Polyhydroxyalkanoate Production by *Cupriavidus necator* with Inedible Rice

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Because of their lower environmental impact, biodegradable polymers such as polyhydroxyalkanoates (PHAs) produced within cultured bacteria represent promising alternatives to petroleum-based plastics. PHA production in flasks yielded optimal results with a carbon-to-nitrogen ratio of 22. The 5-L scale experimental results revealed that when glucose was used as the carbon source, *Cupriavidus necator* could produce 4.74 g/L PHA with 77.6% of PHA content in the microorganism 72 h after the initiation of the experiment. When the hydrolysis liquid from inedible rice was used as the carbon source, the highest concentration of PHA and ratio of PHA content in the microorganism were 4.82 g/L and 68.6%, respectively, after 72 h. Using the hydrolysis liquid from inedible rice as the carbon source reduced the culture cost and shortened the culture time, without affecting the structure of the PHA during production. Using the hydrolysis liquid from rice as the carbon source in PHA production (by *C. necator*) yielded optimal results, and the results may serve as a reference for applications involving other PHA-producing bacteria. Employing alternative carbon sources to culture bacteria might become a means of increasing the productivity and ensuring the quality of PHA products in the future.

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INTRODUCTION

Plastics play a pivotal role in human activities. Since 1950, the use of plastics has increased by an average of 4% annually. In 2010, approximately 4.8 to 12.7 million tons of plastic waste reportedly occupied the ocean, and this problem was predicted to worsen by 2025 (Wabnitz and Nichols 2010; Jambeck *et al.* 2015). Unfortunately, most of these petroleum-based plastic products are not easily decomposed by microorganisms. Some are whole plastic products, and some can be decomposed into debris, but in either form they will nonetheless remain in the environment for a long time. When these plastics enter the ocean, the well-known problem of microplastic pollution occurs (Eagle *et al.* 2016). These petrochemical plastics possess several characteristics, including being lightweight, rigid, not easily decomposed, and resistant to corrosion, heat, and electricity. Since the coronavirus outbreak in 2019, an estimated 1.6 million tons of global plastic waste, such as masks, gloves, protective clothing, and goggles, have been generated daily (Benson *et al.* 2021).

In response to these worsening problems, the production of bioplastics (in addition to reducing, reusing, or recycling petrochemical plastic products) has gradually attracted attention. Among bioplastic materials, polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) are the most promising. Compared with PLA, which can be composted or burned, PHA exhibits the advantage of superior biodegradability and can be 98% decomposed in the soil of a natural environment (test location: Vietnam, average temperature: 29 °C, average humidity: 80%, duration: 300 days) (Boyandin *et al.* 2013).

To achieve the goal of environmental protection, the current trend is to use environmentally friendly technologies in biomass refining to transform biomass sources into high value and biodegradable products (Dietrich *et al.* 2017). In Taiwan, rice harvested during typhoon season or during raining periods is prone to mold and not recommended for human consumption (*i.e.*, inedible rice). The rice industry is thus a prominent source of biomass, with several examples of rice biomass residues (*e.g.*, the straw or husk) being used to produce ethanol, lactic acid, charcoal, and ultrahigh-performance concrete or brick (Tuan *et al.* 2011; Panhwar *et al.* 2019). The biomass residues from the rice industry can also be converted into energy by employing thermochemical or biochemical processes. Such thermochemical processes can be divided into two types: (1) direct combustion and (2) conversion of the biomass into another form that can be subsequently used as an energy source. The biochemical processes are often designed to convert biomass into useful products such as ethanol, hydrogen, and methane. However, few studies have explored the application of rice in such biochemical processes.

