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Research Article

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Bioconversion of Sago Starch to Bioplastic Poly-β-Hydroxybutyrate (PHB) by Local Strain Bacterial *Bacillus megaterium* PSA10

Nur Arfa Yanti* and Nurhayani H. Muhiddin

Department of Biology, Faculty of Mathematics and Natural Science, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia

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ABSTRACT

The potential local strain Bacillus megaterium PSA10 in bioconversion sago starch to bioplastic, Poly-β-hydroxybutyrate (PHB) was investigated in this study. The bacterial strain was grown in fermenter using sago starch minimal media, furthermore was observed enzymatic activity, the products and its growth pattern. Amylase enzyme activity measurements performed using 3'5-dinitrosalicylic acid (DNS) and PHB synthase enzyme activity using Valentin and Steinbuchel methods. Reducing sugar and PHB levels were detected using spectrophotometric method and the biomass growth was measured by gravimetric method. The highest specific activity of the enzyme amylase and reducing sugar concentration were obtained at 18 hours of incubation, i.e. 170.72 U/mg protein and 14.08 g/L, respectively while the highest specific activity of PHB synthase enzyme and the PHB concentration were obtained at 36 hours of incubation i.e. 85.72 U/mg protein and 2.86 g/L, respectively. The growth maximum of B.megaterium PSA10 was obtained at 42 hours of incubation and the strain bacteria was able to accumulate PHB as much as 85.04% wt/wt of the cell dry weight at 36 hours of incubation. Therefore, B.megaterium PSA10 converts sago starch into bioplastic PHB via direct bioconversion.

Keywords: Bioconversion, Sago starch, Bioplastic, PHB, *Bacillus megaterium* PSA10

INTRODUCTION

Synthetic plastics are important for society due to their characteristics of versatility, durability and resistance to degradation. However, there are many environmental problems resulting from the use of petrochemical derived polymers making necessary to find alternatives to replace these bio recalcitrant materials. One of solution to this problem is the use of biodegradable polymers, which are polymers with the desirable properties of conventional plastics but also with rapid biodegradation in the environment after discarding [1]. One such biodegradable type of plastic is poly-β-hydroxybutyrate (PHB), which is accumulated intracellularly by bacteria as carbon or energy reserves, mainly when there is a limitation of an essential nutrient, such as nitrogen, phosphorus, oxygen or sulphur [2].

The commercial use of PHB is still limited mainly due to its relatively high production cost. Therefore, the use of low cost substrates is an important factor, along with the need to obtain bacteria strains for efficient substrate to polymer conversion [3,4]. Low cost substrates include starchy substrates, which are important compounds in food industry by-products [5, 6]. Sago starch is one of the potential substrate for PHB production [7].

The utilization of starchy substrate for producing PHB still require treatment hydrolysis of starch into simple sugars, so that can be used to produce PHB. Some studies using enzyme or chemical compounds to hydrolyze starch before it is used as a substrate to produce PHB [5. 8, 9]. However, the use of enzymes or chemical compounds to hydrolyze starch before it is used as the substrate, is not efficient in terms of cost and time. The selections of suitable bacterial strains, inexpensive carbon sources, efficient fermentation and recovery processes are important aspects that should be taken into consideration for the commercialization of PHB [3]. Hence, requires bacteria strains that can hydrolyze

starch substrate at once capable of synthesizing PHB. *Bacillus megaterium* PSA10 is a local bacterial strain capable of hydrolyzing sago starch and is also capable of synthesizing PHB [10]. Therefore, this study aims to investigate the bioconversion process sago starch into bioplastic PHB conducted by *B. megaterium* PSA10, as a means to facilitate the development of a biotechnology that not only provides an environmentally friendly treatment approach with produce degradable bioplastic, but also has the potential benefits of lowering PHB production costs and off-setting biological treatment costs through the recovery of PHB.

