Polyhydroxyalkanoate Production from Food Packaging Waste Paper

Wei-Lin Tu, Yu-Chuan Hsiung, Chen-Hsien Liang, Jung-Mei Huang, Chung-Mao Ou, and Gia-Luen Guo *

This study evaluated the production of marine biodegradable plastics, specifically polyhydroxyalkanoates (PHAs), using waste paper from food containers as a novel material source. The results showed that adding dilute sulfuric acid as a pretreatment may have a negative impact on enzyme hydrolysis efficiency. Without pretreatment, the highest glucose concentration was observed in the 50-min heating group. In the experimental group with 1% dilute sulfuric acid as a pretreatment, the highest average glucose concentration was observed in the 25-min treatment group. In flask scale experiments, the C/N ratio was controlled at 10, 20, and 30. The results showed that when the C/N ratio was 10, the PHA/CDM ratios were 16.3 and 23.6 at 48 and 72 h, respectively. After 96 h of cultivation using hydrolysis liquid from the waste paper container as the sole carbon source in a 5-L scale experiment, the PHA/CDM ratio was 28.7 and the PHA concentration was 0.95 g/L. The potential bacterial strain in this study was confirmed to be a *Bacillus* genus bacterium after strain identification. The signal peaks indicated that the PHA obtained from the *Bacillus* sp. production process was PHB.

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Contact information: Department of Chemistry, National Atomic Research Institute, No. 1000, Wenhua Road, Jiaan Village, Longtan District, Taoyuan City, 32546, Taiwan;
** Corresponding author: glguo@nari.org.tw*

INTRODUCTION

Traditional plastics are organic polymers synthesized from petroleum raw materials (Andrady and Neal 2009). Since the invention of plastics in the early 1900s, the use of plastics has increased rapidly, and its applications have continued to expand. This has led to the development of various polymers and additives with different chemical and physical properties, and the range of products produced has expanded from disposable products such as plastic bags, food packaging, and plastic bottles to more diverse applications such as clothing, fishing nets, and synthetic fibers. However, plastic waste has become a major environmental concern due to its persistence in the environment and its adverse effects on wildlife and human health (Wabnitz and Nichols 2010; Verma *et al*. 2016). Therefore, there is an urgent need to develop sustainable alternatives to traditional plastics, such as biodegradable plastics made from renewable resources (Moshood *et al*. 2022).

The trend of eating out has led to an increasing number of people using non-disposable utensils, such as paper lunch boxes, instead of plastic plates. However, although the primary material of waste paper lunch boxes (cups, plates, bowls) is paper pulp fibers, they should be classified as "waste paper containers" when recycling, and should not be mixed with waste paper. This is mainly because the paper container is coated with a layer

of polyethylene (PE) or treated with wax on the surface to prevent water and oil stains. If the public does not properly classify the waste paper containers for recycling, and sends them to general paper mills for processing along with waste paper, the plastic film separated from them will be considered garbage and sent to incinerators or boilers for burning, increasing the difficulty and cost of recycling and reusing paper mill waste. The main component of waste paper containers is wood cellulose, which can be considered a type of bio-based material. With appropriate physical, chemical, or biological treatments, the cellulose can be depolymerized into fermentable sugars and transformed into higher-value green products using biotechnological fermentation techniques. The technological aspects that require evaluation include composition analysis, pretreatment, and catalytic conditions for wood cellulose in paper containers, production of hydrolyzed cellulose fibers, enzymatic hydrolysis, and the application of biotechnological fermentation techniques (Dutta *et al*. 2022).

Polyhydroxyalkanoates (PHAs) are a type of renewable and biodegradable bio-based polymer. Many microorganisms can accumulate PHA in the form of intracellular granules in environments with excess carbon source and limited nitrogen source. PHA is stored as a polyester and serves as a carbon source and energy reserve. Together with another type of bio-based plastic, polylactic acid (PLA), PHA is considered a green polymer with significant potential for future applications. This is mainly because bio-based plastics including PHA and PLA have the potential to replace conventional petrochemical plastics with physicochemical properties similar to those of polypropylene (PP) and low-density polyethylene (LDPE).

