CHAPTER V
IMPROVEMENT OF PHB PRODUCTION FROM SWEET SORGHUM JUICE (SSJ) VIA FED-BATCH FERMENTATION UNDER DIFFERENT C/N RATIOS

5.1 Introduction

Since discovery in 1925 (Lemoigne 1926), polyhydroxybutyrate (PHB), a biopolyester, has been found in several microorganisms such as yeast, fungi but mostly found in bacterial strains. Typically, PHB is accumulated as carbon and energy reserve in the microorganism cells under imbalance conditions as excess in carbon source but deficient in some nutrients such as nitrogen and phosphorous. Recently, a biopolymer of PHB was considered to be an alternative bioplastic showing its properties similar to synthetic plastic of polypropylene (PP). However, commercial PHB production still limited due to high production cost attributed to the cost of carbon source, type of fermentation process and also the downstream process to recovery the product (Choi & Lee, 1999).

A suitable production strategy resulting in high volumetric productivities and polymer yields by the organisms is significant factor to allow a commercially possible production of PHB (Choi & Lee 1999). Various number of organisms accumulate the polymer, however, only a limiting number are considered as good candidates for industrial production of PHB. Among them, *Cupriavidus necator* (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*), *Alcaligenes latus*, *Azotobacter vinelandii*, *Bacillus* spp. and recombinant *Escherichia coli* have shown the highest potential for PHB production (Reddy, Ghai, & Kalia, 2003). A bacterial strain of *C. necator* is widely used to synthesize PHB, nutrient limitation in medium was necessary for PHB synthesis to reach a high polymer yields. This strain accumulates most PHB during stationary phase of growth (Kim, Lee, Lee, Chang, Chang, & Woo, 1994). Hence, this strain requires an expensive two stage cultivation (Byrom, 1990; Grothe & Chisti, 2000; Du, Chen, Yu, & Lun, 2001). For other bacteria including

A. latus, *A. vinelandii*, *Bacillus* spp. and rec. *E. coli* do not require nutrient limitation to initiate PHB synthesis, although the limitation of a nutrient in the culture medium favors high PHB accumulation by the cells. The genera *Bacillus* being identified as one of the first Gram-positive bacteria capable of PHA production (Lemoge, 1999), offers several advantages for PHA fermentation studies (Thakur et al., 2001; Yilmaz et al., 2005; Valappil et al., 2007; Adwitiya, Ashwini, Avinash, Badri, Kajal, Vomsi, et al., 2009; Faccin et al., 2009; Kulpreecha et al., 2009). These include chemoorganotropic features, lack of lipopolysaccharide endotoxin (Singh et al., 2009), secretion of a large number of amylases proteases and urease. These features of *Bacillus* spp. explore the possibility of utilising various agricultural raw materials as a carbon source and a cheap nitrogen sources for production of different metabolites. Some *Bacillus* spp. are a growth-associated producer of PHB; hence, a single-stage fermentation is sufficient. For the production of this biopolymer on a large scale, a high cell concentration (high cell density culture) must be obtained during the growth phase, paralleled by a production phase with a high polymer percentage in the cell.

In our previous research, *Bacillus aryabhattai* PKV01 was isolated form sugarcane plantation soil, which could produce PHB from sweet sorghum juice (SSJ) during exponential growth phase without hydrolysis method. It was remarked that this strain did not require nutrient limitation. It may be produced PHB in single step fermentation. Hence, this strain might be suitable to enhance the growth parallel with PHB accumulation by fed-batch fermentation.

The purpose of this study was to compare the efficiency of PHB production from sweet sorghum juice (SSJ) using batch and fed-batch fermentations by *Bacillus aryabhattai* PKV01. The effects of different C/N ratios of SSJ feed medium in fed-batch cultivation on the efficiency of PHB production were also evaluated.

5.2 Materials and Methods

5.2.1 Materials

Sweet sorghum stems was kindly provided from Faculty of Agricultural Science, Khon Kaen University, Thailand. The stems then were squeezed by roller mills and the juice was obtained after filtering by cotton sheet. Here after, it called as sweet sorghum juice or SSJ then was kept at -20 °C prior to use. Other chemicals used throughout this study were analytical grade.

5.2.2 Microorganism

A bacterial strain of *B. aryabhattai* PKV01 isolated from sugar cane plantation soils in Chaiyabhumi province, Thailand, was used in this study.

