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Poly-β-Hydroxybutyrate (PHB) Production By Amylolytic *Micrococcus* sp. PG1 Isolated From Soil Polluted Arrowroot Starch Waste

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Abstract

Poly-β-hydroxybutyrate (PHB) production from amylolytic *Micrococcus* sp. PG1. Poly-β-hydroxybutyrate (PHB) is an organic polymer, which synthesized by many bacteria and serves as internal energy. PHB is potential as future bioplastic but its price is very expensive due to glucose usage in PHB industry. The development of PHB production using starch as an alternative carbon source has been conducted to reduce the dependence of glucose in PHB production. In this study, amylolytic bacteria from arrowroot processing site were screened quantitavely based on amylase specific activity and PHB producing ability. The result of the study showed that among of 24 amylolytic isolates, 12 isolates of them were able to accumulate PHB ranged from 0,68-11,65% (g PHB/g cdw). The highest PHB production from substrate arrowroot starch was PG1 and after optimization resulted in increasing of PHB production up to 16,8% (g PHB/g cdw) 40 hours incubation time. Based on morphological, biochemical and physiological characters, the PG1 isolate was identified as *Micrococcus* sp. PG1. Result of the FTIR analysis of produced polymer by *Micrococcus* sp. PG1 was indicated as poly-β- hydroxybutyrate (PHB)

Keywords: Poly-β-hydroxybutyrate (PHB), indigenous amylolytic bacteria, arrowroot starch

Introduction

Poly-β-hydroxybutyrate (PHB) is a polymer, which naturally synthesized by many bacteria and serves as internal energy. PHB is biodegradabel and biocompatible (Chen, 2009) and its physical and chemical characteristics are similar to polypropylene (Flieger *et al.*, 2003; Reddy *et al.*, 2003). PHB is potential as future bioplastic but its price is very expensive due to glucose usage in PHB industry. The development of PHB production using alternative carbon source such as starch, canola industrial wastes, dairy wastes, and bit has been conducted to reduce the dependence of glucose in PHB production (Koller *et al.*, 2010). Among those, starch is potential to develop as alternative carbon source for PHB production because it is cheap, vary and many in supply (Koller *et al.*, 2010; Gonzalez-Garcia *et al.*, 2011).

Research of starch as carbon source for PHB production has been explored during years with various of bacteria. Margino *et al.* (2000) use tapioka starch to produce PHB by *Bacillus* and *Pseudomonas* while Yanti (2011) use sago starch and isolate *Bacillus* PSA 10. The results suggested that there is possibility to use other starches as carbon source for PHB production. One of alternatives is arrowroot (*Maranta arundinaceae*) starch which has carbon (C) content 57,69%, nitrogen (N) content 0,33%, phosphorous (P) content 0,004% and kalium (K) content 0,35 %.

In order to optimize arrowroot starch as carbon source in PHB production, exploitation of amylolytic bacteria which can

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also synthesize PHB is required. Hoping that by optimization measures the production of PHB from arrowroot starch be more effective and efficient. Amylolytic bacteria which can hydrolyzed arrowroot starches are potential to be found from arrowroot processing starch. Therefore, screening of indigenous amylolytic bacteria is done to get the potential isolate.

Materials And Methods

Isolation of bacteria from starch production site

Three different samples being liquid waste, solid waste and raw arrowroot starch were collected from arrowroot starch processing site in Sendang Sari, Kulon Progo, Yogyakarta, Indonesia. Ten grams of each samples were serially diluted in 90 ml distilled water and placed onto arrowroot starch nutrient agar plates (Joetono *et al.*, 1978). Cultures were incubated at room temperature for 24-72 hours. Various colonies of different morphologies were individually picked and sub cultured on nutrient agar plate added by arrowroote starch.

Microorganisms screening for amylolitic activity, amylase activity and PHB activity

Determination of amylolytic activity on starch agar media was based on the presence of clear zone around the bacterial colony upon flooding with I_2KI solution. The hydrolytic ability value is the ratio of hydrolysis zone (clear zone) diameter (cm) formed by a bacterium colony and its colony diameter (cm) (Jamilah *et al.,* 2009).