PHAs are promising substitutes for petrochemical plastics. Some microorganisms can produce PHAs, which are accumulated in the microorganism cells and then provide the energy to those microorganisms for reproduction and division. The intracellular products are usually extracted using either physical or chemical methods to weaken or directly break up the cells. Physical methods include the following. Bacteria can be treated at high temperature and under high pressure. For example, the *pseudomonas* strain is treated at 120 °C for 1 minute to denature the outer membrane protein (De Koning and Witholt 1997). Second, a freezing method can be used for cell disruption; the principle is to disrupt the bacterial cells through ice crystal formation. This method can be combined with a sodium dodecyl sulfate method to obtain a more effective disruption effect (Dong and Sun 2000). However, this method is expensive and difficult to apply in commercial settings. Finally, sodium chloride solution can be used to change the osmotic pressure and then destroy the cell structure of a bacterium. Research has indicated that copper-greedy bacteria require approximately 140 mM sodium chloride solution for the disruption of their cells (Khosravi-Darani *et al.* 2004). The most common chemical method is the alkali treatment method, which requires at least 0.12 kg of strong sodium hydroxide per 1 kg of bacteria to effectively disrupt the bacterial cells (Tamer *et al.* 1998). PHAs can be roughly divided into short- and medium-chain PHAs. Short-chain PHAs refer to PHAs with five or fewer carbon monomers in their hydroxy acid functional groups, whereas medium-chain PHAs refers to PHAs with six or more carbon monomers in their hydroxy acid functional groups. The short-chain PHAs generally have properties similar to thermoplastics. By contrast, the properties of medium-chain ones are similar to those of rubber (Solaiman *et al.* 2006). The most common PHA type is poly-3-hydroxybutyrate (P3HB), which is also the simplest form of PHAs and possesses characteristics similar to those of polypropylene (PP) (Thirumala *et al.* 2010). P3HB has a melting point of 180 °C and a crystallinity of 55% to 80% (Sudesh *et al.* 2000). Its molecular weight is between 1×10^4 and 3×10^6 g/mol (Grage *et al.* 2009).

Because PHAs can be produced through various biological metabolic pathways, they are a common product accumulated in bacteria and archaea (Lu *et al.* 2009; Poli *et al.* 2011). Therefore, PHAs can be produced by converting various carbon sources, such as sugars (*e.g.*, fructose, lactose, or malt), n-alkanoic acids (*e.g.*, acetic, propionic, butyric, or valeric acids), and n-alkanol (*e.g.*, methanol, ethanol, or glycerol) (Anderson and Dawes 1990; Verlinden *et al.* 2007). PHAs can be produced using many strains, but mostly as intracellular products, which must be extracted by disrupting bacterial cells to obtain high-purity PHAs. Only a small proportion of strains, such as *Alcanivorax borkumensis*, can produce extracellular PHAs (Sabirova *et al.* 2006).

In the present study, inedible rice was used as biomass to produce PHAs, a technique that not only increases the value of expired rice but also reveals a new use for inedible rice in countries prone to rice overproduction. This technique is also relatively convenient. Compared with other techniques that use rice husk or straw as carbon source, it does not have to use complex chemical or physical pretreatment to process the inedible rice and can get more glucose from the biomass.

EXPERIMENTAL

Strain and Preservation Conditions

Cupriavidus necator (Bioresources Collection and Research Center item no. 17389) was purchased from the Food Industry Research and Development Institute, Hsinchu, Taiwan. The culture was initially reactivated in nutrient broth and cultivated with lysogeny broth agar. The bacterial strains were spread onto nutrient-rich agar plates containing 10 g/L peptone, 10 g/L meat extract, and 2 g/L yeast extract. The stock was prepared by adding pure glycerol to the culture broth after 24-h culture, and the final glycerol concentration was adjusted to 20%. The stock was then preserved at −80 °C.

Rice Hydrolysis in 5-L Bioreactor

A flowchart of the rice hydrolysis is presented in Fig. 1.