According to the statistics of the United States Environmental Protection Agency (USEPA), in 2015, the amount of waste paper containers landfilled was 1.9 million tons, while the recycling rate of recycled paper products and cardboard in the United States was about 78.2%. The recycling rate of waste paper in India is only 25 to 27%, which is significantly lower than 70 to 80% in advanced countries, indicating that the recycling of paper containers is a problem that is being taken seriously around the world. In addition, the burning of paper and ink may have environmental impacts. When paper is burned, it releases a significant amount of toxic chemicals. These toxic substances can affect the lungs and heart. Furthermore, when the ink on the paper is burned, it releases toxic gases. Anyone inhaling these fumes may experience irritation in the lungs and throat (Adebona *et al.* 1998; Zhang *et al*. 2017). Moreover, the leaching of chemicals may also cause groundwater pollution. Therefore, the proper reuse of waste paper containers after recycling is of great importance. Recycling paper containers and paper can reduce the carbon footprint of the product and protect trees from being cut down. In 2020, the amount of waste paper containers recycled in Taiwan was 159,000 tons, and from January to June 2021, the amount was 74,087 tons, an increase of 7.85% compared to the same period in 2020 (Environmental Protection Administration 2021). It is a valuable resource with a considerable production volume and recycling channel. Therefore, increasing the recycling percentage of waste paper containers and properly utilizing their short-fiber pulp to produce biomass products is an urgent and necessary issue.

Concerns about climate change caused by greenhouse gas emissions have forced the shift from unsustainable fossil fuel resources to sustainable and renewable energy sources (Amin *et al*. 2022). Therefore, the process of converting biomass into sugar raw materials, fuels, or chemicals through appropriate pretreatment, enzyme action, or microbial transformation techniques is called biorefinery (Cherubini 2010), and has become the mainstream direction for developing renewable energy resources.

Because the past biorefinery industry relied heavily on edible sources such as starch and sucrose, there were concerns about competing with food sources (Himmel *et al*. 2007). Therefore, non-edible lignocellulosic biomass raw materials, which are considered to be very promising for developing renewable chemicals and fuels and obtaining abundant sources with potential low-cost opportunities, are now being used for biorefinery production (Alvira *et al*. 2010). Lignocellulosic waste can come from various sources such as industrial waste (*e.g.* sawdust, paper mill waste, and food industry waste), forestry waste (*i.e.* hardwood, *etc.*), agricultural residues (*e.g.* rice straw, straw, and non-food seeds), and household waste (*e.g.* kitchen waste, sewage, and garbage) (Kumar *et al*. 2009). Therefore, lignocellulose can be regarded as a sustainable raw material that can be supplied in the long term to meet the growing demand for energy resources in the future and to mitigate environmental issues. Most lignocellulosic raw materials are mainly composed of cellulose, hemicellulose, and lignin (Garcia-Maraver *et al*. 2013). Cellulose and hemicellulose are polymers of monosaccharides such as glucose, mannose, galactose, xylose, arabinose, and rhamnose (Peng *et al*. 2012), which can be fermented into biofuels (*e.g.* bioethanol) and biochemicals (*e.g.* succinic acid, lactic acid, dimethyl terephthalate, and polyhydroxyalkanoates (PHAs)) (Qaseem *et al*. 2021). Currently, the cost of raw materials, which is dominated by carbon source cost, is the main factor affecting the final production cost of PHA (Castilho *et al*. 2009). The carbon sources currently used mainly come from corn starch, sucrose, and edible oils, while the use of lignocellulose as a carbon source for PHA production is quite challenging due to the need to break down its tough structure to obtain cellulose hydrolysates.

From the above, it can be seen that lignocellulosic materials are a promising source of materials, but the acquisition of lignocellulosic materials still faces problems such as a dispersed supply source, difficult transportation, and high raw material costs. Therefore, based on the fact that waste paper containers also contain cellulose components, they can be regarded as a source of "lignocellulosic biomass," and how to effectively reuse them is also an urgent issue to be addressed in domestic waste management. Therefore, this study aimed to explore the efficiency of obtaining cellulose hydrolysates from waste paper containers that are rich in cellulose by using the pretreatment and enzymatic hydrolysis techniques used for lignocellulosic materials. At the same time, the feasibility of producing biodegradable PHA from cellulose hydrolysates of waste paper containers using biological fermentation methods was also evaluated.

