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The Effects of Dissolved Oxygen Tension and Ammonium Concentration on Polyhydroxybutyrate Synthesis from Cassava Starch by *Bacillus cereus* IFO 13690

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Abstract

Attempting to get low price of raw material for producing polyhydroxybutyrate is always studied. Tapioca starch is one of the raw material with low price. The objective of this research was to study the effects of initial ammonium concentration and dissolved oxygen tension (doT) on producing PHB by Bacillus cereus IFO 13690 with tapioca starch as the carbon source. This fermentation was carried out in 5 L fementors with a 2 L working volume, temperature of 30 °C, and agitation of 500 rpm. The pH medium was controlled at 5.6 after it came down from the initial pH of 6.8. Meanwhile, the initial doT was 100 % air saturation and also came down to and maintained at doT of experiment, i.e. 1 , 5 , or 10 % air saturation. The best result was obtained when the initial ammonium concentration was 5 g / L and the doT value maintained at 5 % air saturation. By this conditions, the cell growth reached 5,457 g cell dry weight / L containing PHB of 2.42 % cell dry weigh after 29 hours fermentation.

Key words : polyhydroxybutyrate , ammonium, dissolved oxygen tension, *Bacillus cereus* IFO 13690

Introduction

Polyhydroxyalkanoate (PHA) is a polyester synthesized by microbial as carbon storage intracellularly (Solaiman and Ashby, 2005). PHA is a biopolymer (Figure 1) and classified as biodegradable plastic. One of the potential member of PHA and has been studied widely is polyhydroxybutyrate (PHB). PHB has similar characteristics with polypropylene (PP). It is good candidate for substituting such petroleum based plastic. The characteristics similarity of PHB and PP were described in Table 1.

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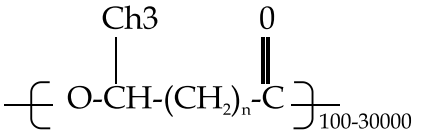

Figure 1. Formula of PHB (Ojumu *et al*., 2004).

Some potential bacterial strains for PHB production are *Alcaligenes eutrophus*, *Alcaligenes latus* (Wang and Lee, 1997), dan *Recombinant of Escherichia coli* (Ahn et al., 2000; Ahn *et al*., 2001; Park *et al*., 2002). Productivity of the bacteria were 2,42 – 5,13 g / L.h with PHB fraction of 74 – 89 % cell dry weight (Kim et al., 1994; Lee *et al*., 1997; Wang and Lee, 1997, Choi and Lee, 1999; Ahn *et al*., 2000, 2001; Shang *et al*., 2003). Carbon substrate for cultivation of these bacteria were glucose (*A. eutrophus*), sucrose (*A. latus*), and whey (Recombinant *E. coli*).

Tabel 1. The Properties of Polyhydroxybutyrate (PHB) and Polypropylene (PP)

Properties	PHB	PP
Crystalline melting point, oC	175	176
Molecular Weigt	5 x 105	2 x 105
Density, (g/cm3)	1,25	0,905
Flexural Modulus (GPa)	4,0	1,7
Tensile Strength	40	38
Ultraviolet Resistance	poor	good

The high price of biodegradable plastics is the main reason why it will not be widely used by people. Based on this problem, lowering production cost is the only way to get biodegradable plastic with lower price. One of factors included in production cost is raw material cost. Raw material cost take high fraction in production cost i.e. 40% of total cost (Kim and Chang, 1998). Cassava starch is one of the potential substrates with low price. Unfortunately, the well known bacteria strains can't be cultivated by this carbon source because of non amylolitic.

Most of *Bacillus* sp. are known as amylolytic bacteria. Furthermore, some *Bacillus* sp. have been proven as PHB producer. *Bacillus mycoides* RLJ B-017 was able to accumulate PHB 55-81,6 % cell dry weight during 24 hours fermentation (Borah *et al*., 2002). The other bacteria are *Bacillus* sp. JMa5 which synthesized PHB 25 – 35 % cell dry weight (Wu *et al*., 2001). *B. cereus* UW85 was also studied and it was the good PHB producer, namely it could accumulated PHB 2,32 - 24,6% cell dry weight (Labuzek dan Radecka, 2001). Valappil et al. (2007) researched on producing PHB by *B. cereus* SPV and it accumulated 29 % cell dry weight. These good results were obtained by non starch substrates as the carbon sources.

