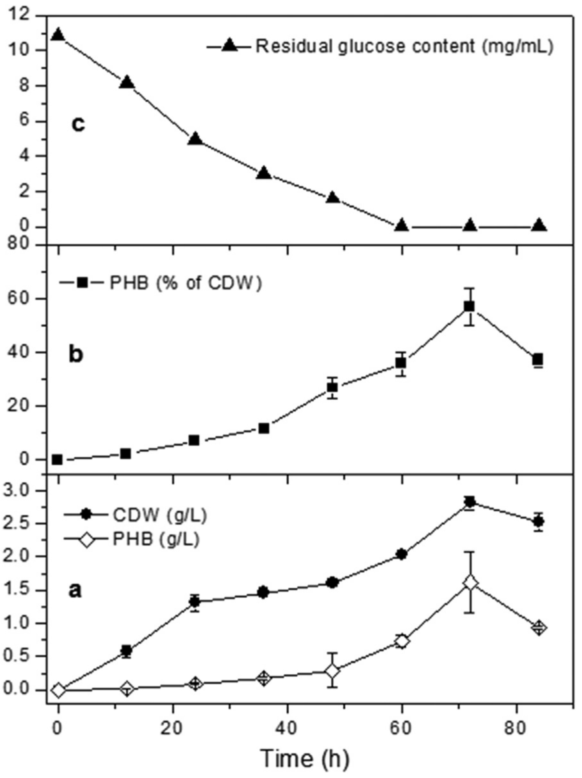

Fig. 2. Fermentation result of *C. necator* utilizing glucose as carbon source: (a) residual glucose concentration (mg/ml) in culture medium and cell dry weight (CDW) (g/L), (b) PHB (%) and (c) PHB (g/L) for different time intervals.

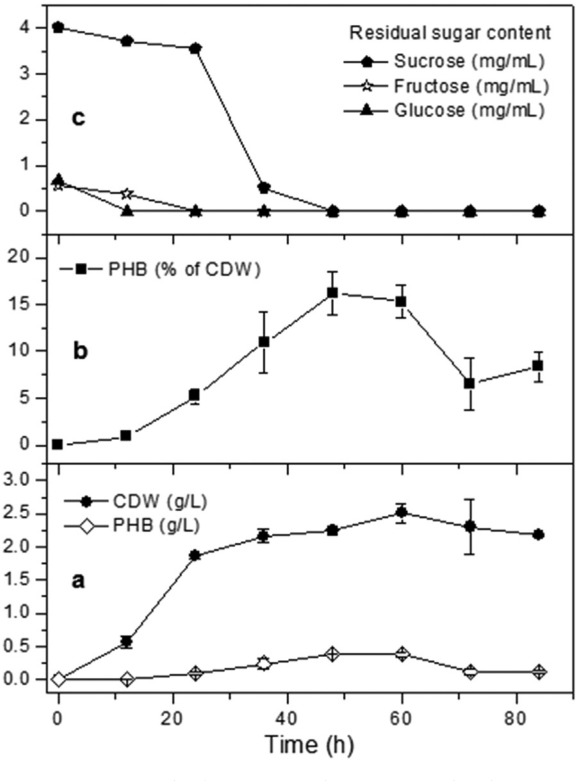

Fig. 3. Fermentation result of *C. necator* utilizing untreated molasses as carbon source: (a) residual glucose concentration (mg/ml) in culture medium and cell dry weight (CDW) (g/L), (b) PHB (%) and (c) PHB (g/L) for different time intervals.