EXPERIMENTAL

Bacterial Strain

The selection samples were collected from the wastewater treatment plant in Yilan, Taiwan. The samples were then prepared for further analysis. Nile red dye stock solution was prepared by dissolving Nile red powder (Product Number: 72485, SIGMA-Aldrich, St. Louis, USA) in DMSO to a concentration of 0.25 mg/mL. The solution was then sterilized by passing it through a 0.22-μm filter and stored in the dark at room temperature. R2A medium containing 2.5% glucose was prepared and sterilized by autoclaving. After autoclaving, the medium was cooled down to 50 °C, and the Nile red dye stock solution was added to the medium to a final concentration of 0.5 μg/mL. The medium was then poured into sterile petri dishes and allowed to solidify. Sewage sludge samples were collected and diluted with PBS. The diluted samples were inoculated onto the Nile red-

containing R2A plates and incubated at 30 °C for 2 to 7 days until single colonies were formed. The colonies with strong Nile red fluorescence were selected using UV light and streaked onto fresh R2A plates for further analysis. Exposure to UV light enabled the identification of the brightest single colony from the Nile red-containing medium as the potential bacterial strain source.

The strain selected by screening on Nile red-containing medium was further screened for PHA-producing strain using PCR. The amplification of *phaC* gene fragments with all primer combinations was performed using the following program: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 45 seconds, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The primer sets used were 5'-CCGCCSTGGATCAACAAGT-3' and 5'-GTGCCGCCGAYGCAGTAGCC-3'. All reactions were carried out using the thermocycler Eppendorf Mastercycler Gradient, with a reaction mixture of 20 µL containing 1× PCR amplification buffer (Invitrogen), 0.2 mM of each dNTP, 0.5 µM of each primer, 1U Taq DNA polymerase (Invitrogen), 2 mM $MgCl_2$, and the template DNA. The PCR amplicons were resolved by electrophoresis on 1% agarose gel and visualized under a UV transilluminator after staining with ethidium bromide.

A colony capable of producing PHA was identified by first conducting a polymerase chain reaction (PCR) to sequence the 16S rDNA and subsequently performing BLAST analysis on the sequenced rDNA using the GenBank database maintained by the National Center for Biotechnology Information.

Enzymatic Hydrolysis in 250-mL Flasks

Waste paper food containers were washed and dried until the moisture content was less than 5%, then pulverized using a grinder. A total of 10 g of the pulverized material was weighed and placed in a hydrolysis flask, followed by the addition of 50 mL of 0%, 1%, and 3% dilute sulfuric acid solutions. The mixtures were then treated at 121 °C using an autoclave for 25, 50, and 120 min, respectively. To determine the optimal pretreatment method for PHA production from waste paper containers, an enzymatic hydrolysis procedure was carried out in 250-mL flasks using 50 mM sodium acetate buffer (pH 4.8) and 10% dry matter (w/v) with an appropriate amount of CTec3 cellulase enzyme (Novozymes, Bagsværd, Denmark). The hydrolysis process was conducted for 72 h at 50 °C on an orbital shaker at 100 rpm. The 10% dry mass slurry was prepared by measuring the moisture content of the waste paper container hydrolysate and weighing the hydrolysate on a wet basis. The weighed pretreated waste paper container hydrolysate was then mixed with sodium acetate buffer to achieve a final volume of 50 mL for the enzymatic hydrolysis. In this study, 15 FPU/g of cellulose of CTec3 was used to determine the best pretreatment condition, with the pH adjusted to 5.0 using 10 M NaOH. High-performance liquid chromatography (HPLC) (Agilent 1200; Agilent, Santa Clara, CA, USA) was employed to analyze the glucose concentration in the reaction solutions. Triplicate reactions were performed for each sample.

Enzymatic Hydrolysis in a 5-L Bioreactor

To obtain a larger quantity of glucose-containing hydrolysate for subsequent PHA production, a larger 5L reactor was used for enzymatic hydrolysis reaction. The enzymatic hydrolysis was conducted at 250 rpm in a 5-L bioreactor with a working volume of 3 L with pH control. Waste paper container hydrolysates from the pretreatment process were mixed with deionized water and commercial CTec3, with a solid-to-liquid ratio of 10%

(w/v) and enzyme activity of 15 FPU/g for cellulose. The pH of the hydrolysis medium was adjusted to 5.0 using 10 M NaOH, and the temperature was set at 50 °C. After hydrolysis for 48 h, the glucose-rich liquid fraction was separated from the hydrolysis medium by centrifugation at 8000 rpm for 15 min and subsequently used in the fermentation process.

