CHAPTER IV
AN ALTERNATIVE APPROACH TO THE FERMENTATION OF SWEET SORGHUM JUICE INTO BIOPOLYMER POLY-β-HYDROXYALKANOATES BY NEWLY ISOLATED *Bacillus aryabhattai* PKV01

4.1 Introduction

The public seriously concerns on huge petroleum-based plastics waste accumulated in environments. The increasing of non-biodegradable plastics are not only negative impacts on life quality of human but also animals as well as single cell lives because of their persistence in environment. Several communities are now more concerned with the impact of discarded plastic on the environments and searching for new resources capable of biodegradability instead of petroleum-base plastics. Nowadays, a wide variety of bio-based polymers are produced in worldwide. Among the various biodegradable plastics, a class that is drawing considerable attention is poly-β-hydroxyalkanoates (PHAs), intracellular storage compounds that are accumulated as discrete granules in cytoplasm of cells as carbon and energy reserve in several kinds of microorganisms but mostly found in bacteria. PHAs are biodegradable thermoplastic polyester (Anderson & Dawes, 1990), compatible compound with mammalian cell (Philip et al., 2007), possessing a widely physical properties similar to synthetic polypropylene. The applications of PHAs as biodegradable plastics are widely used in multi purposes such as veterinary practice, food packaging, soil cover, medicines, tissue engineering as implant materials, etc. However, their use is currently limited due to the cost of manufacturing process of this biodegradable plastic attributed to the cost of carbon source, fermentation process and also the downstream process (Choi & Lee, 1999). Considerately, carbon source alone costs up to 50% of the overall production costs of the PHAs (Halami, 2007). Thus, the use of inexpensive carbon sourced such as agro-industrial raw materials, by-product, waste and even other cheap renewable sources could contribute

as much as 40-50% reduction in the overall production cost (Ramadas et al., 2009; Santimano et al., 2009).

Previously various cheaper renewable substrates were used for PHAs production such as carbon dioxide (Ishizaki et al., 2001), molasses (Gouda et al., 2001; Kulpreecha et al., 2009), methanol (Kim et al., 2003; Mokhtari-Hosseini et al., 2009), maple sap (Yezza et al., 2007) sucrose (Tanamool et al., 2011) and recently, sugar cane (Suwannasing et al., 2011). However, another sugar plant of sweet sorghum would appear to be an alternative substrate for PHAs production due to several advantages including low price, grow in short time approximately 3-4 months, well adapted to sub-tropical and temperate regions of the world, water efficient (Almodares & Hadi, 2009) and high total sugar content in forms of sucrose with other sugars in small amounts, especially glucose and fructose. With these properties, it would be attractive for using as an alternative carbon source medium for microbial growth to produce added value products such as ethanol (Mamma et al., 1995; Sipos et al., 2009; Laopaiboon et al., 2009), hydrogen, (Antonopoulou et al., 2008), lactic acid (Hetényi et al., 2010) and unsaturated fatty acids like docosahexaenic acid (DHA) (Liang et al., 2010). However, only our group has played attention and previously reported the production of PHAs by Gram-negative bacteria of *Alcaligenes eutrophus* TISTR 1095 from sweet sorghum juice (SSJ), although the production yield was still low (Kaewkannetra et al., 2008). Thus, we have tried to screen novel bacterial strains which are able to grow in SSJ. A Nile blue A was directly added into sweet sorghum agar medium and observed their growth under UV light (Spiekermann et al., 1999). The results showed that, the isolate strain S4 was the best to produce PHAs as compared to the other strains and further identified as *Bacillus aryabhattai* (Tanamool & Kaewkannetra, 2011). In addition, this strain, screened from sugar cane plantation soil, can produce PHAs and grew well in SSJ medium (Tanamool & Kaewkannetra, 2011) but the kinetics of production had never been studied. Previous researchers described the benefit in the use of Gram-positive bacteria instead of Gram-negative (Valappil, Peurusm, Langley, Herniman, Boccaccini, Bucke, & Roy, 2007; Singh et al., 2009; Tanamool & Kaewkannetra, 2011). These include utilising various agricultural raw materials as a carbon source for the production of different metabolites and lack of endotoxin as lipopolysacharide which are pryogenic in human

beings (Singh et al., 2009). This feature is undesirable for biomedical applications of the PHAs. Hence, the Gram-positive strain as *Bacilli* was recommended for exploitation in the PHAs production.

In the present study, we explore the feasibility of the bioconversion of mixed sugars in the sweet sorghum juice to produce PHAs through fermentation process by Gram-positive bacteria of *B. aryabhattai* PKV01. In addition, the optimisation of PHAs production by using response surface methodology (RSM) and the properties of the PHAs produced from sweet sorghum juice were also reported.