EXPERIMENTAL SECTION

Bacterial strain : The local bacterial strain of *Bacillus megaterium* PSA10 used for this study was obtained from sago starch processing area in Konawe regency, Southeast Sulawesi, Indonesia [10]. This strain was maintained in starch nutrient agar slants and stored at 4^{o}C.

Sago starch minimal medium : The minimal medium (per liter) consisted of sago starch, 20 g; $Na_2HPO_4.7H_2O$ 6,7 g; KH_2PO_4, 1,5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0,2 g; Ferrous Amonium Citrat, 0,06 g; $CaCl_2.2H_2O$, 0,01 g and trace elements solution 1 ml.

Preparation of inoculum*:* The inoculum was prepared in 100 ml erlenmeyer conical flasks containing 50 ml of the sago starch minimal medium. The flasks were cotton plugged and autoclaved at 121^{o}C for 15 min. The sterilized medium was allowed to cool to ambient temperature and large amount of the bacterial (*B.megaterium* PSA10) was scraped from the cultured slant and added aseptically to the medium in the seed medium of the flasks. The flasks were transferred to the gyratory incubator shaker and growth was achieved at a temperature of 37^{o}C at agitation rate of 150 rpm for 48 hours for proper adaptation of the microorganisms to the medium.

Fermentation*:* The fermentation was carried out aerobically in a bench scale bioreactor (Applikon ADI 1030, *Bio controller*)**.** The fermenter vessel containing 1 liter of the minimal media was sterilized in the autoclave at 121^{o}C for 15 min. The media in the fermenter vessel was inoculated with inoculum with a volume of 50 ml and was properly agitated and aerated for about 30 min before making the initial withdrawal. The agitation speed of 150 rpm. The pH of the system was maintained at 7 and controlled by addition of 0.5 M HCl or 0.5 M NaOH solution as desired. The temperature was controlled and maintained at 37^{o}C. Sample was withdrawn at 6 h interval. Each of the samples collected were centrifuged and the supernatant was analyzed for reducing sugar and was used as amylase crude enzyme. Cell pellet was disrupted to obtain PHB synthase crude enzyme and cell also was used for analyze PHB concentration.

Sample Analysis.

Reducing sugar concentration : The reducing sugar concentration of the fermentation broth was estimated by analyzing the glucose in the supernatant using the DNS method of Miller [11].

Amylase enzymatic assay : amylase enzyme activities was determined using 3'5-dinitrosalicylic acid [12]. One unit of amylase activity was defined as the amount of enzyme that produced 1 mmol maltose per minute. Protein was estimated by Bradford method using bovine serum albumin as the standard [13].

PHB synthase enzymatic assay : PHB synthase activity was assayed following the method of Valentin and Steinbüchel [14]. *B.megaterium* PSA10 cells were centrifuged, washed 2–3 times with 25 mM Tris-HCl buffer (pH 7.5) and resuspended in 5 ml of the same buffer containing 5% (v/v) glycerol, called Tris–glycerol buffer. Cells were disrupted by sonication with B-Braun 2000 U manufactured by Labsonic at 40 W for 10 min. Cell debris was removed by centrifugation (4°C) at 16.000 *g* for 20 min. The supernatant (crude extract) was diluted with Tris-glycerol buffer to a concentration of 1 mg ml^{-1} protein and was used for enzyme assay. The assay mixture (1.0 ml) contained 800 ml of enzyme extract, 100 ml of DL-β-hydroxybutyryl-CoA and 100 ml 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction was started by addition of DL-β-hydroxybutyryl-CoA as the substrate. The optical density of the thiobenzoate anions resulting from the reaction of CoA and DTNB was measured at 30°C for 10 min at 412 nm with a Specord S100 spectrophotometer (Perkin Elmer). Protein estimation was carried out by Bradford method [13].