PHA Production in 250-mL Flasks

To optimize the culture conditions for polyhydroxyalkanoates (PHAs) production, potential bacterial strains were aerobically grown in 50 mL of basal mineral salt medium (MSM) in 250-mL Erlenmeyer flasks at 30 °C for 16 h. The composition of the initial culture broth consisted of 2.0 g/L $(NH_4)_2SO_4$, 1.7 g/L citric 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 solution was prepared by dissolving 10 g/L $FeSO_4 \cdot 7H_2O$, 3 g/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 $CuSO_4 \cdot 5H_2O$ in 1 L of deuterium-depleted water. The initial dry cell weight (DCW) of 0.8 g/L was used. The culture was performed in triplicate under three different carbon-to-nitrogen (C/N) ratios of 10, 20, and 30, with agitation set at 150 rpm and temperature maintained 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. The culture medium with a C/N ratio of 10 was prepared by dissolving 10 g of glucose and 2.2 g of $(NH_4)_2SO_4$, and the C/N ratio of 10 was calculated using the formula: $(10 \div 180 \times 6) \div (2.2 \div 132.14 \times 2)$. In this formula, 10 represents the amount in grams of glucose, 180 is the molecular weight of glucose, 6 represents the number of carbon atoms in the glucose molecular formula; 2.2 represents the amount in grams of ammonium sulfate, 132.4 is the molecular weight of ammonium sulfate, and 2 represents the number of nitrogen atoms in the ammonium sulfate molecular formula. The pH was adjusted to 7.0 using 1 M NaOH. The MSM was sterilized at 121 °C for 15 min, and the trace element solution was sterilized by filtration through a 0.22-μm filter.

PHA Production in 5-L Bioreactor

PHA production was conducted in 5-L bioreactors with a working volume of 3 L, using a Rushton turbine for agitation at a rate of 150 rpm. The culture temperature was maintained at 30 °C for the potential bacterial strain, with an initial DCW of 0.8 g/L. The seed culture was prepared using the same conditions as the PHA production in 250-mL flasks. The culture process was carried out in triplicate, with a C/N ratio of 10, agitation of 150 rpm, and temperature of 30 °C for 48 h. Glucose from hydrolysis liquid was used as the carbon source, obtained from the glucose-rich liquid fraction, while ammonium in $(NH_4)_2SO_4$ served as the nitrogen source.

Analysis Methods

The procedure was described previously in Tu *et al*. (2022). Liquid samples were passed through a 0.45-μm filter and diluted in deionized water. Glucose was quantified by a high-performance liquid chromatography system equipped with a refractive index detector set at 45 °C, using a Coregel-87H3 column (Transgenomic, San Jose, CA, USA) 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 determined by absorbance at 600 nm using a U-3000 spectrophotometer (HITACHI, Tokyo, Japan). All data are the average of the results of three independent experiments.

To determine the dry cell weight (DCW) of the biomass, a 10 mL sample of the

culture broth centrifuged at 12,000 rpm for 10 min at 25 °C. The pellet was resuspended in 10 mL of distilled water and subjected to a second centrifugation. The resulting pellet was transferred to a pre-weighed 2-mL Eppendorf tube and oven-dried at 80 °C until it reached a constant weight. The polyhydroxyalkanoate (PHA) concentration was obtained using gas chromatography (GC) with an AT-WAX fused silica capillary column (Alltech Italia s.r.l., Milan, Italy) and a flame ionization detector. Helium was used as the gas carrier. The injection port, detector, and oven temperatures were 250, 270, and 150 °C, respectively. The GC device was programmed to maintain the oven temperature at 90 °C for 1 min, after which it was increased at a rate of 5 °C/min until a final temperature of 150 °C was reached and held for 6 min. The split ratio was set to 10:1. Benzoic acid was used as the internal standard, while 3-hydroxybutyric acid (Sigma-Aldrich, Italy) and a P(3HB-co-3HV) copolymer (Biopol; Imperial Chemical Industries, United Kingdom) were used as the external standards. Freeze-dried biomass (100 mg) was mixed with 2 mL of acidified methanol (3% v/v sulphuric acid, 2.5 g/L methyl-benzoate), and 2 mL of chloroform in glass tubes. The tubes were then sealed with screw caps and incubated at 100 °C for 3 h. After cooling with ice, 4 mL of distilled water was added to the tubes, which were vortexed for 10 s. Subsequently, 1 μL of the chloroform phase was injected into the GC machine (Thermo Finnigan Corporation, Milan, Italy), using a Restek capillary column (Rtx-5MS; 30 m × 0.25 mm × 0.25 μm) with a gas flow rate of 1.5 mL/min. Helium and nitrogen were used as the carrier and make-up gases, respectively. The molecular structure of PHB was determined using 1H NMR spectra. Samples (4 g) were dissolved in 50 mL of chloroform, and sealed in 250-mL serum bottles, shaking for 15 min. Then, 30 mL of water was added and a separatory funnel was used to remove the lower chloroform layer. The chloroform phase was heated at 60°C to evaporate it, obtaining a crude extract. The crude extract was cut into small pieces, then stirring it with 100-mL methanol (99%) for two hours. The solid product was left after removing the methanol. The solid product was washed with 50 mL deionized water, then the water was drained and dried in a 60°C oven for one hour. The solid product (30 mg) was dissolved in 0.8-mL chloroform-d (product 434876, Sigma-Aldrich, St. Louis, USA) and analyzed using a Bruker AV-400 NMR device at 400 MHz.