On preliminary study, we obtained *Bacillus cereus* IFO 1360 was able to synthesize PHB and it was also amylolytic bacteria. This research was carried out to study the effects of initial ammonium concentration and dissolve oxygen tension (doT) on the cell growth and PHB accumulation by *B. cereus* IFO 13690. Tapioca starch was used as carbon substrate in this research.

Materials and Methods

Bacteria and medium

Bacillus cereus IFO 13690 was obtained from Food and Nutrition Culture Collection of Gadjah Mada University, Yogyakarta. The medium compositions referred medium used by Ramsay *et al.* (1990) with some modifications. The medium contained (1 / L): tapioca starch, 20 g ; $Na_2HPO_4.7H_2O$, 5 g ; KH_2PO4, 1,5 g ; $(NH_4)_2SO_4$, 5 g ; $MgSO_4.7H_2O$, 0,2 g ; $CaCl_2.2H_2O$ 0,01 g ; $FeSO_4$, 28,5 mg ; KCl , 3 g ; dan *trace element*, 1 mL. Trace element compositions contained (1 / L) : H_3BO_3 0,3 g ; $CoCl_2.6H_2O$ 0,2 g ; $ZnSO_4.7H_2O$ 0,1 g ; $NaMoO_4.2H_2O$ 30 mg ; $NiCl_2.6H_2O$ 20 mg ; $CuSO_4.5H_2O$ 10 mg ; and $MnCl_2.4H_2O$ 10 mg.

Inoculum development

The inoculum was developed in 2 steps. Inoculum 1 was 20 mL medium containing *nutrient broth* 8 g / L and glucose 5 g / L. One ose of bacteria from agar slant was inoculated to the medium and incubated in incubator shaker with speed of 150 rpm and atmosphere temperature (about 30 °C) for 24 hours. Inoculum 2 was developed in 180 mL fermentation medium. This medium was inoculated with 20 mL of inoculum 1 and incubated in incubator shaker at 150 rpm and atmosphere temperature for 48 h.

Fermentor culture

This fermentation was carried out in 5 L fementors with a 2 L working volume. The fermentors and 1.8 L medium was sterilized at 121°C for 20 min. The pH medium was checked before inoculation and adjusted to 6.8 using 3 N NaoH. The initial of dissolved oxygen tension (doT) was set at 70% air saturation. 0.2 L inoculum was inoculated aseptically and running at medium

temperature of 30 °C and speed of agitation at 500 rpm. Initially, air supply, doT and pH control were not active. Dissolved oxygen tension came down and maintained at the experiment value (each of experiment was 1 , 5 , or 10% air saturation) by doT controller. pH value also came down and maintained at 5.6 automatically. The cell optical density, starch, PHB, and ammonium concentration were monitored periodically throughout the run.

The variables of experiment were doT and initial ammonium concentration in the medium. The doT of each experiment were 1 , 5 , or 10 % air saturation and the initial ammonium concentration was 1 , 3 , or 5 g / L of ammonium sulphate for each of experiment.

Chemical analysis

The cell dry weight was analyzed as optical density. PHB concentration was analyzed based on Senior *et al.* method (1972). For the extraction of PHB, 4 mL of fermentation broth was centrifuged at 13000 rpm and 3 minutes centrifugation. The residue was suspended by 1.5 mL of buffer phosphate solution pH 7.0 and 0.5 mL commercial hypochloride solution. This cell suspension was incubated in incubator shaker at 150 rpm and 30 °C for 24 h. The suspension was centrifuged at 13000 rpm and the residue was washed by distilled water. One half mL acetone added to the residue and dried at room temperature. Finally, the residue was dissolved by 3 mL concentrated sulphuric acid and boiled about 10 min. After the solution was cool, the absorbance of the solution was checked by spectrophotometer of Shimadzu UV-160A at 238 nm. The cell density was measured by optical density at 600 nm. Starch was analyzed as total sugar by Dubois method (1956). Ammonium concentration was analyzed by phenate method (APHA, AWWA, and WPCF, 1976).

Results and Discussions

The ability of bacteria to synthesize PHA can be divided into two groups (Lee, 1996). The first group is the bacteria which synthesize PHA during stationary phase. This type of bacteria requires nutrient limitation , N, P, Mg, and oxygen for example, and excess of carbon sources. The well known bacteria of PHA producer, *Ralstonia eutropha*, is belonging to this group. The other group is the bacteria which can synthesize PHA during growth phase or called as growth associated product. The bacteria of this type include *Alcaligenes latus* and *Escerishia coli* recombinant.

The present experiments showed *Bacillus cereus* IFO 13690 is belong to the second group. It accumulated PHB during growth phase. This pattern is shown in Figure 1.