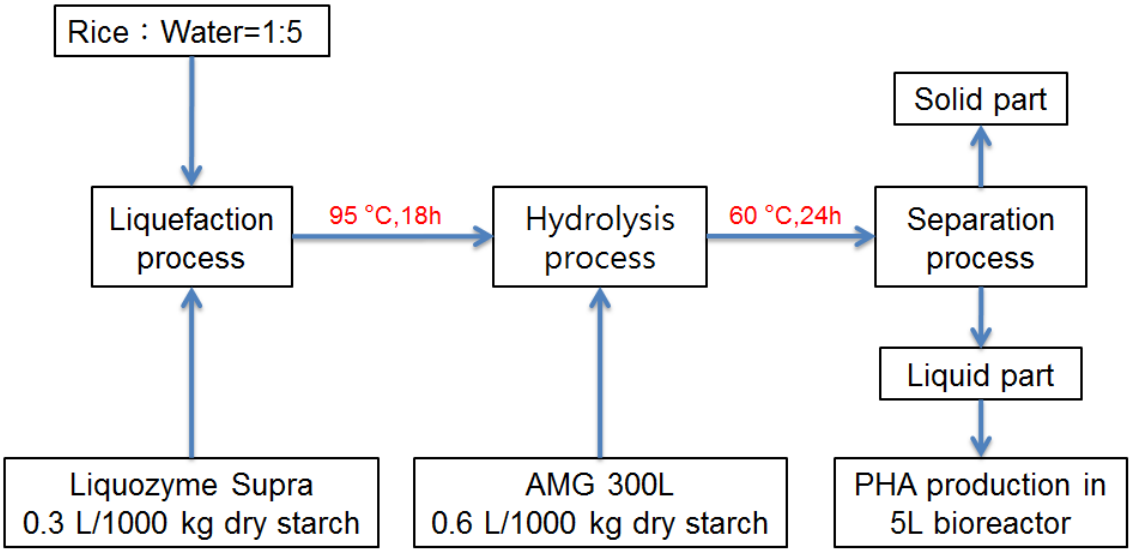

Fig. 1. Flowchart of the rice hydrolysis

The enzymatic hydrolysis was performed at 250 rpm in a 5-L bioreactor with a working volume of 3 L and pH controlled at 5.0 by 10 M NaOH and 85% phosphoric acid. The stored rice was mixed with deionized water and commercial Liquozyme Supra and AMG 300 L. A solid-to-liquid ratio of 20% (w/w) was established. For the liquefaction process, the dosage of Liquozyme Supra was set at 0.3 L/1000 kg dry starch (DS), and the temperature was maintained at 95 °C for 18 h. For the hydrolysis process, the dosage of AMG 300L was 0.6 L/1000 kg DS, and the temperature was maintained at 60 °C for 24 h. After the hydrolysis process, the glucose-rich liquid fraction was separated from the hydrolysis medium through a centrifugation process at 8000 rpm for 15 min. The liquid fraction was then used in the subsequent fed-batch culture process.

PHA Production in 250-mL Flasks

To obtain optimal culture conditions for producing PHAs, the seed cultures of *C. necator* were aerobically grown in 50 mL of basal mineral salt medium (MSM) inside 250-mL Erlenmeyer flasks at 30 °C for 16 h. The initial culture broth contained 2.0 g/L $(NH_4)_2SO_4$, 1.7 g/L citrate acid, 1.2 g/L $MgSO_4 \cdot 7H_2O$, 0.54 g/L KH_2PO_4, and 10 mL/L trace element. The trace element contained 10 g/L $FeSO_4 \cdot 7H_2O$, 3g/L $CaCl_2 \cdot 2H_2O$, 2.2 g/L $ZnSO_4 \cdot 7H_2O$, 0.3 g/L H_3BO_3, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.15 g/L $Na_2MoO_4 \cdot 2H_2O$, and 1 g/L $CuSO_4 \cdot 5H_2O$ dissolved in 1 L of deuterium-depleted water. An initial dry cell weight (DCW) of approximately 0.8 to 1.2 g/L was used. The culture processes were performed in triplicate with three different carbon-to-nitrogen (C/N) ratios of 22, 44, and 73 and with 150 rpm agitation at 30 °C for 72 h. Glucose was used as the carbon source and ammonium in the $(NH_4)_2SO_4$ was used as the nitrogen source. Preparation of the culture medium with a C/N ratio of 22 involved preparing 20 g of glucose and 2 g of $(NH_4)_2SO_4$. The calculation formula for the C/N ratio of 22 was as follows: $(20 \div 180 \times 6) \div (2 \div 132.14 \times 2)$. The pH was adjusted to 7.0 by using 1 M NaOH. The MSM was sterilized at 121 °C for 15 min. The trace element solution was sterilized using filtration (0.22-μm mfilter).

PHA Production in 5-L Bioreactor

PHA was produced in 5-L bioreactors with working volumes of 3 L at 150 rpm. Agitation was applied using a Rushton turbine. The culture temperature was set at 30 °C for the *C. necator* with an initial DCW of 0.7 g/L. The air flow rate was adjusted to 1 vvm. The seed culture conditions were in accordance with the aforementioned production of PHA in 250-mL flasks. The pH of the culture medium was adjusted to 7.0 by using 1 M NaOH. The culture processes were performed in triplicate with the C/N ratio of 22, agitation of 150 rpm, and temperature of 30 °C for 72 h. Glucose from the glucose-rich liquid fraction was used as the carbon source at a concentration of 200 g/L. Ammonium in the $(NH_4)_2SO_4$ was used as the nitrogen source.

