

Isolation of Poly- β -hydroxybutyrate (PHB) Producing Bacteria from Sago Dregs and Sugarcane Solid Waste

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Abstract. Soil is the habitat of thousands of microscopic organisms, especially bacteria. Soil containing sago waste is rich in carbon sources and potentially as a raw material for the manufacture of Poly- β -hydroxybutyrate (PHB). Biodegradable plastic is one of the eco-friendly plastic materials, this type of plastic is made with Poly- β -hydroxybutyrate (PHB) as the raw material. Sago waste is usually not utilized to the maximum. The purpose of this study was to obtain isolates of poly- β -hydroxybutyrate (PHB) producing bacteria from sago pulp waste without the addition of bacterial agents from the outside environment. This research was conducted in the Laboratory of Microbiology FMIPA UNM. Soil samples with sago dregs were obtained from a sago processing plant in Takalar, South Sulawesi. Bacteria are isolated by dilution serialized three times then grown on medium Nutrient Agar and purified on medium Strach Agar then fermented with Ramsay medium. The results showed the presence of biodegradable plastic formed in the breeding medium.

Keywords: Sago Waste, Cane Waste, Insulation, Poly- β hydroxybutyrate Bacteria

INDONESIAN JOURNAL OF FUNDAMENTAL SCIENCES

E-ISSN: 2621-6728

P-ISSN: 2621-671X

Submitted: January, 8th 2021

Revised: February 24th, 2021

Accepted: March, 2nd 2021



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INTRODUCTION

Plastics are among the materials we encounter in everyday life. The use of plastic is already very widespread in communities and large industries. Plastic materials are selected because they are considered strong, lightweight, and waterproof and have low selling value. However, nowadays the use of plastic particularly in Indonesia is a major problem and is understated by governments. After all, plastic is so difficult to decompose by nature that it causes environmental pollution. The perishable nature of plastic litter is the carcinogenic processing that causes toxicity to become a major reason for environmental contamination.

The management of plastic litter is a problem because it is a material that cannot naturally decompose (non-biodegradable). Incinerating plastic waste management can adversely affect the environment in which air pollution occurs especially carcinogenic dioxin emissions. Another form of plastic garbage can be managed by recycling plastic garbage into another form, but this recycling process will only change plastic garbage into a new one instead of containing the volume of plastic garbage so that when a plastic recycling product loses its function, it will return to plastic garbage (wahyudi, etc., 2018).

Poly- α -hydroxybutyrate (PHA) is a polyester microbe produced by various microorganisms including cyanobacteria. PHA includes most of the biodegradable polymers that have been extensively researched in recent years because of their chemical and physical properties which are similar to polypropylene and also some special properties such as non-toxicity, biocompatibility and biodegradability, the most studied types of PHA are PHB (Gopi et al. 2014).

Poly- β -hydroxybutyrate (PHB) is a microbial bio plastic, including the polyester group which has similar properties to conventional plastics. The formation of PHB is a natural way for bacteria to store carbon and energy, when the supply of nutrients is imbalanced. This polyester is formed when bacterial growth is limited by depletion of the amount of nitrogen, phosphorus or oxygen and there is still an excess number of carbon sources. Meanwhile, the most common is nitrogen restriction (Yustinah et al, 2016).

Poly- β -hydroxybutyrate (PHB) is formed from a reserve polymer of carbon and energy produced in bacteria, archaea, and in several other eucaryotes, such as yeast and fungi. The storage molecules are then metabolized under unfavorable conditions when other common energy sources are not available (Thapa et al, 2018). PHB has the same properties as synthetic plastics or petroleum-based plastics, for example, such as polypropylene (Yanti et al., 2010). Chemically, PHB is a polylactic or polyester. Sudan black stain is used in PHB granule staining because it will bind strongly to PHB granules so that if washed with xylol, Sudan black will be washed from the cell cytoplasm, but not from the PHB granules (Haedar et al, 2014).

The bacteria used to produce PHB can be divided into two groups based on their culture conditions. The first group requires limiting the essential nutrients in the form of N, P, Mg, K, O or S, and excess carbon sources. In the second group, nutrient limitation is not required and polymers can still accumulate during the growth phase (Posada, et.al 2011). PHB can be used as a substitute for synthetic

plastics that are difficult to degrade, so that pollution caused by plastic waste can be overcome (Arfa, 2010).