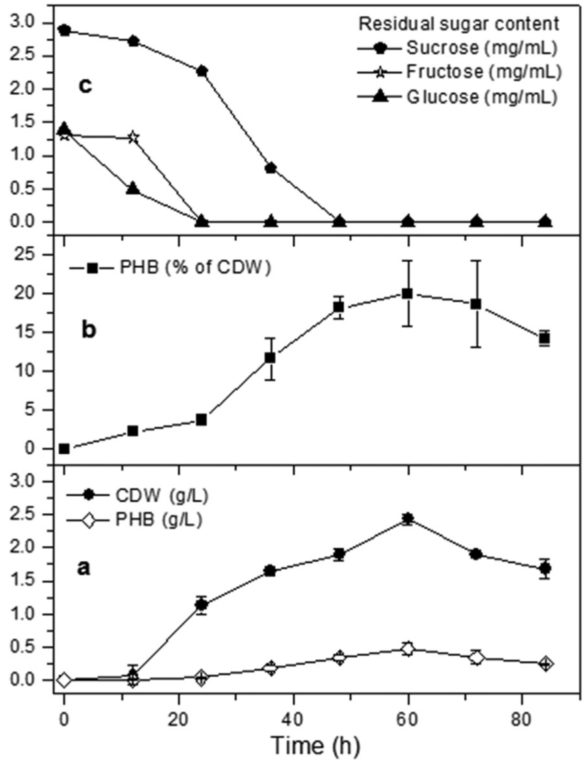

Fig. 4. Fermentation result of *C. necator* utilizing acid pretreated molasses as carbon source: (a) residual glucose concentration (mg/ml) in culture medium and cell dry weight (CDW) (g/L), (b) PHB (%) and (c) PHB (g/L) for different time intervals.

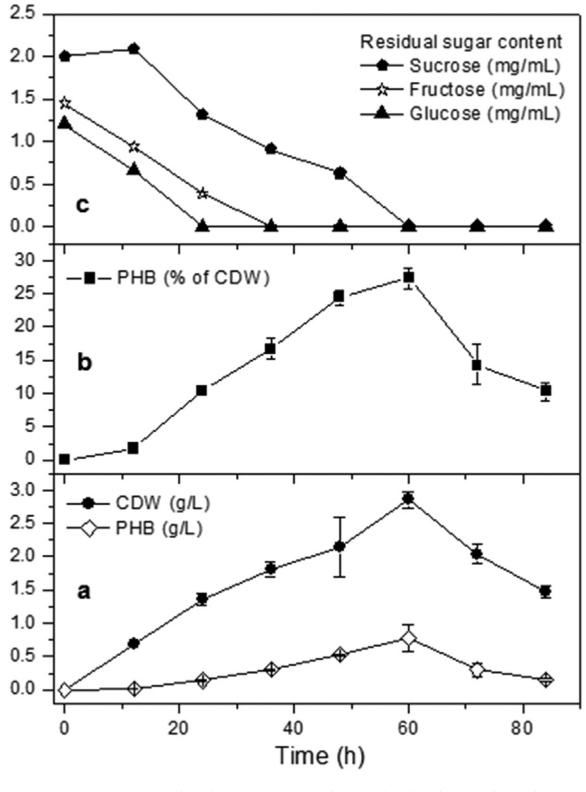

Fig. 5. Fermentation result of *C. necator* utilizing hydrothermal acid pretreated molasses (oven) as carbon source: (a) residual glucose concentration (mg/ml) in culture medium and cell dry weight (CDW) (g/L), (b) PHB (%) and (c) PHB (g/L) for different time intervals.

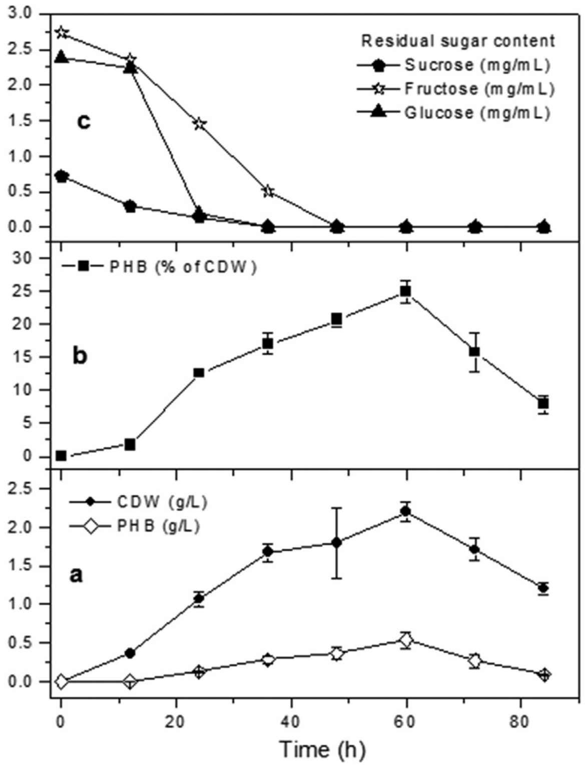

Fig. 6. Fermentation result of *C. necator* utilizing hydrothermal acid pretreated molasses (oil bath) as carbon source: (a) residual glucose concentration (mg/ml) in culture medium and cell dry weight (CDW) (g/L), (b) PHB (%) and (c) PHB (g/L) for different time intervals.