PHB concentration enumeration : The cell pellet was collected by centrifugation at 3000 rpm for 20 min and pellet was digested with 30% (w/v) sodium hypochlorite solution at 30^{o}C for overnight. The residue was collected by centrifugation at 3000 rpm for 20 min and a series of washing steps using water, acetone and finally diethyl ether was performed [10]. The polymer was added with concentrated H_2SO_4 and heated for 10 min at 100^{o}C in a water bath. The resultant crotonic acid was measured spectrophotometrically (Perkin elmer) at 235 nm against H_2SO_4 as blank.

Cell dry weight determination : Biomass content were evaluated by gravimetric method. The bacterial pellet was dried to constant weight (90^{o}C) for 24 hours and weighed. The biomass yield was calculated as the cell dry weight.

RESULTS AND DISCUSSION

Bioconversion of sago starch to Bioplastic PHB by *Bacillus megaterium* PSA10

Process of bioconversion of sago starch into PHB by *B. megaterium* PSA10 is a single-step bioconversion, that is to integrate all process such as hydrolysis sago starch and synthesizes PHB in one step and in one bioreactor (Fig. 1). In the bioconversion process of sago starch into PHB performed by two enzymes, namely amylase enzyme that hydrolyze sago starch (Fig. 2) and PHB synthase enzyme which synthesizes PHB polymer (Fig. 3).

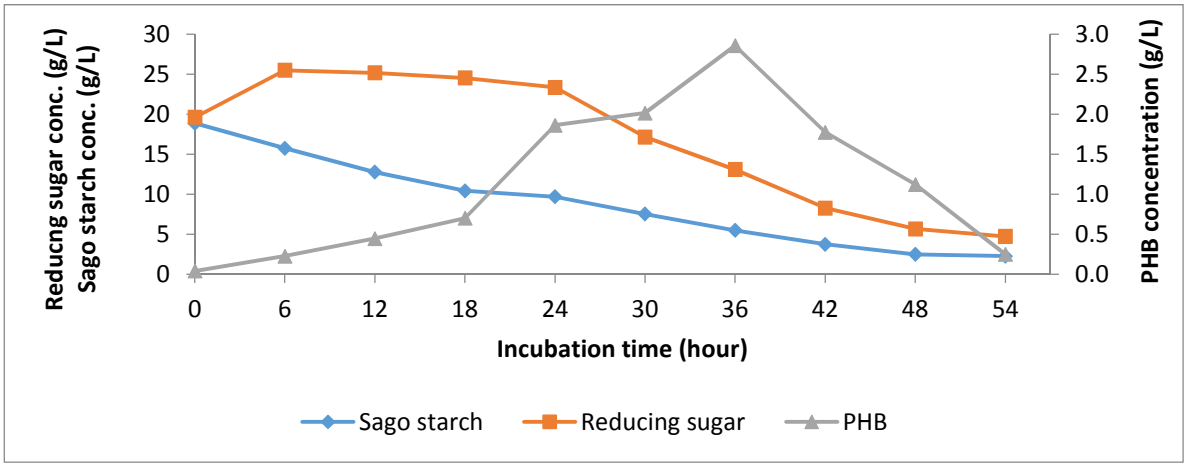

Figure 1. Process of bioconversion of sago starch to PHB by *B.megaterium* PSA10 during cultivation

Sago starch hydrolysis is indicated by a decrease in the concentration of sago starch in cultivation media during incubation time and reducing sugar formed as a hydrolysis product (Fig. 1). Figure 1 shows that the concentration of reducing sugar in the media cultivation has increased from the initial of incubation up to 24 hours of incubation and after that begin to decline. In addition, PHB also began to be produced since the initial of incubation but increased significantly at 18 hours and reached the highest concentration at 36 hours of incubation (Fig. 1).

Bacillus megaterium PSA10 produce the amylase enzymes to hydrolyze sago starch and produce reducing sugar as presented in Figure 2. Process of sago starch hydrolysis by *B.megaterium* PSA10 is shown in Figure 2.