5.2.3 Inocula preparation

A seed starter of *B. aryabhattai* PKV01 inoculums was prepared for 2 steps as follow; the first pre-culture was inoculated with a single colony of *B. aryabhattai* PKV01 from nutrient agar (NA) plates into nutrient broth (NB) and incubated on an orbital shaker at 30°C for 18 h. The second pre-cultures were inoculated with the first pre-culture (2% v/v) into the medium for batch culture (See Table 5.1) and incubated on a rotary shaker at 30°C for 18 h. The 2 % of the second pre-culture were used for inoculation in 3 L bioreactor.

5.2.4 Batch experiment

The media composition for batch cultivation was showed in Table 5.1. The batch fermentation was carried out in 3 L bioreactor, with working volume 1 L. The medium was sterilized *in situ* at 110 °C for 40 min. Two percent inoculums size were added to fermenter and operated at 30 °C, air flowed 2 vvm. The dissolve oxygen was maintained above 40 % of air saturation by automatic increasing agitation speed. pH was maintained throughout of fermentation at 6.5 (pH stat) by addition of 6 N NaOH and 6 N HCL. The silicone oil was used as antifoaming when need. Samples were collected at regular intervals and were analysed for biomass and residual sugars.

5.2.5 Fed-batch experiment

To improve the production yield, only effect of different carbon to nitrogen (C/N) ratios of feed medium was studied. The feeding medium contains a highly concentration of total sugar (195 g/L) in the SSJ were prepared base on different C/N ratios by adding urea as a nitrogen source (Table 5.1). The continuous feeding strategy was used for fed-batch fermentation to keep the constant flow rate, which was calculated from the data obtained from batch experiment with the general equation of fed-batch culture (Eq.1). The initial batch cultivation was conducted under the same as in batch experiment. Fed-batch experiment was started after midlog phase of initial batch cultivation and terminated until the final working volume was 1.5 L.

$$F_s = \frac{\mu X_t V_0}{S_f Y_{X/S}} \qquad (1)$$

Where μ is specific growth rate, X_t is biomass content at that time, V_0 is initial fermentation volume, S_f is substrate concentration in feed medium, $Y_{x/s}$ is biomass yield coefficient on total sugar consumption and F_s is substrate feeding rate.

Table 5.1 Medium components using in batch and fed-batch cultivation

Component	Batch	Fed-batch Feed medium		
	C/N =5	**C/N= 10**	**C/N= 20**	**C/N= 30**
Total sugar in sweet sorghum juice (g/L)	20	195	195	195
Urea (g/L)	4	19.5	9.75	6.5
KH_2PO_4 (g/L)	1	1	1	1
Na_2HPO_4 (g/L)	4	4	4	4
$MgSO_4 .7H_2O$ (g/L)	0.1	0.1	0.1	0.1
Trace element (mL/L)	1	1	1	1

5.2.6 Analytical methods

1) Sugar concentration

The concentration of glucose, fructose and sucrose in the SSJ were analysed by High Performance Liquid Chromatography (HPLC) (Shimadzu, Japan) equipped with refractive index detector (RID) using Vertical GES-NH_2 HPLC column (4.6x250 mm, 5 µm). A solution of 70% acetonitrile in double distilled water as the mobile phase. The constantly flow rate was controlled at1 mL/min. The injection volume was 20 µL. Glucose, fructose, and sucrose concentration were calculated by calibration curves built for these three sugars using standard chemicals.

2) Biomass and PHB content

During fermentation, culture was withdrawn and the biomass was collected after centrifugation (10000 rpm, 10 min). Then, supernatant was discarded; the pellets were washed for twice with distilled water and dried in hot air oven at 60°C until constant weight**.** PHB then was extracted from wet cell pellets. In brief, wet cell was digested by 6 % active chlorine (commercial grade bleach) in form of sodium hypochlorite at 37°C for 1 h. PHB concentration was determined according method of Law and Slepeky (1961).