RESULTS AND DISCUSSION

Preparation of Hydrolysate from Waste Food Packaging Paper

This experiment investigated the effect of pretreatment of waste good packaging material by enzymatic hydrolysis reactions. Adding dilute sulfuric acid as a pretreatment may have a negative impact on enzyme hydrolysis efficiency. The results showed that in waste paper containers without pretreatment, the average glucose concentration after 24 h of hydrolysis was 4.87 g/L (Fig. 1). In the untreated experimental group heated for 50 min, the highest glucose concentration after 24 h of hydrolysis was observed, with an average glucose concentration of 8.30 g/L. However, as the heating time increased, the average glucose concentration in the 120-min treatment group decreased to 7.15 g/L. In the experimental group with 1% dilute sulfuric acid as a pretreatment, the highest average glucose concentration was observed in the 25-min treatment group, with an average glucose concentration of 7.73 g/L after 24 h of hydrolysis. However, with an increase in the pretreatment heating time, the average glucose concentration in the hydrolysate did not increase, but instead was 7.44 and 6.75 g/L in the 50-min and 120-min treatment groups, respectively. Finally, in the experimental group with 3% dilute sulfuric acid as a

pretreatment, the highest glucose concentration after 24 h of hydrolysis was observed in the 25-min treatment group, with an average glucose concentration of 6.93 g/L. Based on the experimental results, it can be concluded that waste paper containers without dilute sulfuric acid pre-treatment and heated for 50 min showed the best performance in enzymatic hydrolysis reaction. Conversely, pre-treatment with dilute sulfuric acid may have a negative impact on enzymatic hydrolysis efficiency, leading to a decrease in glucose concentration in the hydrolysate. Moreover, this effect seems to intensify with increasing pre-treatment heating time.

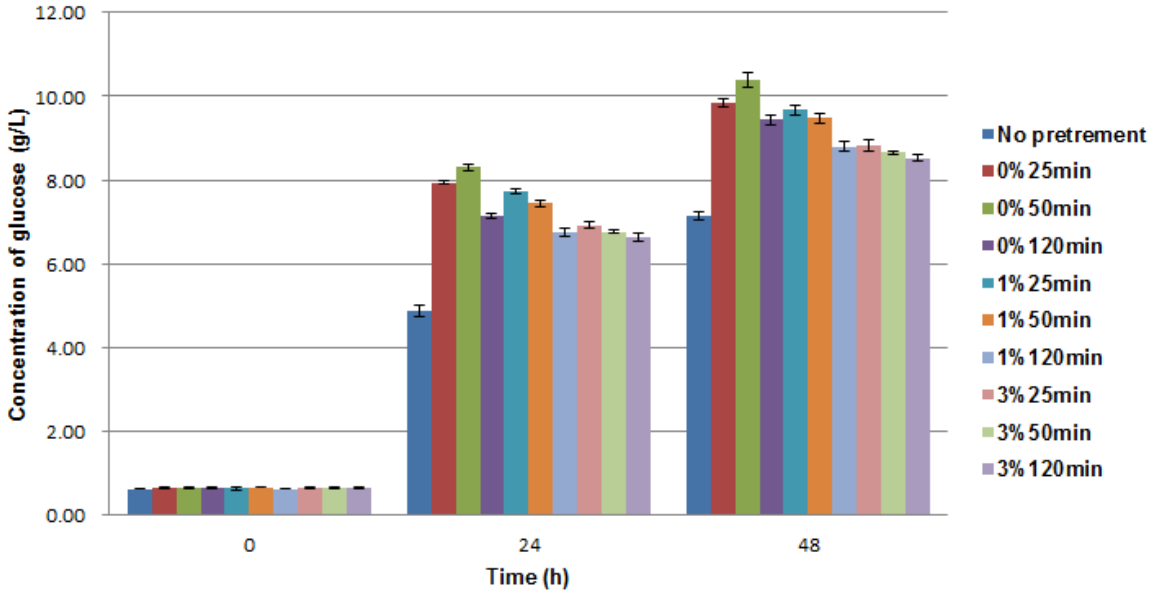

Fig. 1. The increment of glucose concentration with different pretreatment conditions