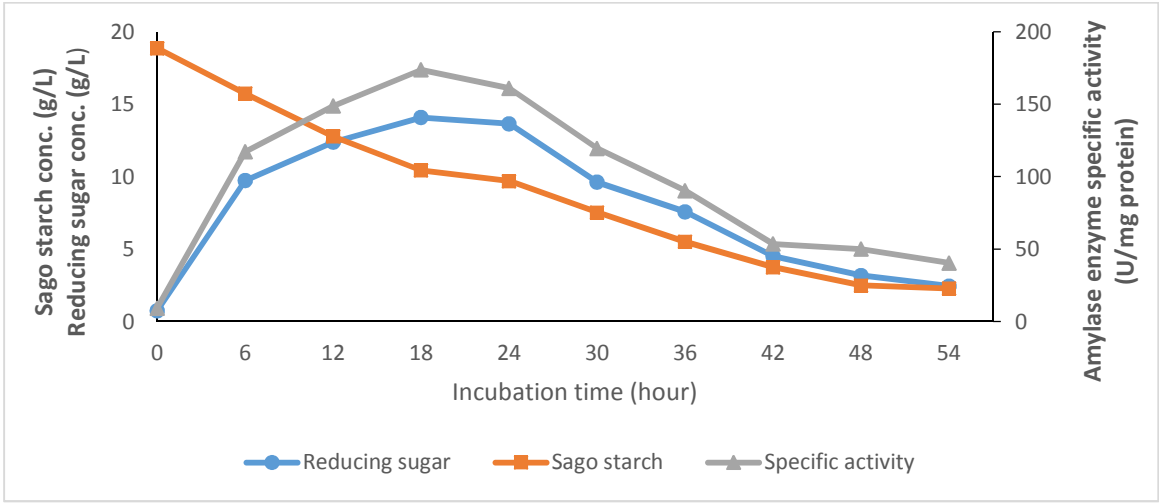

Figure 2. Process of sago starch hydrolysis by *B. megaterium* PSA10 and reducing sugar concentration formed on sago starch minimal media during cultivation

Figure 2 shows that at the time of sago starch content decreases, the amylase enzyme activity was detected anyway. The highest specific activity of amylase enzyme was obtained at 18 h of incubation, i.e. 170.72 U/mg protein, followed by the formation of the highest of reducing sugar concentration, i.e. 14.08 g/L. This indicates that there is a

sago starch hydrolysis process carried out by the enzyme amylase to produce a reducing sugar. Dutta *et al*. [15] suggested that the enzyme amylase is an enzyme that hydrolyze glycosidic bonds in starch and the products with a reducing sugar such as oligosaccharides, maltose and glucose. Reducing sugar produced in the cultivation media, then can be fermented by *B.megaterium* PSA10 into value-added products such as bioplastic PHB.

PHB synthesis process conducted by *B.megaterium* PSA10 on media sago starch occurs with the help of PHB synthase enzyme. PHB synthase enzyme activity of *B.megaterium* PSA10 grown on a substrate of sago starch was determined based on the ability of these enzymes that produce PHB. The results of the analysis of the PHB synthase enzyme using sago starch substrates during 54 hours of cultivation is shown in Figure 3.