Analysis Methods

The rice was supplied by a private farm in Taoyuan, Taiwan. The starch content of the rice was determined after hydrolysis according to the Association of Official Agricultural Chemists' (1995) method 996.11. To determine the protein concentration, an aliquot of the hydrolysis liquid from the rice was used in conjunction with the Bradford (1976) method, with bovine serum albumin as the standard protein.

Each liquid sample was filtered through a 0.45-μm filter and diluted appropriately with deionized water. The quantitative analysis of the glucose was conducted using a high-performance liquid chromatography system equipped with a refractive index detector at 45

°C. The separation was achieved using a Coregel-87H3 column (Transgenomic, San Jose, CA, USA), which was maintained at 65 °C with 8 mM H_2SO_4 as the eluent, at a flow rate of 0.8 mL/min. Cell growth was monitored by measuring the absorbance at 600 nm by using a U-3000 spectrophotometer (HITACHI, Tokyo, Japan). All the data presented in this paper were averaged from the results of the three independent experiments.

To measure the DCW of the biomass, 10 mL of culture broth was centrifuged at 12,000 rpm for 10 min at 25 °C, suspended in 10 mL of distilled water, then recentrifuged, whereupon the pellet was transferred to a preweighed, 2-mL Eppendorf tube and dried at 80 °C in a hot air oven to obtain a constant weight. The concentration of polyhydroxybutyrate (PHB) was determined using gas chromatography (GC) with an AT-WAX fused silica capillary column (Alltech Italia s.r.l., Milan, Italy) and a flame ionization detector. The gas carrier was helium, and the injection port, detector, and oven temperatures were 250, 270, and 150 °C, respectively. The GC device was programmed with the oven temperature at 90 °C for 1 min, after which the temperature was increased at a rate of 5 °C/min up to a final temperature of 150 °C, which was maintained for 6 min. The split ratio was set at 10:1. The internal standard was benzoic acid, and the external standards were 3-hydroxybutyric acid (Sigma-Aldrich, Italy) and a P(3HB-co-3HV) copolymer (Biopol; Imperial Chemical Industries, United Kingdom). Freeze-dried biomass (100 mg) was placed into glass tubes, to which 2 mL of acidified methanol (3% v/v sulphuric acid, 2.5 g/L methyl-benzoate) and 2 mL of chloroform were added. The methylbenzoate was used as an internal standard. The tubes were closed with screw caps and maintained at 100 °C for 3 h. After cooling with ice, 4 mL of distilled water were added to the glass tubes. The samples were vortexed for 10 s, and then 1 μL of the chloroform phase was injected into the GC machine (Thermo Finnigan Corporation, Milan, Italy). A Restek capillary column (Rtx-5MS; 30 m × 0.25 mm × 0.25 μm) was used with a gas flow rate of 1.5 mL/min. The carrier and make-up gases were helium and nitrogen, respectively. The molecular structure of the PHB was determined with ^{1}H NMR spectra. Specifically, samples (10 mg) were dissolved in 1 mL of dimethyl sulfoxide-d_6 and then detected using a Bruker AV-400 NMR spectrometer at 400 MHz.