5.3 Results and Discussion

5.3.1 Batch cultivation in bioreactor for PHB production

Time course of PHB accumulation in pH-stat batch fermentation by *B. aryabhattai* PKV01 using 20 g/L SSJ as carbon source is shown in Figure 5.1. The cells concentration started to gradually increase after 6 h cultivation. The exponential growth phase was appeared from 9 to 15 h. The highest production of PHB was obtained at about 1.96 g/L with 8.83 g/L of DCW at 15 h incubation which as equivalent to 22.20 % (w/w) of PHB content. The total sugar was rapidly decreased during 6 to 18 h fermentation. The specific growth rate (μ) and biomass yield coefficient on total sugar consumption ($Y_{x/s}$) were calculated at mid-exponential growth phase (12 h) and were obtained at 0.67 h^{-1} and 0.79 g/g. The feed rate for fed-batch fermentation was further calculated from the data obtained from batch fermentation.

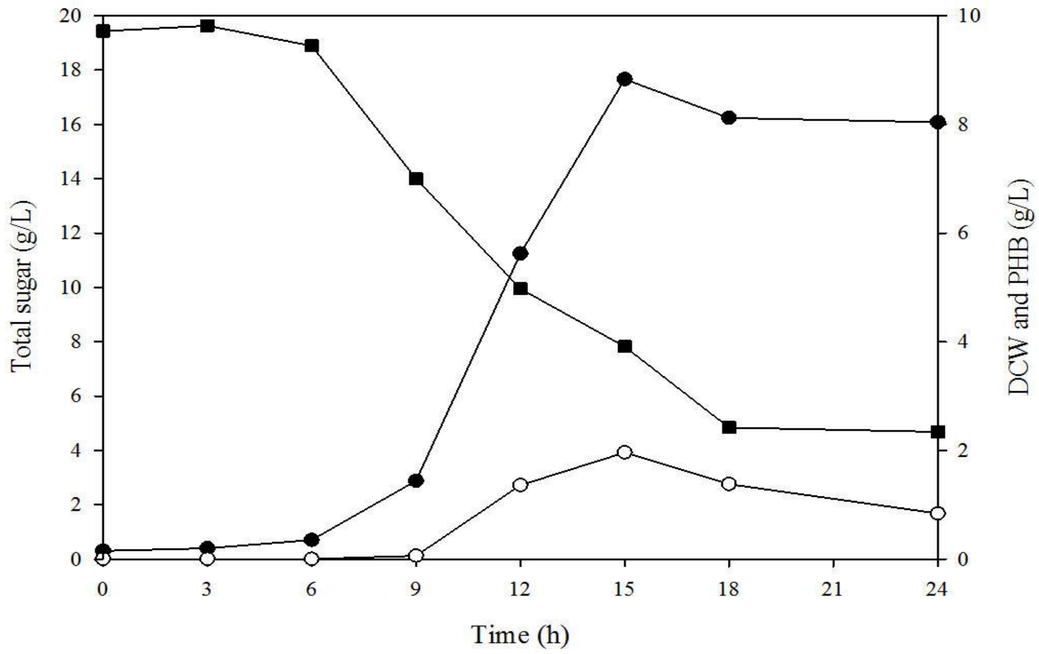

Figure 5.1 The time course of PHB production under batch fermentation from SSJ (■-total sugar, ● DCW, ⊖ PHB)

5.3.2 Continuously fed-batch feeding cultivation in bioreactor for PHB production

Time course of DCW and PHB production under fed-batch fermentation under different feeding C/N ratios (10, 20 and 30) in feed medium is shown in Figure 5.2. The medium were fed with feeding rate calculated from Eq. 1 at mid-exponential growth phase (12 h). The feed was controlled at constant rate of 0.41 mL/min and was stopped after 48 h fermentation when the working volume in bioreactor was registered at 1.5 L. As it can be depicted from Figure 5.2a, the exponential growth was observed from 12 h to 48 h fermentation by using C/N ratios (10 and 20 of feed medium). In case of the C/N ratio of 30 was applied, the exponential growth phase was stopped at 19 h. The highest DCW production was achieved when the C/N ratio of 10 of fed-medium was used. The highest DCW were exhibited in lower C/N ratio because the higher nitrogen sources were used for the synthesis of proteins that was essential for the growth in bacteria (Ramadas et al., 2009). Results of PHB production under fed-batch fermentation are shown in Figure