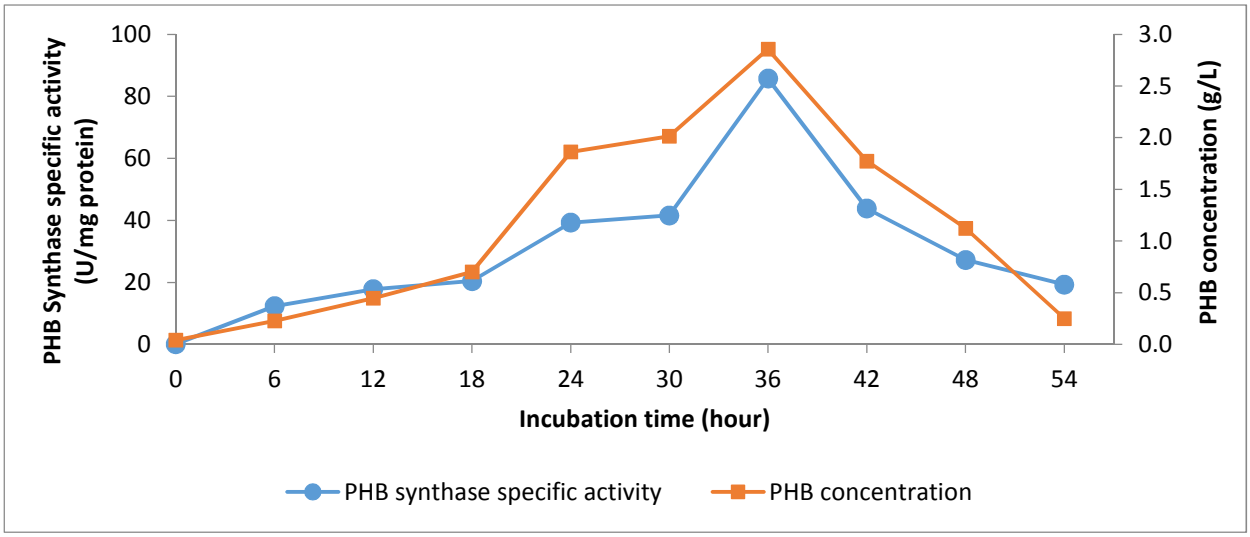

Figure 3. PHB synthase enzyme activity and PHB products were produced during cultivation

Figure 3 shows that the increased activity of PHB synthase enzyme is followed by an increase in the concentration of PHB during cultivation. This indicated that the PHB synthase enzyme responsible for synthesizing PHB. Lenz & Marchessault [2] states that the PHB synthase enzyme acts to catalyze the polymerization of monomers PHB is β-hidroksibutiril-CoA into PHB polymer. The highest PHB synthase enzyme activity produced by *B. megaterium* PSA10 obtained at the 36th hour incubation with a specific activity of 85.72 U/mg protein (Fig. 3). PHB synthase enzyme activity produced by this local amylolytic bacteria, still within the range of PHB synthase enzyme activity of some strains of *Alcaligenes eutrophus* that have been commercialized to produce bioplastic PHB on an industrial scale which ranges from 70-120 U/mg protein [16]. This indicates that *B. megaterium* PSA10 potential to be used as a producer of PHB on an industrial scale.

Mechanisms of sago starch bioconversion into PHB conducted by *B. megaterium* PSA10 through a single-step bioconversion, is fermentation process that is highly efficient in terms of time and productiom cost, because the process is carried out by two enzymes that can work simultaneously. This process not only eliminates the use of enzymes to reduce the production cost but also yield added value by-products via co-culture of bacteria. A single-step bioconversion will reduce contamination and the operation cost resulted from multistage processes of PHB production. This also will reduce energy consumption of the overall process [6,17,18]. The one-step bioconversion can be done by using microorganisms that able to degrade or digest starch into intermediate product such as oligosaccharides and reducing sugar by starch fermenting microorganism and then, the fermentation followed by fermenting the intermediate products into PHB by microbe [6,7].

Production of Poly-β-hydroxybutyrate (PHB) and Growth of *Bacillus megaterium* PSA10

PHB production from sago starch by *B. megaterium* PSA10, was expressed as a percentage of PHB by cells dry weight (cdw), which indicate the ability of the bacterial synthesize and accumulate PHB in the cell. PHB production and growth pattern of *B. megaterium* PSA10 from sago starch is shown in Figure 4.