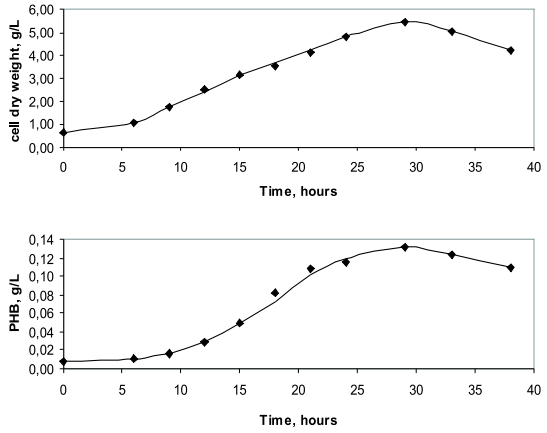

Figure 1. Growth associated product pattern in *B. cereus* IFO 13690 with initial ammonium sulphate of 5 g / L and doT 5 % air saturation.

The others bacteria of *Bacillus* sp. , *B. cereus* SPV, *B. mycoides* RLJ B-017, showed the same type in accumulating of PHB.

The effects of initial ammonium concentration

The initial ammonium concentration has important role on producing PHB by *B. cereus* IFO 13690. This research studied three

various of initial ammonium concentration, i.e. 0.29 , 0.57 , and 1.2 g/L. These concentration was obtained by dissolving 1, 3, and 5 g/L of ammonium sulphate respectively.

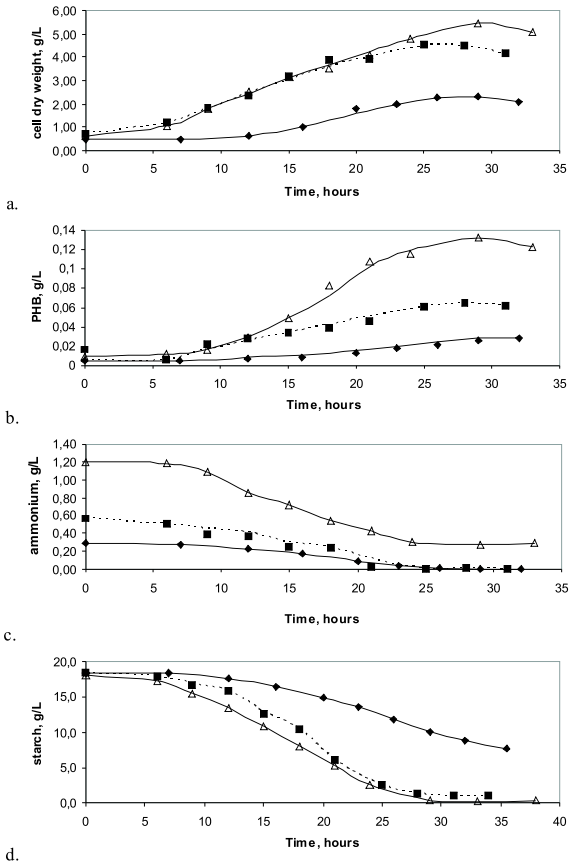

Figure 2. The effects of initial ammonium concentration on growth of cell and PHB. Initial ammonium:

Based on Figure 2, the higher initial ammonium concentration the higher cell growth and PHB accumulation. *B. cereus* IFO 13690 accumulated 0.029 gPHB/L at 32 h fermentation when it was cultivated by initial ammonium of 0.29 g/L. The accumulation reached 0.064 and 0.132 g PHB/L for the initial ammonium concentration of 0.57 and 1.2 g/L when the fermentation time of 28 and 29 h respectively.

Figure 2.A. depicts the same growth of the cell between initial ammonium concentration of 0.57 and 1.2 g/L until 23 h fermentation. The next time, the growth of cell start into stationary phase for initial ammonium concentration of 0.57 g/L, while the growth of cell still continue for initial ammonium concentration of 1.2 g/L. This is because of ammonium depleted for the initial concentration of 0.57 g/L. PHB accumulation has different phenomena, it was higher accumulation for initial concentration of 1.2 g ammonium/L than others. This results showed that the better PHB productivity obtained when *B. cereus* IFO 13690 was cultivated without ammonium limitation. *B. cereus* SPV (Valappil *et al.*, 2007) also showed better productivity without ammonium limitation.

The effects of dissolved oxygen tension (doT)

Dissolved oxygen tension (doT) is one of the important factors on producing PHB. It would be studied in this research, how the influence of doT to the growth of cell and PHB accumulation. It was necessary to get the better doT must be run for the fermentation.