RESULTS AND DISCUSSION

Effect of Carbon-to-Nitrogen Ratio on PHA from *Cupriavidus necator*

In the flask scale experiments, when the C/N ratio was 22, the ratios of PHA on cell dry mass (CDM) were 19.36 and 35.9 at 48 and 72 h, respectively (after the initiation of the experiment); the concentrations of CDM were 0.01, 0.67, 3.77, and 4.93 at 0, 24, 48, and 72 h, respectively; the concentrations of PHA were 0.73 and 1.77 at 48 and 72 h, respectively (Fig. 2a); and the optical density (OD) values were 0.4, 2.4, 13.5, and 17.5 at 0, 24, 48, and 72 h, respectively. When the C/N ratio was 44, the ratios of PHA on CDM were 22.93 and 22.87 at 48 and 72 h, respectively; the concentrations of CDM were 0.11, 2.66, 3.62, and 4.11 at 0, 24, 48, and 72 h, respectively; the concentrations of PHA were 0.83 and 0.94 at 48 and 72 h, respectively (Fig. 2b); and the OD values were 0.41, 9.5, 12.6, and 14.7 at 0, 24, 48, and 72 h, respectively. When the C/N ratio was 73, the ratios of PHA on CDM were 42.03 and 32.85 at 48 and 72 h, respectively; the concentrations of CDM were 0.11, 0.81, 2.07, and 2.77 at 0, 24, 48, and 72 h, respectively; the concentrations of PHA were 0.87 and 0.91 at 48 and 72 h, respectively (Fig. 2c); and the OD values were 0.4, 2.9, 7.4, and 9.9 at 0, 24, 48, and 72 h, respectively.

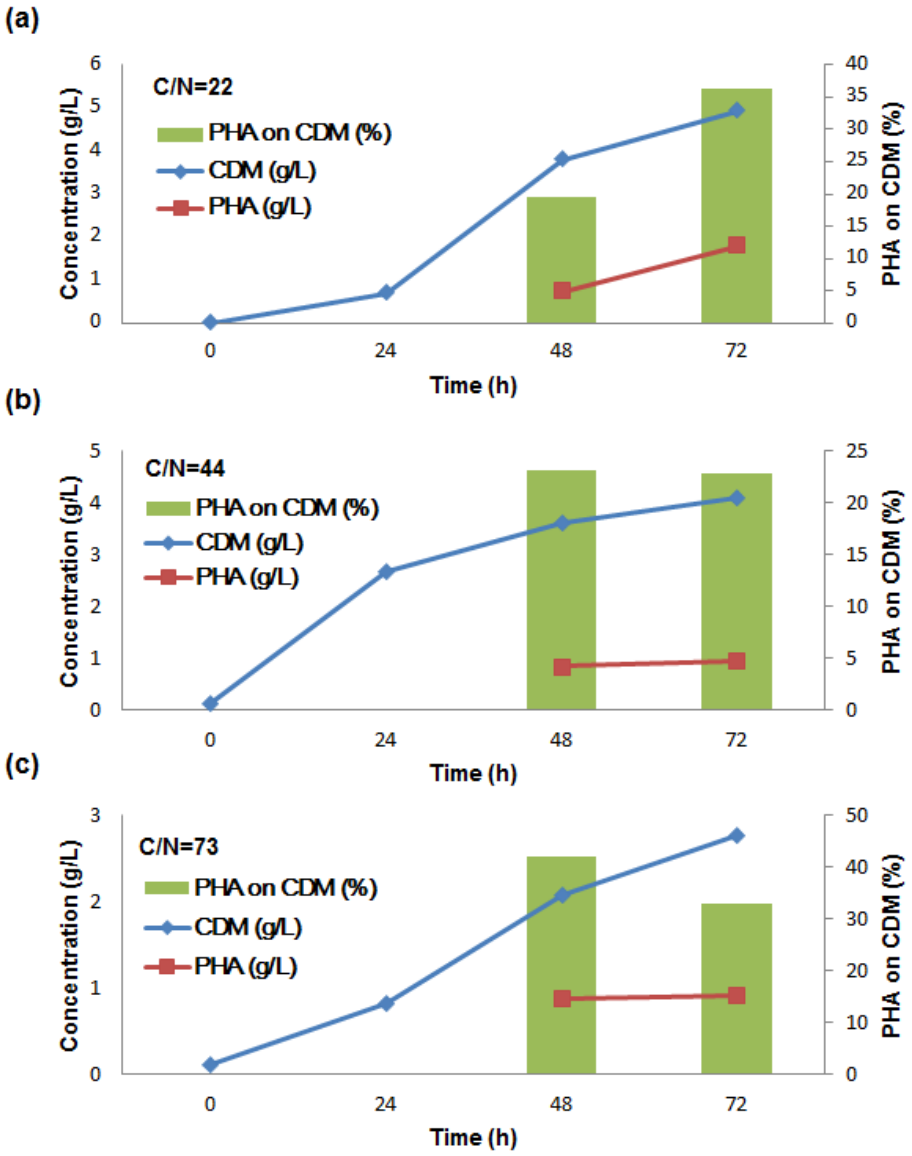

Fig. 2. Variations of PHA on CDM, CDM, and PHA concentration at three different C/N ratios of (a) 22, (b) 44, and (c) 73