Effect of Carbon-to-Nitrogen Ratio on PHA

In the flask-scale experiments, the C/N ratio was controlled at 10, 20, and 30, and the cell dry mass (CDM), PHA concentration, PHA/CDM ratio, and optical density (OD) value were measured at different time points. The results indicated that the C/N ratio had a significant effect on the PHA production and accumulation by the bacterial strain. The highest PHA concentration and PHA/CDM ratio were 0.7 g/L and 23.6% when the C/N ratio was 10, while the lowest PHA concentration and PHA/CDM ratio were 0.4 g/L and 21.5% when the C/N ratio was 30. This suggested that a lower C/N ratio favored biomass growth, while a higher C/N ratio favored the accumulation of PHA. These results are consistent with some previous studies that reported similar effects of C/N ratio on PHA production by different bacterial strains (Ahn *et al*. 2015; Cui *et al*. 2017).

5-L Scale PHA Production Process

As in the 250-mL flask scale experiments, the C/N ratio was set at 10 for the 5-L scale production experiment described here. In this experiment, hydrolysis liquid from the waste paper containers was used as the carbon source. The highest PHA concentration and PHA/CDM ratio were 0.9 g/L and 28.7% after 96 hours of cultivation (Fig. 3). The potential bacterial strain in this study was confirmed to be a *Bacillus* genus bacterium after strain identification.

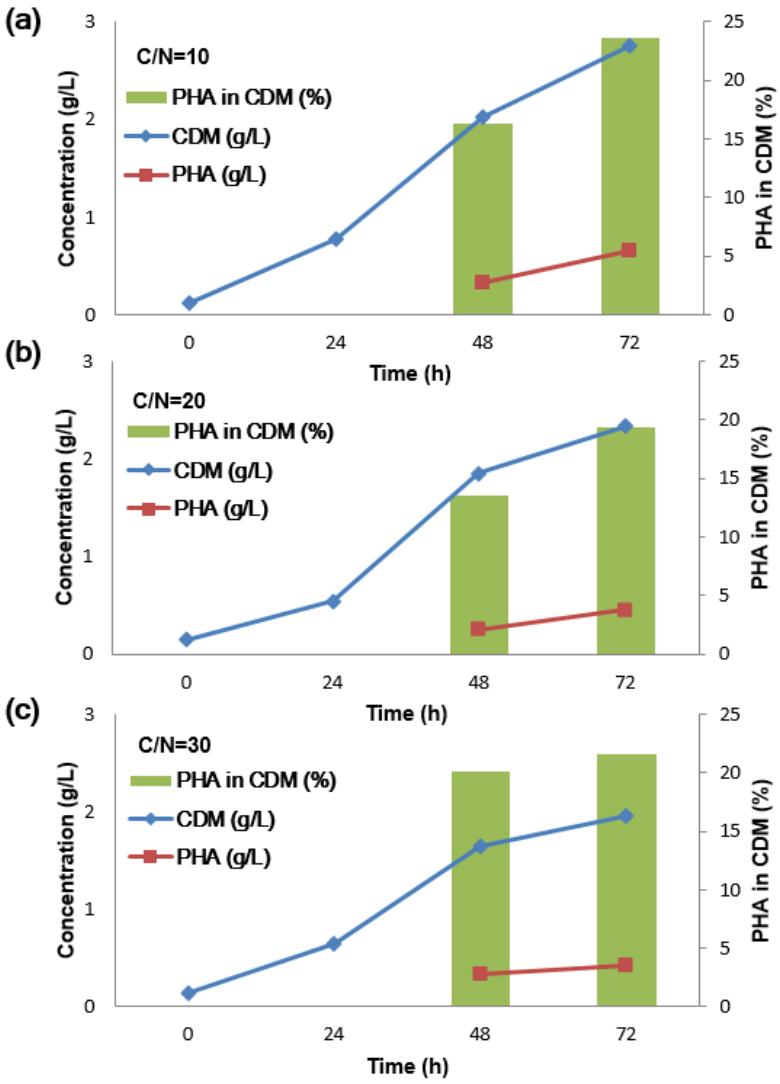

Fig. 2. Variations of PHA on CDM, CDM, and PHA concentration at three different C/N ratios of (a) 10, (b) 20, and (c) 30

