

A Study on Production of Poly- β -Hydroxybutyrate Bioplastic from Sago Starch by Indigenous Amylolytic Bacteria

Nur Arfa Yanti^{1*}, Langkah Sembiring², Sebastian Margino³ and Nurhayani. H. Muhiddin¹

¹Department of Biology, Faculty of Mathematics and Natural Science, Universitas Halu Oleo, Kendari

²Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta

³Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta

Abstract

Bacillus sp. PSA10 and *Bacillus* sp. PPK5 were two indigenous strain amylolytic bacteria from Southeast Sulawesi that have ability to produce bioplastic poly- β -hydroxybutyrate (PHB) from sago starch. The study was attempted to determine the mechanism of PHB production by bacteria amylolytic was grown on sago starch-containing media. Two amylolytic bacteria i.e. *Bacillus* sp. PSA10 and *Bacillus* sp. PPK5 was grown for 168 h in a mineral salts medium with sago starch as carbon source. Growth of amylolytic bacteria was monitored by cell dry weight. Extraction of PHB was done by N-hexane acetone-diethyl ether method and PHB content was quantified with UV spectrophotometer at 235 nm. Glucose level was determined by using kit of glucose GOD 10" and was quantified with spectrophotometer at 500 nm. Sago starch concentration was determined by phenol method using spectrophotometer at 490 nm. The result of the study showed that *Bacillus* sp. PSA10 was produced PHB up to 66,81 % (g PHB/g cell dry weight) at 48 h and *Bacillus* sp. PPK5 up to 24,83 % (g PHB/g cell dry weight) at 84 h. *Bacillus* sp. PSA10 has ability to converse sago starch to be PHB directly without glucose accumulation in the media, whereas *Bacillus* sp. PPK5 have to accumulate glucose as product of sago starch hydrolysis to produce of PHB. PHB synthesis by *Bacillus* sp. PHB production on sago starch of the *Bacillus* sp. PSA10 was found to be growth-associated whereas *Bacillus* sp. PPK5 was found to be non growth-associated. Therefore, two indigenous amylolytic bacteria were having of difference in biosynthesis mechanism of PHB in sago starch medium and their characteristics of PHB synthesis should be considered in developing cultivation methods for the efficient production of PHB.

Keywords : Production, PHB, Amylolytic bacteria, Sago starch.

Introduction

Poly- β -hydroxybutyrate (PHB) is a synthesized by a wide variety of bacteria as intracellular and energi storage materials. These biopolymer is a biodegradable and biocompatible thermoplastic, so that it is an effective substitute for petrochemical plastics in applications in which both thermoplasticity and biodegradability are required (Anderson and Dawes, 1990). However, the higher

production cost of PHB compared with that of petrochemical plastics has hampered widespread use of PHB in everyday live. In PHB production, about 40% of total production cost is for raw material (Kim, 2000). Thus, the use of cheaper carbon source is required in order to reduce the high production cost of PHB.

Sago starch is one of cheaper candidates for cultivation substrate of PHB production. The utilization of sago starch as cultivation substrate for PHB production hopefully can reduce the production cost of PHB while giving an added value to sago starch. Prior to fermentation, sago starch is hydrolyzed to glucose by two step, liquefaction and

*Corresponding author:

Nur Arfa Yanti

Microbiological Laboratory, Department of Biology,
Universitas Halu Oleo, Kendari.

E-mail: arfayanti73@yahoo.com

saccharification. The utilization of sago starch in PHB production, needs enzymatic hydrolysis for sago starch degradation since many microorganism do not produce these enzymes natively (Syamsu *et al.*, 2006). This suggests there is a need for exploitation of bacterial culture for the co-production of sago starch hydrolyzing enzyme (amylolytic bacteria) as well as PHB.