The results revealed that the highest OD value and concentration of PHA in the initial 72 h of production occurred when the C/N ratio was set at 22. However, the highest PHA on CDM ratio (%) was registered in the initial 48 h of production, when the C/N ratio was 73. Thus, when the *C. necator* was cultured under high C/N ratio conditions, it was prone to the accumulation of PHA during the initial 48 h of production, but the highest CDM of the *C. necator* was recorded in the initial 72 h of production, when the C/N ratio was set at 22. This is consistent with previous studies showing that when a cell undergoes a growth period, its growth performance is largely dependent on the concentration of nitrogen, which is essential for protein synthesis during the growth period. Valentino *et al.* (2015) and Cui *et al.* (2017) used glucose as a carbon source and adjusted the C/N ratio in various feeding strategies during the PHA production period by *Haloferax mediterranei.* They also discovered that the biomass increased with higher nitrogen concentrations.

Research has also indicated that myriad initial C/N ratios can be used to determine the optimal culture conditions for bacteria to produce PHAs. Ahn *et al.* used rice straw hydrolysate as a carbon source, allowing *C. necator* to produce PHAs; their group reported that nitrogen was pivotal for new cell production. They also noted that the accumulation rate of PHA was higher in conditions with higher degrees of N-deficient than in those with lower ones. When the C/N ratio of the rice straw hydrolysate was adjusted to 160, the concentration of PHA was 0.36 ± 0.0033 g/L in the initial 12 h of production (Ahn *et al.* 2015). Selecting the optimal C/N ratio during the production process is crucial to the accumulation of PHAs, biomass, and production costs.

5-L Scale PHA Production Process

As in the 250-mL flask scale experiments, the C/N ratio was set at 22 for the 5-L scale production experiment described here. In this experiment, glucose and hydrolysis liquid from the rice were used as the carbon source. The proportions of protein and starch in the rice were 8% and 58.9%, respectively. Moreover, the protein concentration of hydrolysis liquid from the rice was 2.44 g/L. When using glucose as the carbon source, the ratios of PHA on CDM were 61.6, 77.1, 77.6 and 77.3 at 48, 56, 72, and 96 h, respectively (after the initiation of the experiment); the concentrations of CDM were 0.12, 0.31, 1.23, 2.83, 4.82, 5.67, 6.11, and 7.26 at 0, 8, 24, 32, 48, 56, 72, and 96 h, respectively; and the concentrations of PHA were 2.97, 4.37, 4.74, and 5.61 at 48, 56, 72, and 96 h, respectively (Fig. 3a).

When using the hydrolysis liquid from rice as the carbon source, the ratios of PHA on CDM were 55.5, 65.5, 68.6 and 68.6 at 48, 56, 72, and 96 h, respectively; the concentrations of CDM were 0.13, 0.49, 3.41, 4.69, 6.22, 6.72, 7.03, and 8.76 at 0, 8, 24, 32, 48, 56, 72, and 96 h, respectively; and the concentrations of PHA were 3.45, 4.41, 4.82, and 5.53 at 48, 56, 72, and 96 h, respectively (Fig. 3b). As illustrated in Fig. 3b, during the period of 8–48 h after the experiment's commencement, the CDM of the *C. necator* underwent a rapid growth phase. The rate of growth was faster when the hydrolysis liquid from the rice was used than when only glucose was used as the carbon source. This might be because the hydrolysis liquid from the rice contained an additional nitrogen source that increased the C/N ratio in growth period of *C. necator*, thereby aiding in the *C. necator* protein synthesis and, ultimately, the growth rate. These results are consistent with those of previous research, which have also suggested that when the C/N ratio of the culture medium is set at a lower level, the PHA-producing bacteria exhibit superior growth performance during a rapid growth phase.