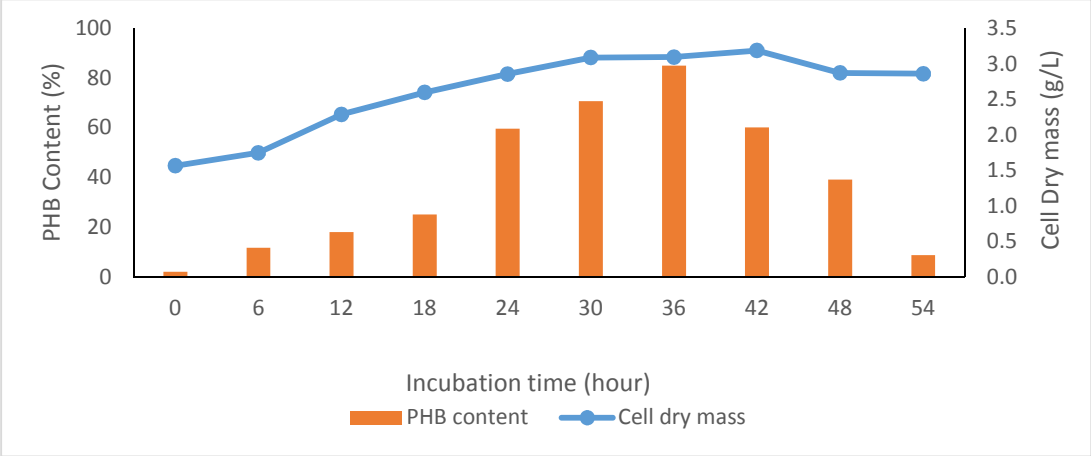

Figure 4. PHB production from sago starch and growth pattern of *B.megaterium* PSA10

Figure 4 shows that the growth of *B. megaterium* PSA10 increased significantly starting at the 6th subsequently entered the stationary phase at the 30th to 42nd hours and began to decline in the 48th hour. PHB production began at the 6th hour or at the exponential phase and the highest production achieved at the 36th hour, i.e. 85,04% (g PHB/g cdw) or at the stationary phase, after that the PHB production begins to decline (Fig. 4). The results of this study indicate that the production of PHB by *B. megaterium* PSA10 is growth-associated. The PHB production which growth-associated, it means PHB was synthesized during the growth (exponential) phase [19,20].

Figure 4 also be shown that the optimum incubation time for producing PHB by *B. megaterium* PSA10 is 36 hours incubation and the incubation time is too long (over 36 hours) would reduce of PHB content. This result indicates that PHB have been used by the bacterial cells as food for growth because of the C source in the cultivation medium decreases. This indication is reinforced by the data presented in Figure 4, which shows that the growth of *B. megaterium* PSA10 was increased to 42 hours of fermentation, but PHB content have declined. The results are consistent with research conducted by Yuksegdag *et al*. [21] and Rodriguez-Contreras *et al*. [22] which states that during the medium conditions have not changed, microbial remains accumulate PHB in the cell but if the concentration C in the medium begins to decrease, the microbes will degrade PHB polymer (depolymerization) to obtain the energy needed for its growth. This causes the levels of PHB in the cell decreased.

The highest PHB value of 85.04% (wt/wt) of the biomass dry weight given by *B.megaterium* PSA10 was comparable to those reported by Gouda *et. al*. [23], Lopez *et al*. [23] and Rodrıguez-Contreras *et al*. [24] of 46,2% (wt/wt), 60% (wt/wt) and 70% (wt/wt) in *Bacillus megaterium* respectively. The slightly higher results could be represented that the local strain bacterial, *B.megaterium* PSA10 has great potential as a producer of PHB used on an industrial scale.

CONCLUSION

Bacillus megaterium PSA10 capable of converting sago starch into bioplastic PHB trough a single-step bioconversion mechanism with produce amylase and PHB synthase enzyme that works silmultaneously. This ability is an advantage because commercial production of value added products such as PHB from sago starch will not only ensure reduction in manufacturing costs, but will also go a long way to solve the problem associated with management of synthetic plastic wastes.

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