Bacillus sp. PSA10 and *Bacillus* sp. PPK5 were amylolytic bacteria isolated from Southeast Sulawesi that can produce PHB in a sago starch medium (Yanti *et al.*, 2009a; Yanti *et al.*, 2009b). *Bacillus* sp. PPK5 appeared to have a higher amylolytic activity than *Bacillus* sp. PSA10, i.e. 99,30 DUN/ml and 42,74 DUN/ml, respectively (Yanti *et al.*, 2009b; Yanti *et al.*, 2009c). The utilization of the amylolytic bacteria for synthesis of PHB from sago starch, hopefully is the effort to overcome the problem of utilization of sago starch as substrate PHB production. Hence, study of mechanism of PHB synthesis have been done to be considered in developing cultivation methods for the efficient production of PHB.

The present study attempted to determine the mechanism of PHB production in sago starch medium by indigenous amylolytic bacteria. PHB might have value as environmentally-degradable thermoplastics for a wide range of agricultural, marine, and medical applications.

Materials and Methods

Bacteria and culture condition

Bacillus sp. PSA10 and *Bacillus* sp. PPK5 were obtained from sago starch processing area in Konawe regency, Southeast Sulawesi (Yanti *et al.*, 2009a). These strains have been used for the current study. Both strains were maintained in starch nutrient agar slants.

The shake-flask experiments were performed in 500 ml conical flasks using 200 ml of chemically-defined mineral salts medium at an initial pH of 7 (Yanti, *et al.*, 2009b). The modified medium contained (g/l) sago starch, 20; Na₂HPO₄·7H₂O 6,7; KH₂PO₄,

1,5; (NH₄)₂SO₄, 1; MgSO₄·7H₂O, 0,2; Ferrous Amonium Citrat, 0,06; CaCl₂·2H₂O, 0,01 and Trace element solution 1 ml/L (Ramsay *et al.*, 1990). The inoculum was prepared in test tube containing 5 ml of sterile nutrient broth and then they were incubated at 30°C for 24 h on rotary shaker at 150 rpm. The seed cultures, 10 ml were transferred into 190 ml of sterile modified mineral salts medium. Cultivation was carried out for 168 h at 30°C with an agitation rate of 150 rev min⁻¹.

Ten ml of samples were taken aseptically from each flask for the analysis of dry cell weight, PHB content, glucose and sago starch level during the growth. The fermentation broth was centrifuged at 3000 rpm for 20 minutes. The bacterial pellet was collected for analysis of PHB content and dry cell weight and the supernatant kept for analysis glucose and sago starch concentration.

Determination of Cell dry weight

Biomass content were evaluated by gravimetry. The bacterial pellet was dried to constant weight (90°C) for 24 hours and weighed. The biomass yield was calculated as the cell dry weight.

PHB Extraction

The pellet was collected by centrifugation at 3000 rpm for 20 min and pellet was digested with 30% sodium hypochlorite solution at 30°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 (Yanti *et al.*, 2009b). The polymer was added with concentrated H₂SO₄ and heated for 10 min at 100°C in a water bath. The resultant crotonic acid was measured spectrophotometrically (Shimadzu UV-6A) at 235 nm against H₂SO₄ as blank.

Analytical Procedures

A series of samples of the growing culture was taken to determine glucose and sago starch levels in the supernatan fluid after centrifugation. The glucose level was

determined by spectrophotometer at λ 500 nm using a kit of glucose GOD 10". Sago starch concentration was determined by the phenol method (Dubois *et al.*, 1956) using Shimadzu UV-Vis 6A spectrophotometer at λ 500 nm.

Results and Discussion

The use of biological system for the production of PHB as biodegradable plastic is becoming important as a solution to the problems concerning non-biodegradable plastic waste and the environment. PHB offers a range of thermoplastics varying in toughness and flexibility (Madison and Huisman, 1999).

From previous work, it is known that organisms such as *Bacillus* strains were able to synthesize PHB as storage material using starch as carbon source (Halami, 2008; Aarthy and Ramana, 2011). For this investigation, *Bacillus* sp. PSA10 and *Bacillus* sp. PPK5 strains were chosen as promising candidates from a number bacteria known to accumulate PHB in sago starch medium (Yanti *et al.*, 2009a), to determine their mechanism of PHB

production in sago starch medium. *Bacillus* sp. PSA 10 and *Bacillus* sp. PPK5 were Gram positive bacteria, rod shaped and spores former (Figure 1).