Despite the growth result of the PHA-producing bacteria recorded in the present study being consistent with previous research (Valentino *et al.* 2015; Cui *et al.* 2017), the concentrations of PHA were higher in 5-L scale production experiments where the hydrolysis liquid from the rice was used as the carbon source. This might be attributable to the content of the hydrolysis liquid. The rice used in this study was a unique species in Taiwan; the hydrolysis liquid might have contained other trace elements such as inorganic elements or vitamins that were conducive to *C. necator* producing higher PHA concentrations. The present study's data suggest that using hydrolysis liquid from rice as a carbon source and providing a supplement of air can not only accelerate the growth rate of *C. necator* but also allow it to produce higher concentrations of PHA during the 72 h after the initiation point of a culture.

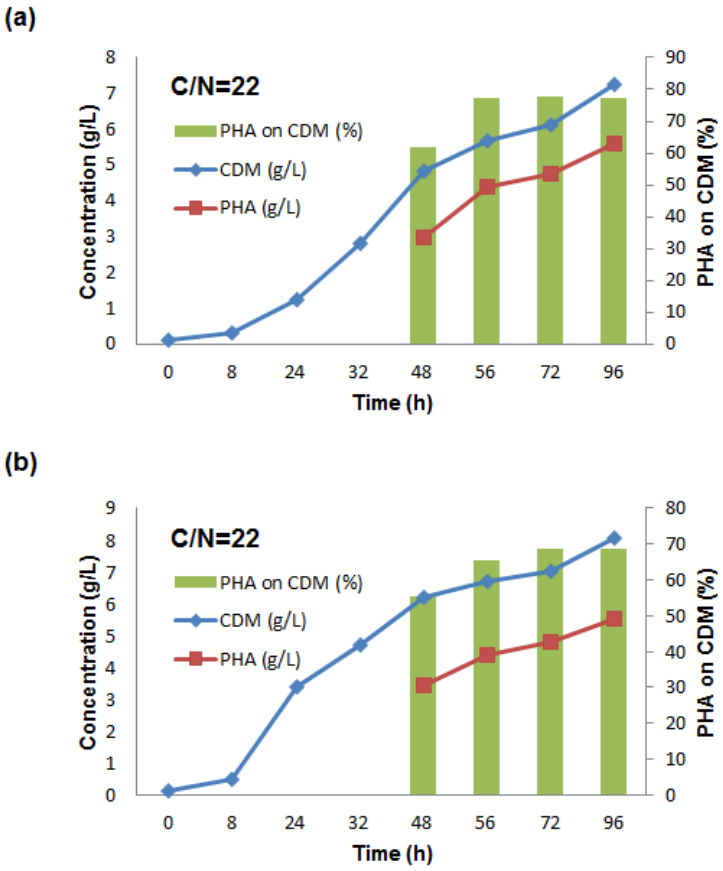

Fig. 3. Variations of PHA on CDM, CDM, and PHA concentrations when using (a) glucose and (b) hydrolysis liquid from rice as the carbon source

Structure of PHA After Production Process

The ^{1}H NMR spectra of the copolymers (produced by the *C. necator*) are displayed in Fig 4. The peak signal registered at approximately 1.26–1.28 ppm was classified as the methyl (CH_3) group in the polymer. This accords with the results obtained in previous research (Lopez-Cuellar *et al.* 2011; Arumugam *et al.* 2018). The peak signal at 2.43–2.64 ppm was classified as the H-atom resonance of methylene group (CH_2) in the polymer. The third peak signal, which was recorded at 5.22–5.29 ppm, was due to the resonance absorption of methine (CH) groups in the polymer. These signal peaks imply that the PHA obtained from production process by *C. necator* was PHB. In addition, the peaks observed at approximately 0.9 and 1.58 ppm suggest the presence of 3-hydroxyvalerate (3-HV) units (Li *et al.* 2009; Zainab-L *et al.* 2018). The monomer parts of each polymer were calculated according to the intensity ratio of the methyl components from the ^{1}H spectra. The results show that the *C. necator* produced the same copolymer of PHB and PHV during the production process, regardless of whether glucose or hydrolysis liquid from rice was used as the carbon source.

The results of 5-L scale experiments indicate that using the hydrolysis liquid from rice did not affect the molecular composition of the PHA during the production process. Because the chemical and physical characteristics of the PHA can be altered by adding different ratios of various PHA types (Albuquerque and Malafaia 2018), producing a single copolymer initially and then using different PHAs at different ratios to develop products with unique chemical and physical characteristics is most beneficial. Previous studies have

indicated that using different carbon sources alters the molecular composition of PHAs (Tsuge *et al.* 2013; Hanik *et al.* 2019). In this study, using the hydrolysis liquid from rice as a carbon source was demonstrated to control the characteristics of eventual PHAs and increase the reliability and quality of products.