Amylolytic isolates were grown in Ammonium Sulphate Starch media (Fred and Waksman, 1928) with inoculum concentration 5% (v/v) and were incubated at agitation rate 125 rpm, temperature 30°C for 72 hours (Lee and Fujio, 1997). Crude enzymes were obtained by centrifugation at 4,000 rpm, temperature 4°C for 20 minutes. One ml of the crude enzyme was added to one ml of 2% arrowroot starch in 0.1 M phosphate buffer (pH 6.5). The mixture was then incubated at 37°C for 1 hour. Reaction was stopped by adding 1.5 ml of I N acetic acid followed by 0.5 ml of Lugol's iodine. Absorbance was read at 700 nm (Lee and Fujio, 1997). The alpha-amylase activity was calculated by (Espino and Tambalo, 1997) :

DUN/ml = $[(R_0-R_1)/R_0]$ [faktor pengenceran] [10]

DUN/ml = *Denitrinizing unit per ml*

R_0 = Blank absorbance

R_1 = Isolate absorbance

Determination of crude enzyme protein was measured by Bradford method (Bradford, 1976). The specific activity of amylase was determined by dividing the volumetric activity (DUN/ml) by the protein content (mg/ml).

Production of PHB was quantitatively measured by using Ramsay media (6.7 g of $Na_2HPO_4.7H_20$; 1.5 g of KH_2PO_4; 1.0 g of $(NH_4)2SO_4$; 0.2 g of $MgSO_4.7H_20$; 60 mg of ferrous ammonium citrate; 10 mg of $CaCl_2.2H_20$, and 1 ml of trace-element solution. Each liter of trace-element solution contained 0.3 g of H_3B0_3; 0.2 g of $CoCl_2.6H_20$; 0.1 g of $ZnSO_4.7H_20$; 30 mg of $MnCl_2.4H_20$; 30 mg of $NaMoO_4.2H_2O$; 20 mg of $NiCl_2.6H_20$ and 10 mg of $CuSO_4.5H_20$)(Ramsay *et al.,* 1990) with arrowroot starches as carbon sources 1% (w/v), inoculum concentration 5% (v/v), temperature 30°C, pH 7, agitation rate 125 rpm and incubation time 72 hours. The culture then subjected to centrifugation for 20 minutes at 3,000 rpm (Hahn *et al.*, 1995). Extraction of PHB from bacteria cells was done by N-hexane acetone-diethyleter method (Senior *et al.,* 1972). Pellet then were changed to crotonic acid by adding 3 ml of sulfuric acid and boiled it at 100°C for 10 minutes. The PHB content was measured by U.V. spectrophotometer at 235 nm.

Growth optimization

The selected isolate was optimized for PHB fermentation condition in terms of

inoculum concentration (2.5; 5; 10; 15% (v/v)), nutrients concentrations such as arrowroot starch (2.5; 5; 10; 20 and 30 g/l), $(NH_4)_2SO_4$(0; 0.5; 1; 1.5 and 2 g/l), $Na_2HPO_4.7H_20$ (0; 2.3; 4.5; 6.7 and 8.9 g/l0) and KCl (0; 0.5; 1; 1.5; 2 g/l); and environmental condition such as pH (5; 6; 7 and 8), incubation temperature (30; 37 and 40°C) and agitation rate (75; 100; 125 and 150 rpm). All optimization parameters were observed in shake flask culture.

PHB production in fermentor (batch)

The production of PHB by selected isolate was carried out in 2 l fermentor with a 1.5 l working volume. The best condition from optimization steps was applied. The agitation rate and temperature was maintained stable. pH of the medium was checked before inoculation. Aeration for this condition was 1.6 l/minute. Samples were withdrawn aseptically at 4 hourly intervals. PHB concentration, cell dry weight (cdw), glucose level and residual starch was observed.

Genus of selected isolate identification and FTIR analysis of the polymer

The selected isolate was further identified based on morphology, biochemical and physiological characteristics (Joetono *et al.*, 1980). The characters was compared with characters in *Bergey's Manual of Determinative Bacteria 9th* (Holt *et al.*, 1994).