Production of PHB by Bacillus sp. PSA10 in sago starch media

In this study, PHB production of the *Bacillus* sp. PSA10 from sago starch was shown in the Figure 2. Change in sago starch content of the medium, glucose level in medium, the growth of *Bacillus* PSA10 and PHB accumulation were measured at selected time intervals (Figure 2a to 2d).

Based on Figure 2a showed that sago starch was reduced by *Bacillus* sp. PSA10. Reduction sago starch content in medium showed that sago starch was hydrolyzed by *Bacillus* sp. PSA10 for its growth and PHB production. Figure 2b showed that the glucose was produced by hydrolyzing enzyme of *Bacillus* PSA10, however glucose level in medium was detected less than 0,1 g/L. That result indicated that glucose in medium was consumed immediately by *Bacillus* sp. PSA10 for its growth and PHB production. This

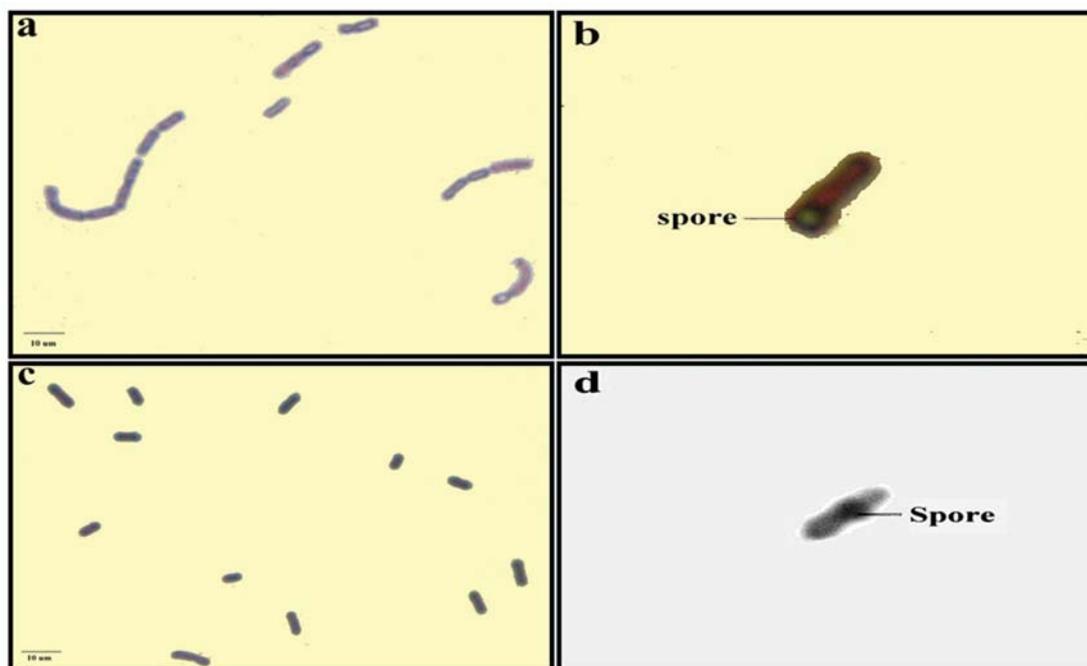


Figure 1. Characteristic features of *Bacillus* sp. PSA10 and *Bacillus* sp. PPK5. **a-b**. Cell shaped and spore formed of *Bacillus* sp. PSA10. **c-d**. Cell shaped and spore formed of *Bacillus* sp. PPK5.