4.2 Material and Methods

4.2.1 Microbial strain

A PHAs producing strain *Bacillus* sp. was isolated from sugar cane plantation soil in north eastern area of Thailand. The strain used throughout for this study was recently identified as *B. aryabhattai* (99.7 % similarly) (Tanamool & Kaewkannetra, 2011). The stock cultures were maintained their growth at 30 °C on nutrient agar (NA) which is contained of beef extract (0.3%), peptone (0.5%) and agar (15%) and kept at 4 °C prior to use.

4.2.2 Raw material

Sweet sorghum KKU 40 (*Sorghum bicolor* L. Moench), a 3 annual and yields large amount of sugars in the stems. Typically, it mainly consists of sucrose (up to 55% of dry weight biomass), fructose and glucose, was harvested in July 2009 and kindly provided from Faculty of Agricultural Science, Khon Kaen University, Thailand. The stems were squeezed and the juice was obtained after filtering by cotton sheet. The SSJ then was kept at -20 °C for preventing microbial contamination.

4.2.3 Media and culture condition

1) Effect of different nitrogen sources on growth and PHAs production

Different nitrogen sources as ammonium chloride, ammonium sulphate, urea, peptone and yeast extract were investigated by adding into mineral salt medium which was consisted of 20 g/L total sugars (sucrose, fructose and glucose) in the SSJ. Other supplements were prepared follow the method explained by Grothe et al. (1990) as different nitrogen sources (1 g/L), Na_2HPO_4 (4 g/L) KH_2PO_4 (1 g/L),

$MgSO_4$ $7H_2O$ (0.1 g/L) and trace element solution (1 mL/L), respectively. After incubation for 24 h at 30 °C and 200 rpm, the culture was withdrawn and biomass and PHAs content were estimated.

2) Effect of differences initial pH on cell growth and PHAs production

The variations of initial pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) in the culture medium SSJ were investigated. The culture was maintained at a desired value by adding 6 N NaOH or HCl. After fermentation, dry cell weight (DCW) and PHAs concentration were determined and the optimal pH obtained was used for further studies.

3) Media optimisation using Plackett–Burman optimisation design

In this study, statistical tool of Plackett-Burman's design was preliminary used to screen medium culture used during fermentation. Six main factors as total sugar (A), KH_2PO_4 (B), Na_2HPO_4 (C), $MgSO_4$ 7 H_2O (D), urea (E) and Trace element solution (F) of media compositions were screened earlier. A design of 12 experiments was formulated for six factors using the Design Expert software (Stat-Ease Corporation, Minneapolis, USA, trial version) and each parameter was tested at two levels, high (+1) and low (−1). The concentration range for the variables was decided on the basis of previous reports for PHAs production (Grothe et al., 1999; Gouda et al., 2001; Khanna & Srivastrava, 2005; Kulpreecha et al., 2009). The experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL media at 30 °C, 200 rpm and for 72 h in duplication. The responses were measured in terms of DCW (G) and PHAs (H) production. The DCW and PHAs obtained in all experiments were subjected to compatible analysis from p-value. The components, giving in a highly significant ($p \leq 0.05$) effect on the responses, were taken to consider by Response Surface Methodology (RSM).

4) Response Surface Methodology (RSM)

Only affected factors, obtained from the Plackett–Burman design, were used for media optimisation by the RSM using central composite design (CCD) via the software Design Expert. However, the insignificant factors were kept at a constant level. In addition, validity of optimised media optimisation was necessary to consider.

5) Batch bioreactor studies

The optimal media composition predicted by the RSM in shake flask was extensively carried out in 3 L fermenter, with 2 L working volume. The medium was sterilized as *in situ* at 110 °C for 40 min and left for cool down. When a 2 % inoculums size were added into the bioreactor, batch fermentation was operated at 30 °C with 1.5 vvm air flow supply and 200 rpm agitation. In addition, a silicone oil was used for antifoaming problem during fermentation. Samples were taken at regular intervals and were analysed for DCW, residual sugars and PHAs production.

4.2.4 Analytical methods

1) Determination of sugar concentration

The SSJ was characterised in terms of glucose, fructose and sucrose using 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) and a 70% acetonitrile in double distilled water was used as the mobile phase. The flow rate and the injection volume were constantly controlled at 1 mL/min and 20 µL. Each sugar concentrations as glucose, fructose, and sucrose was calculated from calibration curves. For other minerals (Na, K, Mg, Ca and Zn) were estimated using Perklin Elmer atomic absorption spectrophotometer (Model 2380, England) as described in the AOAC (1998). In addition, organic nitrogen concentration was measured using total Kjeldahl nitrogen method (TKN).