The polymer produced by the selected isolate was extracted by incubated cell mass with H_2O_2 5% (v/v) and chloroform (1:1) for 2 hours. Pellet was harvested by centrifugation 4,000 rpm for 20 minutes. The bottom part of supernatant was pour on the petri dish to let the chloroform evaporate. The extracted polymer was analyzed by Fourier Transform Infrared spectroscopy (FTIR).

Results and Discussion

Isolation and screening of amylolytic bacteria producing PHB

Twenty four amylolitic bacteria were isolated from arrowroot processing site. Twelve among them which had hydrolytic ability ranging from 2.50-6.00 were screening for amylase activity and PHB production (Table 1). The amylase specific activity ranges from 111.85-2121.52 DUN/mg and PHB productivity capacity ranged from 0.68-11.65% (g PHB/g cdw) for those isolates. Based on this data, PG1 was chosen as the most potential isolate. It has amylolytic activity 2121,52 DUN/mg and PHB production 11.65%.

Growth optimization

The best conditions for PHB production by the isolate PG1 was achieved at 5% (v/v)

Table 1. Hydrolytic ability, amylase activity and PHB production of several amylolytic isolates from arrowroot processing site

Isolate code	Hydrolytic ability	Amylase activity (DUN/ml)	Protein content (mg/ml)	Spesific Activity of Amylase (DUN/mg protein)	PHB content (g/l)	Cell dry weight (g/l)	PHB (%)
ABG 21	3.08	86.59	0.272	318.18	0.0134	0.58	2.31[a]
ABG 22	3.20	86.64	0.319	271.55	0.0050	0.69	0.72[a]
AG 3	2.54	24.56	0.169	145.28	0.0085	0.80	1.06[a]
AG 4	5.95	30.26	0.163	186.03	0.0078	0.38	2.08[abc]
AG 7	3.66	27.07	0.143	189.35	0.0147	0.68	2.16[ab]
AG 8	3.16	46.34	0.154	300.80	0.0118	0.52	2.29[abc]
LC 32	3.k11	86.90	0.297	292.86	0.0090	1.33	0.68[a]
PG 8	3.5	16.23	0.145	111.85	0.0398	0.76	5.23[de]
PG 1	3.71	95.74	0.045	2,121.52	0.0816	0.70	11.65[g]
PG 4	4.48	89.77	0.200	448.80	0.0130	0.67	1.96[ab]
PG 5	3.54	89.94	0.081	1106.31	0.0120	0.57	2.09[ab]
PG 6	2.57	24.05	0.144	167.53	0.1025	1.29	7.98[f]