The main limitations of this study were the low PHA production concentration and yield compared to other studies. For example, Jiang *et al*. (2012) reported PHA productivity could reach 2 g/L per day when using paper mill wastewater as a substrate. Mohanrasu *et al*. (2020) also reported a 5.61 g/L production concentration by *Bacillus megaterium* in a batch culture within 64 h. Yasin and Al-Mayaly (2021) reported that *Bacillus tequilensis* ARY86 produced PHA with a PHA/CDM ratio of 64% and a PHA concentration of 1.66 g/L using 1% lactose as a carbon source in the medium. Thu *et al*. (2021) reported that *Pichia* sp. TSLS24 produced PHA with a PHA/CDM ratio of 43.4% and a PHA concentration of 1.8 g/L using glucose as carbon source. The low PHA production in this study could be attributed to several factors, such as the type and concentration of waste paper fibers, the hydrolysis method, the inoculum size and age, the culture conditions (pH, temperature, aeration), and the extraction method. Therefore, future research should optimize these parameters to enhance the PHA production from waste paper containers by *Bacillus*.

Previous studies have reported the production of PHA by *Bacillus* using various

waste feedstocks, such as food waste (Vu *et al*. 2021), paper mill wastewater (Mohanrasu *et al*. 2020), and lignocellulosic biomass (Munir *et al*. 2015). However, to our knowledge, this is the first study to use waste paper containers as a carbon source for PHA production by *Bacillus*. Waste food packaging paper items are abundant and cheap, and they can be hydrolyzed to glucose by enzymatic methods. Compared to other carbon sources, glucose can be utilized more efficiently in *Bacillus* (Vu *et al*. 2021).

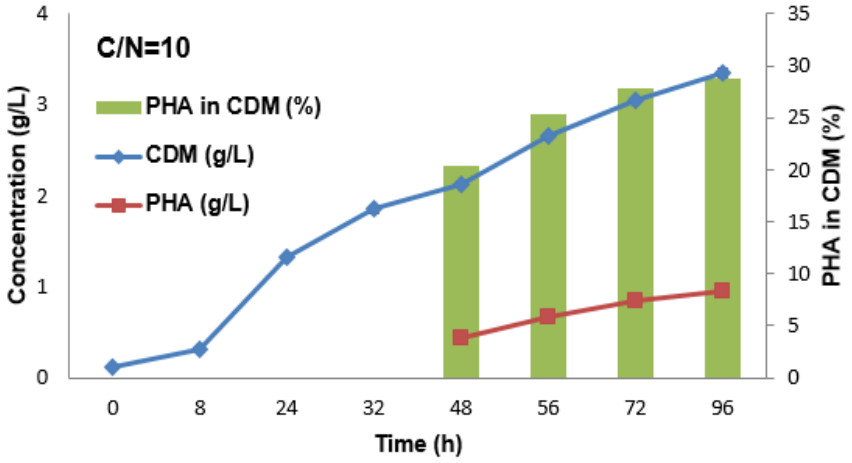

Fig. 3. Variations of PHA on CDM, CDM, and PHA concentrations when using hydrolysis liquid from waste paper container as the carbon source

Structure of PHA After Production Process

The ^{1}H NMR spectra of the copolymers are presented in Fig. 4. The peak signal detected at around 1.2 to 1.4 ppm was assigned to the methyl (CH_3) group in the polymer, which was consistent with the findings reported in previous studies (Lopez-Cuellar *et al*. 2011; Arumugam *et al*. 2018). The peak signal observed at 2.4 to 2.6 ppm was assigned to the H-atom resonance of the methylene group (CH_2) in the polymer. The third peak signal, recorded at 5.2 to 5.4 ppm, was attributed to the resonance absorption of methine (CH) groups in the polymer. These signal peaks indicate that the PHA obtained from the *Bacillus* sp. production process was PHB. These results indicate that the copolymers had a high degree of purity and crystallinity.