5.2b. The highest PHB concentration was obtained at 15.5 g/L under C/N ratio of 20. Meanwhile, the lower PHB concentration of 14.30 and 8.80 g/L were obtained from C/N ratios of 10 and 30. However, the PHB accumulation rate of 59.14 % (w/w) was achieved from C/N ratio 30. Resulting in the higher C/N ratio led to an excess of reduced coenzymes (NADH and NADPH) and a higher carbon flux can be directed toward PHB synthesis pathway (Tavares, da Silva, & da Cruz Pradella, 2004; Anderson & Dawes, 1990). The limitation of nitrogen at a C/N molar ratio of 25 resulted in enhancement of PHB and cell growth by *Bacillus megaterium* BA-019, were reported by Kulpreecha et al (2009). Later, Wei, Chen, Huang, Wu, Sun, Lo, et al (2011) demonstrated a PHB content of 58.81% and a PHB production of 2.44 g/L when the carbon/nitrogen ratio of 8/1 was selected for *C. taiwanensis* 184.

The sugar concentration were analysed during fed-batch production. The mix sugars in the SSJ were mainly glucose, fructose and sucrose, respectively. Figure 5.3 shows the utilisation of sugars by *B. ayabhattai*. The results showed that sucrose could be breakdown faster than that glucose and fructose. The increasing of glucose and fructose were appeared which indicated that this microorganisms was able to hydrolyses sucrose faster than the utilisation of glucose and fructose. In addition, the total sugar profiles is illustrated in Figure 5.3, it was found that increasing of total sugar in fermentation broth resulted from an increasing of glucose and fructose. However, the total sugar remaining in fermentation medium were continuously utilised by this strain and finally total sugar concentration were approximately 2.78, 3.21 and 18.45 g/L in cases of the C/N ratios of 10, 20 and 30, respectively.

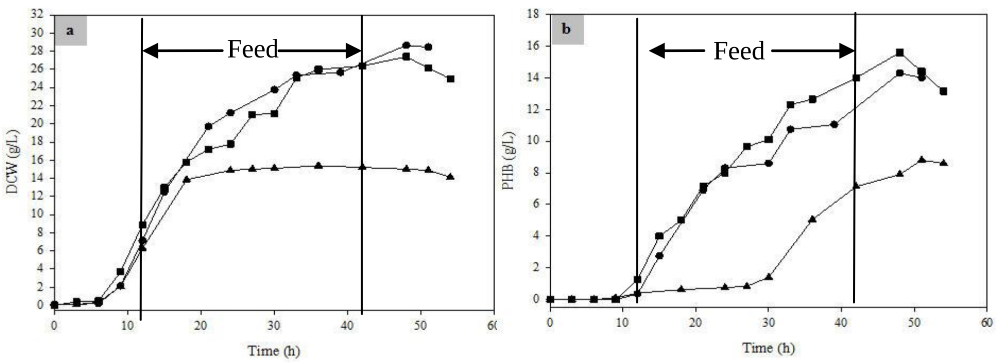

Figure 5.2 DCW (a) and PHB (b) production under fed-batch cultivation with different C/N ratio of feeding SSJ (● C/N=10, ■ C/N=20, ▲ C/N=30)