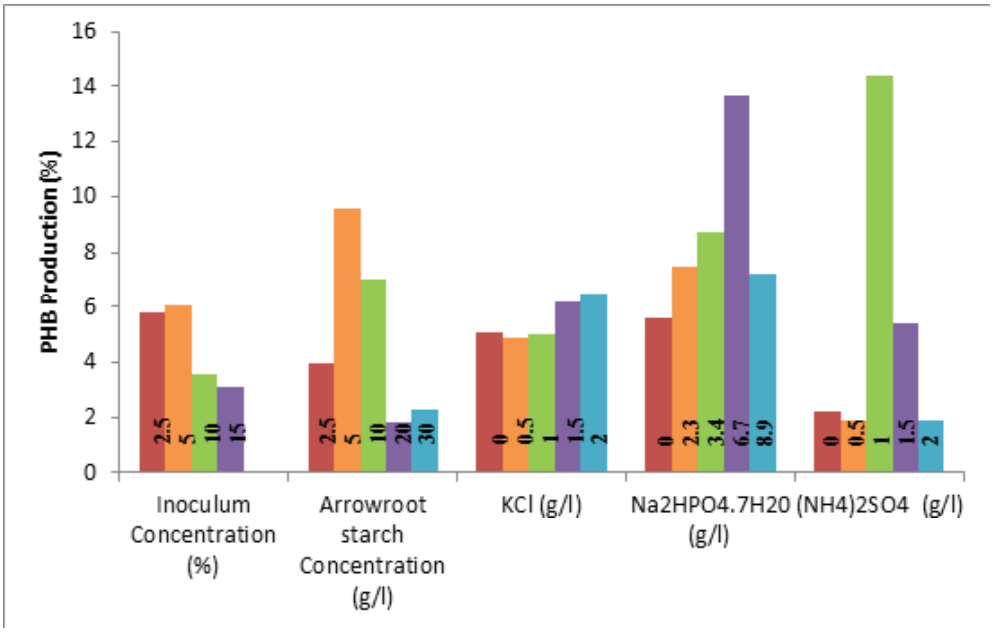


Figure 1. Effect of inoculum and nutrient concentration on PHB production

inoculum concentration, arrowroot starch at 5 g/l, $(NH_4)_2SO_4$ 1 g/l, $Na_2HPO_4.7H_20$ 6,7 g/l and KCl 2 g/l (Figure 1). Whereas the best environmental condition in term of initial pH at 7, temperature at 30°C and agitation speed at 125 rpm (Figure 2). After optimization, PHB production of PG1 was increased from 11,65 to 16,8 %.

PG1 isolate had the highest rate of PHB production by 6% at inoculum concentration 5 % (Figure 1). Inoculum concentration is important to be studied because inoculum concentrations affect the efficiency of PHB production and the incubation time (Yamane *et al.*, 1996; Cetin *et al.*, 2006). While for the nutrient concentration, all parameters were similar with the standart Ramsay's medium, except the KCl concentration. PG1 achieved 9.6% PHB when used 5 g/l arrowroot starch, and respectively 13.7% and 14.4 % of PHB with 6.7 g/l $Na_2HPO_4.7H_20$ and 1g/l $(NH_4)_2SO_4$. For the K component, PG1 isolate required 2 g/l KCL to get the best production of PHB. It was suggested that PG1 isolate required more K for PHB production.

The effect of temperature was studied to find out the best temperature to support production PHB by PG1. PG1 required incubation temperature at 37 °C to achieved 12 % of PHB. Shi *et al.* (2001) mentioned that incubation temperature at 37 °C significantly induced the expression of gene which responsible for PHB production. *Bacillus megaterium* PSA 10 also need incubation temperature at 37 °C for the PHB production (Yanti, 2011).

PG1 produced 14.9 % PHB when incubated with agitation rate 125 rpm. Agitation rate below and above 125 rpm caused a decrease in PHB production, suggest that this agitation rate served the best condition for PG1 in using starch as carbon source for growth and PHB production.

In terms of initial pH, our result (Figure 2) showed consintency with Palleroni and Palleroni (1978) who recommended a pH range of 6.0 to 7.5 for microbial growth and PHB production. Wei *et al.* (2011) also showed the best PHB production by *Cupriavidus taiwanensis* at initial pH 7. PG1 achieved the best PHB production by 16.8 % at initial pH 7.

Production of PHB in fermentor

Optimization result showed that PG1 isolate could produce highest PHB 37 mg/L and cell dry weight 850 mg/L, respectivelly,