Biodegradable plastic materials can retain the desired material properties of conventional synthetic plastics, and can be completely degraded without leaving unwanted residues (Lopez et al., 2012). An important component for the commercialization of bioplastic production is the optimization of conditions for maximum PHB synthesis by varying culture parameters such as temperature, pH and types of carbon sources (Singh and Nikita, 2011). Bacterial isolates that have been tested for their ability to degrade lipids are then characterized to facilitate identification (Elyza et al, 2015).

RESEARCH METHODS

This research is a descriptive study conducted to test the presence of poly- β -hydroxybutyrate (PHB) producing bacteria from solid waste of sago and sugarcane dregs. The research was conducted from November to December 2020 at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, The State University of Makassar. Sources of bacterial isolates were taken from soil samples around the disposal of sugarcane waste from the Takalar Sugar Factory and sago waste from a home factory in Gowa, South Sulawesi, Indonesia. Several stages were conducted in the research, namely bacterial isolation, bacterial testing, bacterial purification and fermentation of PHB bacteria.

- a. **Bacteria isolation** : 10 grams of sample were diluted with 95 mL aquadest, homogeneous for 5 minutes, diluted to 10⁻³, 0.1 mL of the third dilution was taken and spread on a petri dish containing NA medium with a spread rod, incubated for 48 hours.
- b. **Testing of PHB Bacteria**: Colonies obtained from bacterial isolation are dripped with 5 drops of Lugol (Gram B), a positive test is marked by the presence of a clear zone.
- c. **Purification of PHB Bacteria**: Bacterial colonies were taken using a swab, scratched by the bacteria with a quadrant method on the surface of the SA medium, incubated for 48 hours.
- d. **Fermentation of PHB Bacteria**: A single colony was taken from the purification results using a swab, inoculated a single colony that had been extracted into the Ramsay medium, shaker it for 3 days, added 75 mL of acetone then homogenized for 15 minutes, poured into a petri dish and evaporated to leave a thin layer is a biodegradable plastic.

RESULTS AND DISCUSSION

The study used 3 mediums; first, Nutrient Agar (NA) medium. NA medium is used to grow PHB bacteria. According to Supriyati (2011), NA acetate carbon source is the best for these bacteria to grow and produce PHB. Second, Starch Agar (SA) medium, to purify PHB bacteria. Winarno, (2002) said that amylolytic microorganisms will break down starch and glycogen. The starch in the SA medium will then be broken down by amylase. The third is Ramsay medium for fermentation of PHB bacteria. Ramsay, et al (1990) explained that Ramsey medium is a medium used to produce PHB by several bacteria. This explains that the nutrient

concentration in the Ramsay medium can be used for the production of PHB by many strains of members of the bacterial species.

Table 1 Bacterial Isolation

Documentation	description
 <p>Sago</p>	<p>Incubation of NA medium for 48 hours</p>
 <p>Sugarcane</p>	<p>Incubation of NA medium for 48 hours</p>

In the early stages of this research, bacterial isolation was carried out using a multilevel dilution method, namely 10⁻¹, 10⁻² and 10⁻³ dilutions. At 10⁻³ dilution the samples were then transferred to NA medium and then incubated for 48 hours. This is in accordance with Wasteson and Hornes (2009) who say that the purpose of multilevel dilution is to reduce or reduce the number of microbes suspended in the liquid. Determination of the amount or number of levels of dilution depends on the estimated number of microbes in the sample. A 1: 9 ratio was used for the sample and the first and subsequent dilutions, so that the subsequent dilutions contained 1/10 of the microorganism cells from the previous dilutions.

Bacterial testing is carried out to take the target bacteria, namely PHB-producing bacteria. This test uses a lugol solution. A positive result is indicated by the presence of a clear zone on the media. Next, purification was carried out for bacteria that were positive as PHB-producing bacteria. Types of bacteria that are known to produce PHB include *Pseudomonas* sp, *Bacillus* sp, *E.coli*, *Rastonia eutropha*, *Azotobacter*, *Micrococcus*, *Alcaligenes*, *Cupriavidus necator* (Aslim et al, 2002).

Table 2. Testing positive hydrolytic bacteria

Documentation	Description
 <p data-bbox="459 548 526 582">Sago</p>	<p data-bbox="774 409 1276 481">Clear zones indicate positive bacteria PHB</p>
 <p data-bbox="422 869 566 902">Sugarcane</p>	<p data-bbox="774 710 1276 781">Clear zones indicate positive bacteria PHB</p>

The purification process was carried out on agar starch medium (for 48 hours) by means of inoculation with the quadrant scratch method in order to obtain a single colony. This purification (inoculation) is carried out by the quadrant scratch method with the aim of producing bacterial colonies that are well separated from the concentrated suspension, the scratch method used is quadrant scratching which is divided into several regions, and the first area is the initial scratch so that it still contains many microorganisms' cells. The strokes are then cut or crossed from the first stroke so that the number is smaller and finally separated into a single colony (Amalia, Muria & Chairul, 2014).