Figure 3 depicts the influence of doT on growth of cell and PHB accumulation. There are three various of doT were studied, i.e. 1, 5 and 10% air saturation. The figure described that doT of 5% air saturation has better results on PHB accumulation. PHB accumulation reached 0,132 g/L or 2.42% cell dry weight at 29 hours fermentation. This is the better result than others, those are 0.097 and 0.064 g PHB/L for doT 1 and 10% air saturation after 35 and 30 h fermentation respectively.

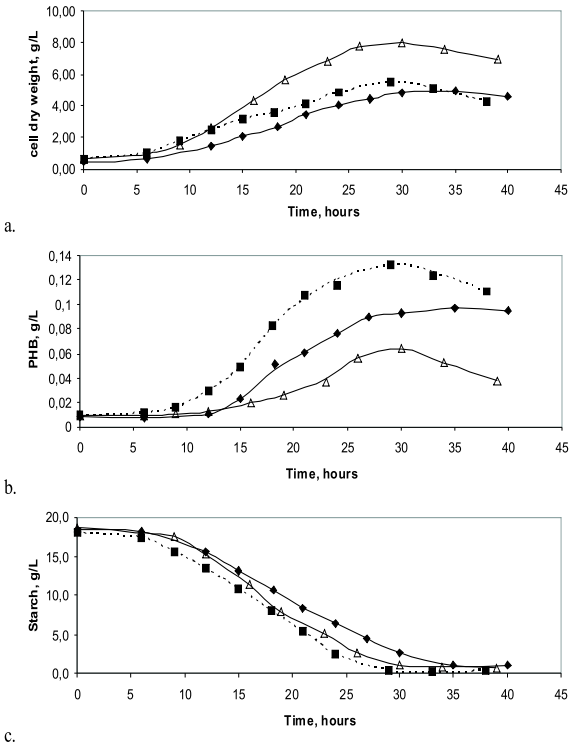

Figure 3. The effects of dissolved oxygen tension (doT) on growth of cell and PHB. doT (% air saturation) : diamond 1 ; triangle 5 ; square 10.

Figure 2 and 3 show the best growth of cell and PHB accumulation was reached by initial ammonium concentration of 5 g/L and doT of 5% air saturation. By this condition, *B. cereus* IFO 13690 accumulated PHB about 0.132 g/L and cell growth reached 5.457 g of cell dry weight / L broth or 2.42% cell dry weight. Other bacteria of the genus Bacillus could accumulated more PHB with substrate of glucose. *Bacillus mycoides* RLJ B-017 was observed able to accumulate PHB reached 56.6% cell dry weight during 24 h of fermentation (Borah *et al.*, 2002). *Bacillus cereus* UW85 was able to synthesize 24.6% cell dry weight during 24 h incubation (Labuzek and Radecka, 2001). The other Bacillus is *Bacillus cereus* SPV, it could accumulate PHB reached 29% cell dry weight during 48 h (Valappil *et al.*, 2007).

This research utilized define medium, while the previous research used undefined medium. Two factors could describe why *B. cereus* IFO 13690 was less productive, namely carbon source and nitrogen source. The undefined medium was concise of rich nutrient for growth, both are carbon source and nitrogen resource. It also had effects of more stimulate PHB accumulation.

Bacillus cereus IFO 13690 produced PHB by growth associated product pattern. It synthesized PHB during exponential growth phase. This present research showed that the initial ammonium concentration and dissolved oxygen tension (doT) influence the productivity. The best conditions was at initial ammonium concentration of 5 g/L and doT 5% air saturation.

Table 2 show the complete results of the influence of initial ammonium concentration and doT on cell growth and PHB accumulation.

Table 2. Influence of initial ammonium and dissolved oxygen tension (doT) on cell growth and PHB accumulation by *Bacillus cereus* IFO 13690.

No.	Time, hours	Initial NH_4^+, g/L	doT, % air saturation	CDW, g/L	PHB, g/L	Productivity, g/L.h.		PHB, %CDW
						CDW	PHB	
1	32	0.29	5	2,090	0,029	0,0653	0,0009	1,36
2	28	0.57	5	4,479	0,064	0,1600	0,0023	1,44
3	29	1.2	5	5,457	0,132	0,1882	0,0046	2,42
4	30	1.2	10	7,989	0,064	0,2219	0,0018	0,80
5	35	1.2	1	4,926	0,097	0,1407	0,0028	1,97

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