The PHA produced by *Bacillus* in this study was mainly composed of poly(3-hydroxybutyrate) (PHB). The synthesis of PHB from *Bacillus* sp. is a promising approach for biodegradable polymer production, as it offers several advantages over conventional methods, such as low cost, environmental friendliness, and high yield. PHB has potential applications in various fields, such as biomedical engineering, packaging, agriculture, and bioremediation (Chavan *et al*. 2021; Moreira *et al*. 2022; Fernandez-Bunster and Pavez 2022). However, PHB has some drawbacks, such as brittleness and low thermal stability, that limit its industrial use. The production of a single copolymer followed by the use of different PHAs at varying ratios enables the modification of the chemical and physical properties of the PHA by incorporating different types of PHA units (Taguchi and Matsumoto 2020; Utsunomia *et al*. 2020). Previous studies have also shown that the molecular composition of PHAs depends on the carbon sources used (Hanik *et al*. 2019; Chen *et al*. 2018). By using the above methods, the limitations of PHB might be overcome, and its toughness and heat resistance may be enhanced. This study demonstrated that *Bacillus* sp. can produce PHB with high purity and crystallinity using waste paper

containers as a carbon source. The ^{1}H NMR spectra of the copolymers confirmed the structure and composition of PHB. The results provide valuable insights for further research on PHB synthesis from waste paper containers and applications.

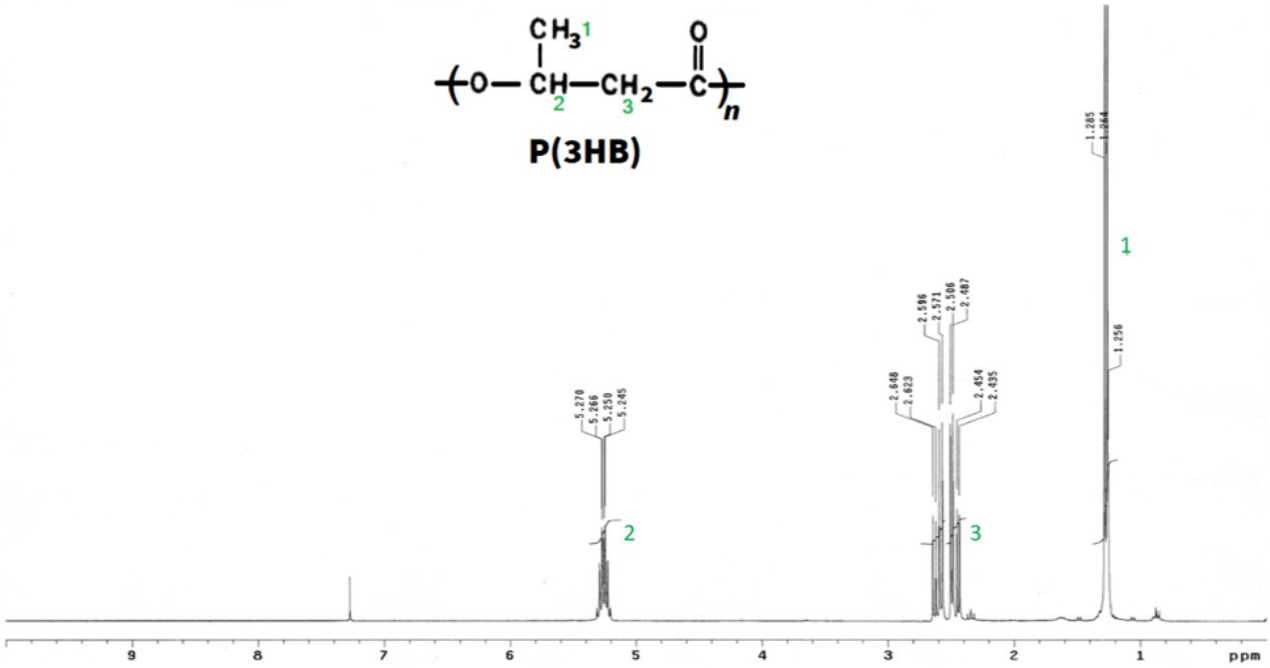

Fig. 4. ^{1}H NMR spectra of PHA derived from the production process by *Bacillus* sp. using hydrolysis liquid from the waste paper container as the carbon source. The block copolymer was P(3HB).

CONCLUSIONS

1. This experiment showed that heating waste paper containers without dilute sulfuric acid for 50 min was the most effective pretreatment for enzymatic hydrolysis, resulting in the highest glucose concentration. Dilute sulfuric acid pretreatment had a negative effect on enzymatic hydrolysis and reduced the glucose yield, especially with longer heating times.
2. The ratio of carbon to nitrogen (C/N) plays a critical role in determining both the optical density (OD) value and the concentrations of polyhydroxyalkanoate (PHA) and cell dry mass (CDM).
3. This study demonstrated the feasibility of using waste paper containers as a carbon source for PHA production by *Bacillus*. The waste paper container is a potential feedstock for PHA production, as they are renewable, abundant, and cheap. However, further optimization and scale-up are required to improve the PHA production efficiency and quality.

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