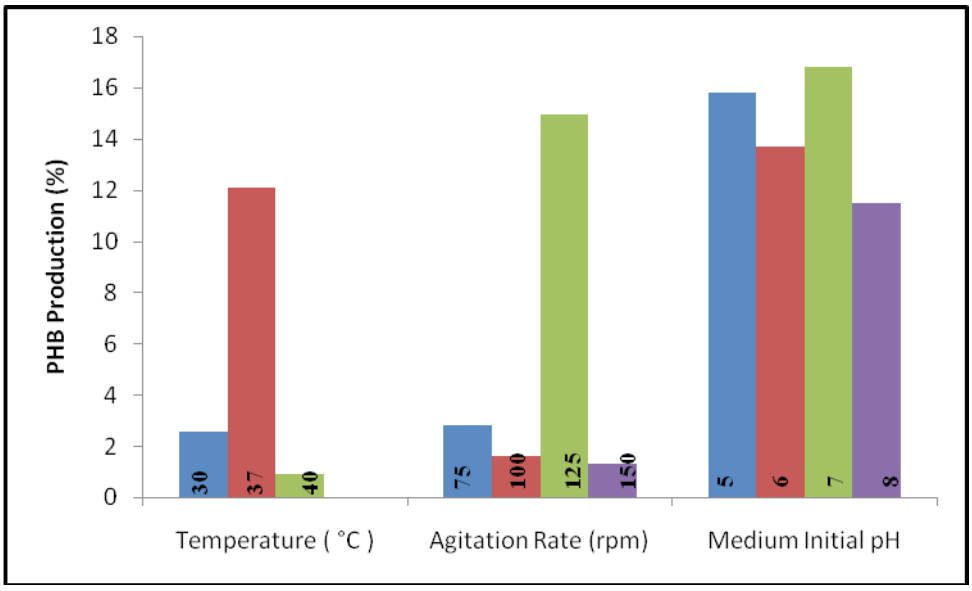


Figure 2. Effect of environmental condition on PHB production

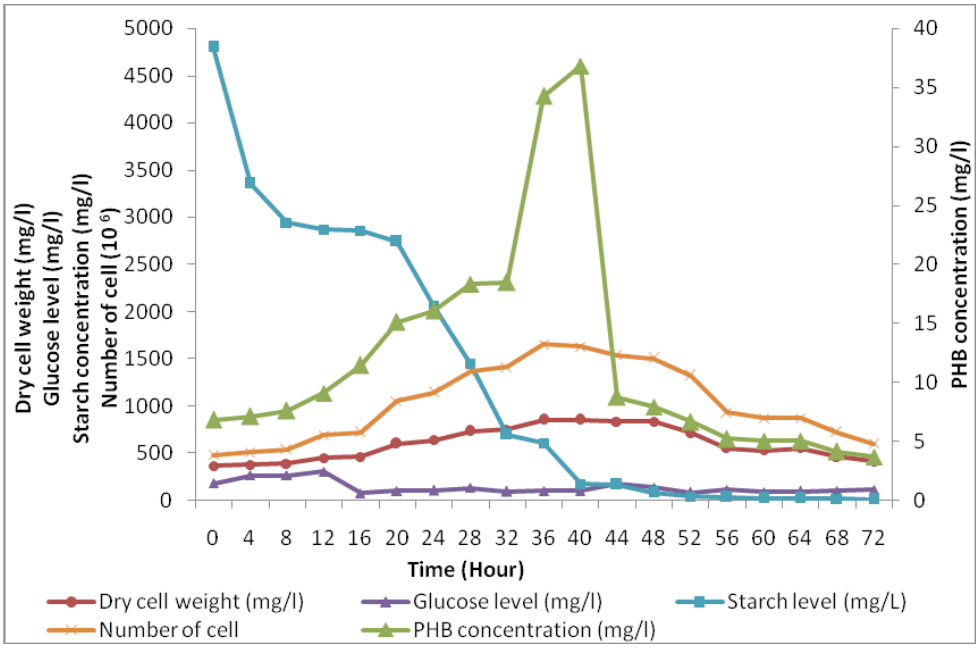
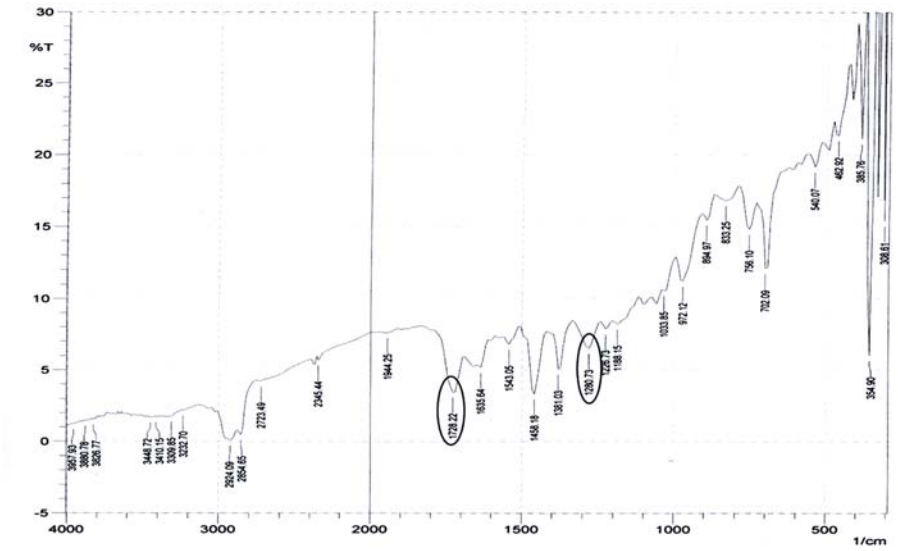