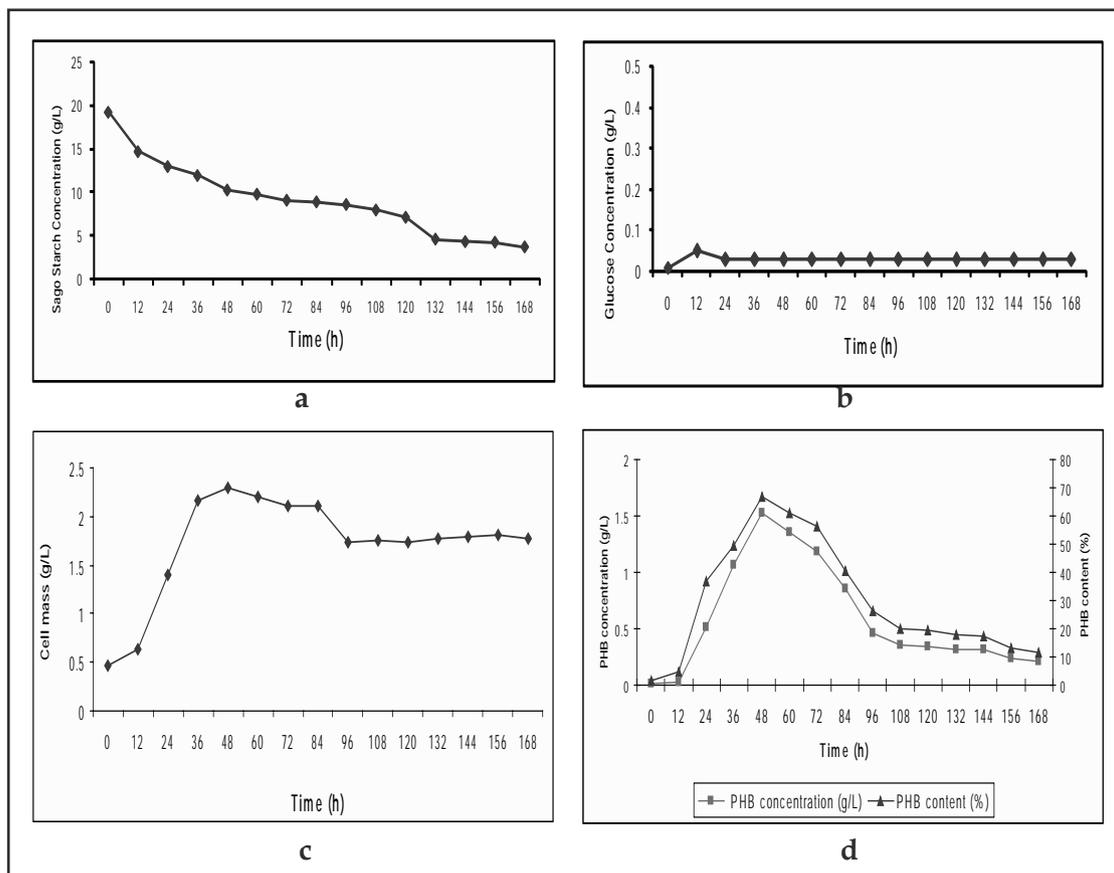


Figure 2. Production of PHB by *Bacillus* sp. PSA10 was grown in sago starch medium under optimal condition. **a.** Substrate (sago starch) reduction, **b.** Glucose level in medium, **c.** Time course of growth, and **d.** PHB accumulation

process term simultaneous saccharification and fermentation (SSF). Taherzadeh and Karimi (2007) was reported that in the process SSF, the glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganism present in the culture. SSF process has many advantages to use as fermentation strategy, they are the risk of contamination is low and the number of vessels required is reduced so that resulting in lower capital cost of the process (Taherzadeh and Karimi, 2007). Hence, *Bacillus* sp. PSA10 most effective utilized for PHB production from sago starch.

Growth of *Bacillus* sp. PSA10 increased steadily, was reached a maximum at 48 h and then declined slowly following a stationary phase (Figure 2c). PHB accumulation began from the early exponential phase at 12 h and

continued till the end of this phase (48 h). (Figure 3d). As expected, PHB production was accompanied by simultaneous decrease in sago starch concentration. PHB accumulation of *Bacillus* sp. PSA10 reached a maximum (1,53 g/l with PHB content 66,81 %) at 48 h of growth could be explained by the maximum cell growth at that stage. PHB is accumulated during the growth phase (exponential phase). This result indicated that PHB production by *Bacillus* sp. PSA10 appeared to growth-associated.