The inoculum aims to adapt microbial cells to fermentation media, with the adaptation it is hoped that the Lag phase as the initial stage of fermentation will be passed. This opinion is also supported by Sarl (2009) and Grothe, Young, Chisti (1999) that the use of the right inoculum can accelerate the fermentation process by reducing lag phase, so that the fermentation time is faster and the resulting alcohol content is getting bigger too.

Microbial fermentation was carried out in Ramsay medium. Ramsay medium is incubated on a shaker for 3 days, this is considered appropriate by referring to the research of Yanti, Margino & Sembiring (2019) and Sari, et al (2008) who both say that the most optimal fermentation time is 36 - 78 hours (about 3-6 days). Meanwhile, according to Kunaepah (2008) the longer the fermentation time, the number of microbes decreases, and will go to the death phase because more alcohol is produced and the nutrients available as microbial food decrease. The function of the shaker in Amalia, Muria & Chairul, (2014) is to facilitate the diffusion of oxygen into the medium and the mixture becomes homogeneous.

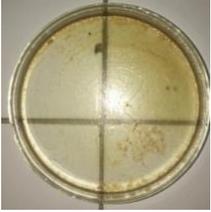
The addition of acetone was carried out into the Ramsay medium as much as 75 mL, then homogenized for 15 minutes. The use of acetone as a solvent is carried out to remove nonpolar compounds from the media. Rusmiati, (2010) states

that the events that occur in the use of acetone are the chemical and physical separation of the simplicia substance using the appropriate solvent.

Next, it is poured into 5 petri dishes (each waste) to be dried and dried for 1 day. Meigaria, Mudianta, & Martiningsih (2017) say that the temperature required to evaporate the sample with acetone solvent is not too high, thus minimizing the possibility of damage to the secondary metabolite content in the sample due to too high heating temperature, and the time required to evaporate the sample is relative short. Seton is a polar organic solvent with a polarity index of 5.1 and a boiling point of 56 ° C, while the polarity index of ethanol is 5.2 and a boiling point of 78 ° C.

Table 3 Microbial fermentation of sago dregs and bagasse

Documentation	Description
Microbial fermentation of sago dregs	
 petri dish 1	a biodegradable plastic is formed
 petri dish 2	a biodegradable plastic is formed
 petri dish 3	a biodegradable plastic is not formed
 petri dish 4	a biodegradable plastic is not formed

	<p>a biodegradable plastic is not formed</p>
<p>Microbial fermentation of bagasse</p>	
 <p>petri dish 1</p>	<p>a biodegradable plastic is formed</p>
 <p>petri dish 2</p>	<p>a biodegradable plastic is formed</p>
 <p>petri dish 3</p>	<p>a biodegradable plastic is not formed</p>
 <p>petri dish 4</p>	<p>a biodegradable plastic is not formed</p>
 <p>petri dish 5</p>	<p>a biodegradable plastic is formed</p>

Based on the practicum that has been carried out, it can be seen that several petri dishes gave positive test results on the hydrolysis test of sago and sugarcane waste starch. This is indicated by the formation of a clear zone on the third day (after evaporation extraction) around the bacterial growth area after being given a few drops of lugol iodine solution, sago petri dishes (1, 2) and sugarcane (1, 2, 5) experiencing this. This provides information that the bacterial isolate can produce the α -amylase enzyme.

The α -amylase enzyme acts to break bonds with the α configuration in starch. The hydrolysis of starch by the α -amylase enzyme is divided into two ways, namely amylose hydrolysis and amylopectin hydrolysis, amylose hydrolysis by α -amylase occurs in two stages. The first stage is the random breakdown of amylose into maltose and maltotriose. This decomposition occurs rapidly followed by a rapid decrease in viscosity. Even though the activities were carried out with reference to reliable sources, the results did not match estimates. We suspect 75% (acetone): 100% (extark) is less than optimal in the process of obtaining bio-degradable plastics produced by PHB microbes (unsuitable solvent dose).

CONCLUSION

Based on the results of research on the isolation of Poli- β -hydroxybutyrate (PHB) producing bacteria, it was obtained that the PHB-producing bacteria originated from sago dregs solid waste isolates and bagasse solid waste, which can be seen in the results of microbial fermentation activities. The results obtained showed the presence of a thin layer of biodegradable plastic in the two samples on the third day after the evaporation extraction process that had been dried to air.

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