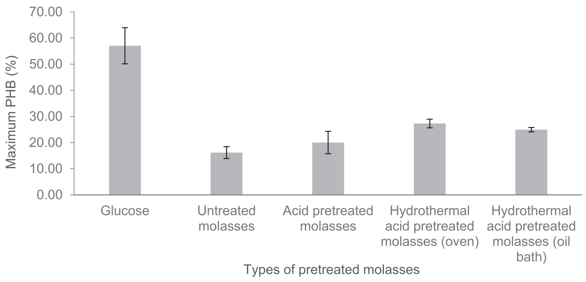

Fig. 7. Comparison of percentage of PHB accumulated in *C.necator* biomass for various types of pretreatment on molasses.

the glucose uptake by *C. necator* is not completely metabolized by the cell. Whereas for the untreated molasses and other pretreated molasses (Figs. 3–6) the highest CDW and PHB yield of *C. necator* was obtained at 60 h. After 72 h of fermentation, the CDW, PHB yield and PHB percentage of *C. necator* using glucose as carbon source decreased, whereas for *C. necator* using untreated molasses and other pretreated molasses, the CDW, PHB yield and PHB percentage decrease after 60 h. The decreased in CDW, PHB yield and PHB percentage of *C. necator* is due to substrate depletion (Figs. 2–6) and toxic by-product accumulation such as hydroxymethylfurfural (HMF) (Aramvash et al., 2015). Figs. 2–6 shows that the PHB production started in exponential phase and continue until the beginning of stationary phase. The percentage of PHB is increased correlating with the CDW, shows that the PHB production of *C. necator* involves growth associate and non-growth associate component. Moreover, from Figs. 3c–6c, the glucose consumption rate by *C. necator* was higher than fructose and sucrose, which indicate the preference of *C. necator* toward glucose compared to fructose and sucrose in the untreated molasses and other pretreated molasses.

Comparison of PHB percentage accumulated in *C. necator* biomass for glucose, untreated molasses and various pretreated molasses was shown in Fig. 7. Table 3 shows the maximum CDW (g/L), maximum PHB content (g/L), space time yield (gPHB/L/h) comparing various types of pretreatment on molasses for *C. necator* fermentation. Fig. 7 shows the *C. necator* utilizing glucose as carbon source accumulate the highest PHB percentage compared to other pretreated molasses which is 57.07 ± 6.93%. This obtained data is slightly lower from the literature due to various factors, such as the presence of impurities in the raw cane molasses, different strain, different culture condition (agitation speed, oxygen concentration) and different glucose concentration, and to some extend it will affect the PHB accumulation. The PHB accumulated by *C. necator* utilizing glucose as carbon source is 3.53 and 2.09 times higher than the *C. necator* utilizing untreated molasses and hydrothermal acid pretreated molasses (oven). This observation can be explained by correlating the sugar content in the molasses solution, which consist only 45–50% of the molasses total mass and the existence of complex chemical component may affect the PHB accumulation. Among all pretreatments condition, fermentation utilizing hydrothermal acid pretreated molasses (oven) accumulate the highest PHB percentage which are 27.30 ± 1.64% or 1.68 times higher than the untreated molasses. The reason may possibly due to the fructose and glucose content in the hydrothermal acid pretreated molasses (oven) is 2.48 times and 1.93 times higher than untreated molasses. Furthermore, acid pretreatment using sulfuric acid shows the capability to remove the inorganic inhibitors that affect the cell growth from the molasses.