The decrease in PHB accumulation observed after the cessation of cell growth at 48 h incubation and after that the concentration cell remained constant (Figure 3c and Figure 3d). This result indicated that PHB could be consumed as energy and carbon source by *Bacillus* sp. PSA10, causing an unsuitable

condition due to inadequate carbon source in the medium. Yuksekdag *et al.* (2004), reported that PHB accumulation was decreased after the stationary phase since the bacteria used PHB as a carbon and nitrogen source.

Production of PHB by *Bacillus sp.* PPK5 in sago starch media

PHB production of the *Bacillus sp.* PPK5 from sago starch was shown in the Figure 3. Change in sago starch content of the medium, glucose level in medium, the growth of *Bacillus sp.* PPK5 and PHB accumulation were measured at selected time intervals (Figure 3a to 3d).

Figure 3a and 3b showed that *Bacillus sp.* PPK5 was hydrolized sago starch to glucose and glucose was accumulated in

media. The glucose level in media was increased and reached a maximum level (4,12 g/L) at 24 h of growth and then decreased (Figure 3b). This result indicated that the glucose was produced from sago starch by the hydrolizing enzymes of *Bacillus sp.* PPK5 was not consumed immediately by *Bacillus sp.* PPK5 for PHB production.

Growth of *Bacillus sp.* PPK5 increased until 48 h and then declined slowly following a stationary phase (Figure 3c). PHB accumulation began from the end exponential phase (36 h) and continued until the stationary phase (Figure 3d). PHB production was accompanied by simultaneous decrease in sago starch concentration (Figure 3a) and after glucose accumulation at 24 h of growth (Figure 3b). The strain of *Bacillus sp.* PPK5 reached stationary