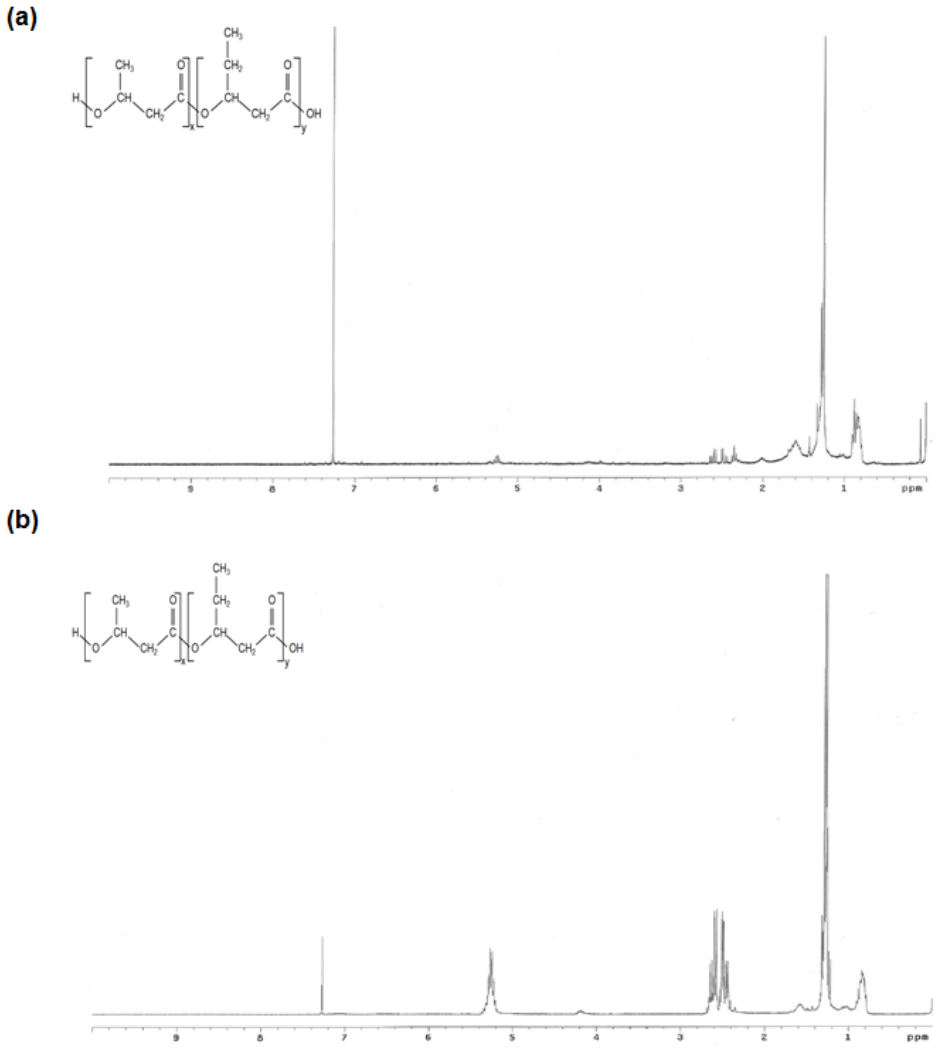

Fig. 4. ^{1}H NMR spectra of PHA derived from the production process by *C. necator* using (a) glucose and (b) hydrolysis liquid from rice as the carbon source. The block copolymer is PHB-co-PHV.

CONCLUSIONS

1. The C/N ratio is critical in determining the optical density (OD) value and the concentration of polyhydroxyalkanolate (PHA) and cell dry mass (CDM).
2. When using hydrolysis liquid as a carbon source, the additional nitrogen and other nutrients in the hydrolysis liquid potentially catalyze a higher growth rate and PHA concentration than using only glucose as the carbon source to produce PHA by *C. necator*.

3. Using hydrolysis liquid from inedible rice as a carbon source during the PHA production process does not affect the eventual PHA structure of PHA, which may be beneficial for applications in the bioplastics industry.

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