Figure 3. Time course of PHB production by *Micrococcus* sp. PG1

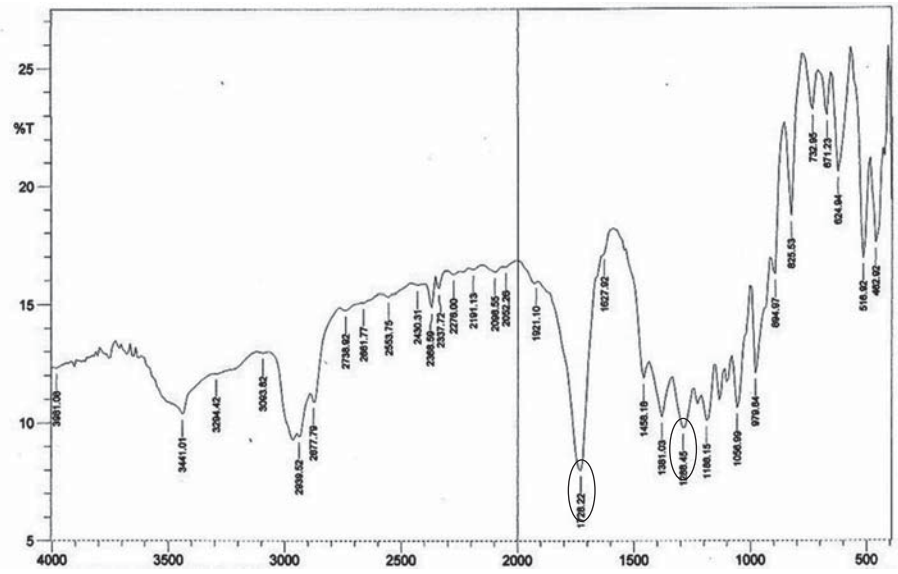
for 40 hours incubation time (Figure 3). The PHB production sharply increase when the level of starch was sharply decrease. This condition was linear with (Anderson & Dawes, 1990) that mentioned the production of PHB occurred when the nutrients is unbalanced because of the lack of carbon source. This result showed that PG1 synthesized PHB since exponential phase. PHB production during exponential phase means the PHB production is growth associated (Lee, 1996; Grothe *et al.*, 1999).

Identification of PGI Isolate

Based on the morphological, biochemical, and physiological characters



(a)



(b)

Figure 4. FTIR spectroscopy of the extracted polymer synthesized by PG1 (a) and PHB standard (b)

PGI isolate was identified as *Micrococcus* sp. PG1 (data unpublished).

FTIR analysis

FTIR analysis of polymer produced by PG1 using arrowroot starch as subtrates was investigated along with PHB obtained from commercial source (Sigma). FTIR spectra of the extracted polymer showed the intense peaks 1728.22 $cm^{-1}$and 1280.73 cm^{-1} corresponding to specific rotations around carbon atoms specific to certain functional groups (Figure 4). The peak at 1728.22 cm^{-1} corresponds to ester carbonyl (C=O) stretch of the ester group present in the molecular chain of highly ordered crystalline structure. The peak of at 1280.73 cm^{-1}corresponds to -CH group (Figure 4). These peaks are corresponding to the peaks obtained for the standard PHB (Sigma) at 1728.22 cm^{-1} and 1280.73 cm^{-1}

exactly confirming that the extracted polymer was poly-β-hydroxubutyrate (PHB) (Hong *et al.*, 1999).

Conclusion

The best condition for PHB production by the isolate PG1 was achieved at 5 % (v/v) inoculum concentration, arrowroot starch 5 g/l (w/v), $(NH_4)_2SO_4$ 1 g/l, $Na_2HPO_4.7H_20$ 6,7 g/l and KCl 2 g/l; whereas the best environmental conditions in term of initial pH was 7, temperature 37°C and agitation speed 125 rpm. Optimization of PG1 resulted in increasing of PHB production up to 16.8% (g PHB/g cdw) for 40 hours incubation time. FTIR analysis of the polymer indicated that produced polymer by *Mirococcus* sp. PG1 was a poly-β-hydroxybutirate.

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