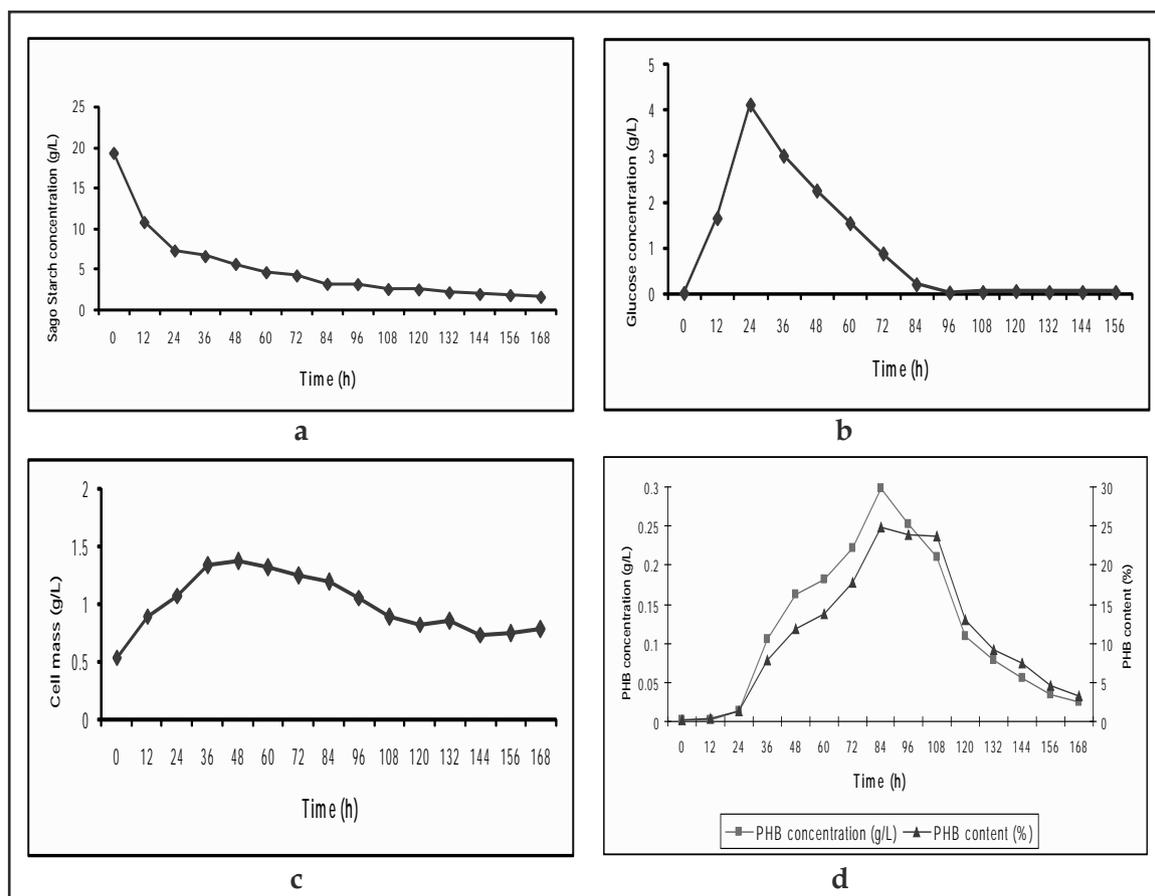


Figure 3. Production of PHB by *Bacillus sp.* PPK5 was grown in sago starch medium under optimal condition. a. Substrate (sago starch) reduction, b. Glucose level in medium, c. Time course of growth, and d. PHB accumulation

phase after 48 h of growth (Figure 3c) and PHB accumulation reached a maximum (0,298 g/l with PHB content 24,83 %) at the end stationary phase (84 h of growth) (Figure 3d). Accumulating PHB during stationary phase indicated that PHB production by *Bacillus* sp. PPK5 appeared to non growth-associated. Beside that, PHB production was observed after reduction of glucose. This phenomenon might showed that PHB production by *Bacillus* sp. PPK5 requires an excess glucose as the carbon source.

Lee (1996) was reported that bacteria able to synthesize PHB can be divided into two groups based on the culture conditions required for PHB synthesis. The first group, accumulating the polymer during stationary growth phase (non growth-associated), requires limitation of an essential nutrient such as N, P, Mg, K, O or S and an excess of the carbon source for the efficient synthesis of PHB. The most important microorganism for industrial polymer production, *Ralstonia eutropha* (Lee, 1996), *Bacillus subtilis* 25 and *B. megaterium* 12 (Yuksekdag, 2004) belongs to the this group. The second group, accumulating the polymer during the growth (exponential) phase (growth-associated), includes *Alcaligenes latus* (Lee, 1996), *Bacillus mycoides* (Thakur *et al.*, 2001) and *B. sphaericus* NCIM 5149 (Ramadas, *et al.*, 2009). Our strains in this study, *Bacillus* sp. PSA10 belongs to the second group, since it was accumulate of PHB during the exponential phase while *Bacillus* sp. PPK5 belongs to the first group since it was accumulate of PHB during the stationary phase. Therefore, these characteristics should be considered in developing cultivation methods for the efficient production of PHB.

Based on data obtained in the present work, *Bacillus* sp. PSA10 was capable of PHB accumulation up to 66,81 % of dry cell weight (dcw) at 48 h incubation and *Bacillus* sp. PPK5 was capable of PHB accumulation up to 24,83 % dcw at 84 h incubation. The bacteria was selected as candidate for industrial PHB production should be easy to grow, accumulated high levels of PHB, short time

incubation and capable used an inexpensive the carbon source (Lee, 1996).

The type of PHB synthesis by *Bacillus* sp. PSA10 was not required excess of glucose (sago starch hydrolized product) as carbon source but *Bacillus* sp. PPK5 was required excess of glucose for synthesized of PHB, so that incubation time for PHB production of *Bacillus* sp. PSA10 was to be shorter than *Bacillus* sp. PPK5. Therefore, *Bacillus* sp. PSA10 was the most potential as a candidate for PHB production from sago starch on industrial scale since it was produced PHB at higher amount (66,81 %) and at a short incubation time (48 hour).

Acknowledgement

The research was supported a Fundamental research grant by Indonesian Directorate General of Higher Education, No. 097-2/PPK/Unhalu/V/2013, Ministry of Government of Indonesia.