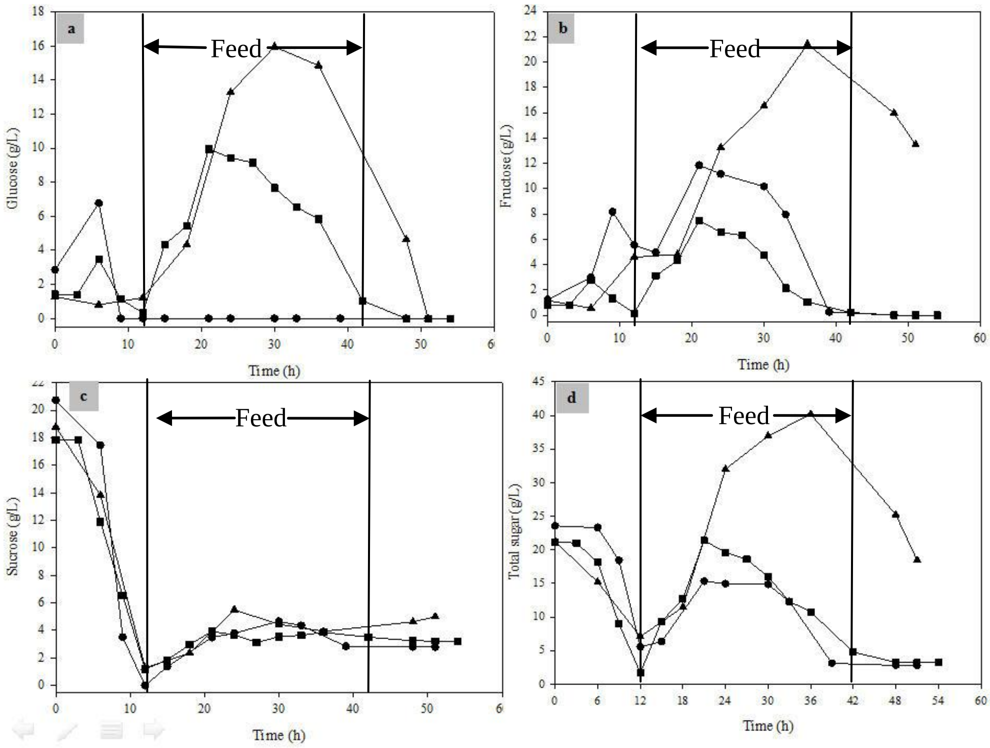

Figure 5.3 The sugar utilising profiles (glucose, a; fructose, b; sucrose, c; total sugar, d) in fed-batch cultivation with different C/N ratio of feeding SSJ (● C/N=10, ■ C/N=20, ▲ C/N=30)

According to the sugar utilising profile, it could be stated that bacterial strain of *B. aryabhattai* showed a high efficiency to use sucrose as a carbon source. Compared among various kinds of sugar, sucrose is the most inexpensive sugar which is mostly contained as carbohydrate form in sugar plants including sugar cane, sweet sorghum, maple sap and etc. Hence, this strain could be used for production of biopolymer of PHB.

5.3.3 PHB production in batch and fed-batch fermentations

In Table 5.2 summarises the results obtained in batch and fed-batch experiments under various C/N ratios of feed medium. In batch fermentation, the highest cell, PHB concentration and PHB content were 8.83, 1.96 and 22.20% (w/w) while for fed-batch fermentation, the highest cell and PHB concentration were reached at 28.65 and 15.60 g/L after C/N ratios of 10 and 20 were used. The highest PHB content of 59.14 % was obtained in case of C/N ratio of 30. The results showed that DCW and PHB in fed-batch cultivation increased approximately 3.24 and 7.96

folds compared to those of the batch cultivation. On another way, the PHB content and PHB productivity were also increased about 2.66 and 2.54 folds when it was carried out in batch fermentation. Moreover, the fed-batch fermentation of *B. aryabhattai* showed substantially higher DCW (27.40 g/L) in turn, high PHB accumulation (15.60 g/L) (56.93% w/w) among other *Bacillus* strains as reported before including *Bacillus sp.* INT005 (35.30%) (Tajima, Igari, Nishimura, Nakamura, Satoh, & Munekata, 2003), *B. cereus* SPV (41.90%) (Valappil et al., 2007), and *B. cereus* CFR06 (46.0%) (Halami, 2008).

However, strategies for increasing high cell density have many drawbacks such as limitation of substrate, cell damage by agitation shear force, high demand for oxygen during the process due to the high specific oxygen uptake rate, and formation of growth inhibitor byproducts (Lee, 1996). Therefore, further studies are required to supply pure oxygen and optimum substrate concentration of feeding medium to improve the PHB yield and to reduce the cost of production media along with suitable PHB induction media components.

Table 5.2 PHB production in batch and fed-batch fermentations

Fermentation method	DCW (g/L)	PHB (g/L)	PHB content (%w/w)	PHB productivity (g/L.h)
Batch	8.83	1.96	22.20	0.13
Fed-batch				
C/N=10	28.65	14.30	49.91	0.30
C/N= 20	27.40	15.60	56.93	0.33
C/N= 30	14.88	8.80	59.14	0.17

5.4 Conclusion

In this study, fed-batch fermentation of the SSJ showed a great improvement for 7.96 folds PHB production as compared to batch production. The accumulation of PHB as high as 56.93 % w/w in *B. aryabhattai* cells was achieved by continuously SSJ feeding with C/N ratio of 20. Successfully use single stage fed-batch for enhancing PHB production by the strain of *B. aryabhattai* was obtained in this study. This high accumulation is favorable for cost effective PHB production. In addition, the SSJ can also be utilised as a suitable and cheap substrate for PHB production thereby increasing the possibility of using SSJ into biodegradable green plastics.

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