In terms of maximum CDW and maximum PHB concentration, the fermentation of *C. necator* using hydrothermal acid pretreated molasses (oven) produces the highest CDW (2.86 ± 0.82 g/L) and PHB concentration (0.78 ± 1.35) compared to other molasses pretreated molasses samples. Despite both acid pretreated molasses and hydrothermal pretreated molasses have similar fructose and glucose content, the CDW and PHB produced by the *C. necator* using the hydrothermal acid pretreated molasses (oven) is higher than acid pretreated molasses. This observation is due to the higher fructose content in the hydrothermal acid pretreated molasses (oven) compared to acid pretreated molasses. A similar observation was reported by Aramvash et al. (2015), with the PHB production by *C. necator* utilizing fructose is higher than glucose, which are 2.16 g/L and 1.38 g/L respectively.

Other factors such as the presence of toxic by-product in the pretreated molasses also affects the cell biomass and PHB production. Although hydrothermal acid pretreated molasses (oil bath) contains the highest fructose and glucose compare to other pretreated molasses, the CDW and PHB yield are lower compared to hydrothermal acid

Table 3
Comparison of maximum cell dry weight (CDW) (g/L), maximum PHB content (g/L), Space time yield (gPHB/L/h) glucose, untreated molasses and different pretreated molasses for *C. necator* cultivation.

Types of pretreatment on molasses	Time, h	Maximum CDW, g/L	Maximum PHB content, g/L	Space time yield, gPHB/L/h
Glucose	72	2.81 ± 0.10	1.62 ± 0.45	0.022 ± 0.012
Untreated molasses	60	2.53 ± 0.15	0.38 ± 0.24	0.006 ± 0.004
Acid pretreated molasses	60	2.43 ± 0.08	0.49 ± 0.35	0.008 ± 0.006
Hydrothermal acid pretreated molasses (oven)	60	2.86 ± 0.82	0.78 ± 1.35	0.013 ± 0.022
Hydrothermal acid pretreated molasses (oil bath)	60	2.20 ± 0.12	0.55 ± 0.10	0.009 ± 0.002

Table 4
Comparison of PHB production by various bacterial species utilizing cane molasses as carbon source from previous studies.

Bacterial strain	Cane molasses concentration (%)	Fermentation mode	Harvest time (h)	PHB (%)	PHB yield (g/L)	References
Cupriavidus necator	1	batch	60	27.30	0.78	This study
Bacillus megaterium	2	batch	48	46.30	0.60	Gouda et al. (2001)
Bacillus megaterium ATCC 6748	4	batch	45	35.00	1.23	Chaijamrus and Udpuay (2008)
Bacillus thuringiensis IAM12077	1	batch	24	23.81	0.47	Shivakumar (2012)
Cupriavidus necator DSMZ 545	5	batch	70	–	1.35	Baei et al. (2009)

pretreated molasses (oven). This observation may due to the high temperature used in the hydrothermal acid pretreated molasses (oil bath) will promote the 5-hydroxymethylfurfural (HMF) production, which is toxic to the *C. necator* (Aramvash et al., 2015).

Table 4 shows the comparison of PHB production by various bacterial species utilizing cane molasses as carbon source from previous studies. Gouda et al. (2001) reported the highest PHB yield in the *Bacillus megaterium* cell biomass utilizing 4% cane molasses as carbon source is 0.60 g/L. Whereas the highest PHB yield was reported by Baei et al. (2009) which is 1.35 g/L utilizing 5% cane molasses as carbon source. In this study, the obtained PHB percentage and PHB yield is within the range of previously reported study, however, optimization of the fermentation process is required to further improve the PHB production.

4. Conclusion

The results obtained from the pretreatment experiment shows the types of pretreatment does affect the sucrose hydrolysis, glucose, and fructose concentration in the molasses. The hydrothermal acid pretreatment using oil bath as a heating source achieves the highest sucrose conversion and produce the highest fructose and glucose concentration in molasses. Furthermore, the hydrothermal acid pretreated molasses either using the oven or oil bath as the heating source produces a higher fructose concentration than glucose concentration. Although acid pretreated molasses and hydrothermal acid pretreated molasses (oven) have similar amount of glucose and fructose, the PHB and CDW of *C. necator* using hydrothermal acid pretreated molasses (oven) is higher than acid pretreated molasses, due to the higher fructose content. Fructose and glucose content in molasses was one of the main factors in molasses that affect the PHB accumulation and cell biomass production. Other components in molasses may also affect the PHB accumulation and cell biomass production.

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Conflict of interest

The authors declare no conflict of interest.

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