References

- Aarthy, N. and Ramana, K.V., 2011. Identification and Characterization of Polyhydroxybutyrate producing *Bacillus cereus* and *Bacillus mycoides* strains. *Int. J. of Environ. Sci.*, **1** (5), 744-756.
- Anderson, A.J. and Dawes, E.A., 1990. Occurrence, Metabolism, Metabolic Role and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiological Reviews*. **54** (4) : 450-472.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, F.A. and F. Smith. 1956. Colorimetric Method for Determination of Sugars and Related of Substances. *Analytic Chemistry*, **18** (33), 350-358.
- Halami, P.M., 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World Journal Microbiology and Biotechnology*, **24**, 805-812.
- Kim, B.S., 2000. Production of poly(3-hydroxybutyrate) from inexpensive substrates. *Enzyme and Microbial Technology*, **27**, 774-777.

- Lee, S.Y., 1996. Plastic Bacteria? Progress and Prospect for Polyhydroxyalkanoate Production in Bacteria. *Tibtech*, **14**, 431-438.
- Madison, L.L. and Huisman, G.W., 1999. Metabolic Engineering of Poly (3-Hydroxyalkanoates) : From DNA to Plastics. *Microbiology and Molecular Biology Review*, **63** (1), 21-53.
- Ramadas, N.V. Singh, S.K., Soccol, C.R. and Pandey, A., 2009. Polyhydroxybutyrate Production using Agro-industrial Residue as Substrate by *Bacillus sphaericus* NCIM 5149. *Brazilian Archives of Biology and Technology*, **52**, 17-23.
- Ramsay, B.A., Lomaliza, K., Chavarie, C., Dube, B., Bataille, P. and Ramsay, J.A., 1990. Production of Poly-(β -Hydroxybutyric-Co- β -Hydroxyvaleric) Acids. *Applied and Environmental Microbiology*, **56** (7), 2093-2098.
- Syamsu, K., Fauzi, A. M., Hartoto, L., Suryani, A and Nur Atifah., 2006. Utilization of Hydrolyzed Sago Starch as Main Substrate for the Production of Bioplastic Poly Hydroxy Butyrate (PHB) By *Rasltonia eutropha* on Fed Batch Cultivation System. *Proceedings The Fifth Regional UNINET Conference and International Seminar 2006. The 21st Century: The Era of Bioscience Challenge and Hope. 22 -23 June 2006, Medan North Sumatra, Indonesia.*
- Taherzadeh, M.J. and Karimi, K., 2007. Enzyme-based Hydrolysis Processes for Ethanol form Lignocellulosic Materials : A Review. *Bioresouces*, **2** (4), 707-738.
- Thakur, P.S., Borah, B., Baruah, S.D. and Nigam, J.N., 2001. Growth-Associated Production of Poly- β -hydroxybutyrate by *Bacillus mycoides*. *Folia Microbiology*, **46** (6), 488-494.
- Yanti, N.A., Sembiring, L. and Margino, S., 2009a. Amylolytic Bacteria Producing The Raw Material of Bioplastic. *Berkala Penelitian Hayati*, **3C**, 95-99.
- Yanti, N.A., Sembiring, L. and Margino, S., 2009b. Production of Poly- β -hydroxybutyrate (PHB) from sago starch by the native isolate *Bacillus megaterium* PSA10. *Indonesian Journal of Biotechnology*, **14** (1), 1111-1116.
- Yanti, N.A., Sembiring, L. and Margino, S., 2009c. Amylolytic Bacteria Isolated from sago starch Processing Area. *Proceeding in Seminar Nasional Biologi, Ilmu Lingkungan dan Pembelajaran. Universitas Negeri Yogyakarta, Yogyakarta, 4 Juli 2009.*
- Yuksekdag, Z.N., Aslim, B., Beyatli, Y. and Mercan, N., 2004. Effect of Carbon and Nitrogen Sources and Incubation Times on Poly-Beta-hydroxybutyrate (PHB) Synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. *African Journal of Biotechnology*, **3** (1), 63-66.