2) DCW (biomass) measurement

After fermentation, the culture broth was centrifuged at 10000 rpm for 10 min and supernatant was discarded. The pellets were washed with distilled water for twice and dried in hot air oven at 60°C until constant weight and finally the biomass was obtained.

3) PHAs recovery and quantification

PHAs then was extracted directly from wet cell pellets. In brief, wet cell was disrupted by 6 % sodium hypochlorite (commercial grade bleach) at 37°C for 1 h. The white pellets were collected after centrifugation and then washed with distilled water and acetone: ethanol (1:1). When a soxlhet apparatus was built, the pellets was transferred into chloroform was used to extract intracellular PHAs. After 2 h, the mixed solution was casted on clean Petri disk glass and dried under

room temperature until constant weight. PHAs concentration was determined by method of Law and Slepeky (1961).

4) PHAs characterisation

The extracted PHAs were characterised in their thermal properties as differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) while their structure were measured using Nuclear Magnetic Resonance (NMR) technique. Then, they were compared to the polymer standard in term of poly-β-hydroxybutyrate (PHB) (Sigma-Aldrich, USA). It was noted that PHAs are mostly found in form of PHB. For the DSC and TGA measurements were followed the method explained by Tanamool et al. (2011).

4.3 Results and Discussion

4.3.1 Sweet sorghum juice (SSJ) composition

Composition of the SSJ composition is shown in Table 4.1. The SSJ consisted of high total sugar content in forms of sucrose and in small amounts of glucose and fructose. In this case, the SSJ showed 175.97, 12.32 and 5.75 g/L for the contents of sucrose, glucose and fructose, respectively. This is on agreement and comparable to previous studies (Mamma et al., 1995; Kaewkannetra et al., 2008; Laopaiboon et al., 2009; Sipos et al., 2009) who reported that sugar contents found in the SSJ was varied. The order sugar contents found in the SSJ were sucrose › glucose › fructose, respectively as in the same way of the results reported by Liang et al. (2010). The different observation may be found due to variety, location, and harvest time. In addition, the first two factors are obviously and easy to understand however harvest time has been shown to be critical for sugar composition (Liang et al., 2010). Other trace elements found in the SSJ are also shown in Table 4.2. Minerals of potassium (P) and calcium (Ca) were dominant following by nitrogen (N), magnesium (Mg), sodium (Na) and zinc (Zn), respectively.

According to the composition of SSJ showed in Tables 4.1-4.2, we have made an assumption that the SSJ may contains of suitable components for microbial growths and they could directly use those sugars without pretreatment process as hydrolysis. The high total sugar concentration is one of advantages for increasing the efficient fermentation. However, some elements still had to add into the

SSJ based medium (e.g. nitrogen). Since, a lower concentration was appeared when the SSJ was diluted to the desired condition.

Table 4.1 Comparison of sweet sorghum juice composition from various crop locations

Crop location	Harvest month	Sugar composition (g/L)			Total sugar (g/L)	References
		Sucrose	Glucose	Fructose		
Thailand	July	175.97	12.32	5.75	194.04	This study
USA	October	143.30	39.30	61.00	242.60	Liang et al. (2010)
Thailand	NA	124.10	20.90	16.80	161.80	Laopaiboon et al. (2009)
Hungary	September	75.10	25.00	18.10	118.20	Sipos et al. (2009)
Thailand	December	124.05	14.22	11.46	149.73	Kaewkannetra et al. (2008)
Greece	November	211.90	20.10	-	232.00	Mamma et al. (1995)

Table 4.2 The various elements content in sweet sorghum juice

Component	Concentration (mg/L)		
	This study	Laopaiboon et al. (2009)	Massoud & Abd El-Razek, (2011)
N	410*	21.4	N.D
K	5175	1790	3028
Ca	639.3	166	66.5
Mg	396	194	N.D
Na	200	170	2710
Zn	0.31	1.4	N.D

*Determined by TKN method, N.D = not determine

4.3.2 Effect of nitrogen sources on PHAs production

Nitrogen plays a major role in the metabolism of microorganisms. The cell needs nitrogen for building up its structures. Considering in term PHAs

production, type of nitrogen sources affected on the PHAs accumulation in the cells as well. Table 4.3 shows the DCW, PHAs concentration and PHAs content obtained from 5 nitrogen sources tested. The highest DCW (4.0 g/L) and PHAs concentration (2.10 g/L) were obtained when urea was used. Considerably, PHAs content, maximum PHAs content of 54.3 % (w/w) was gained in case of yeast extract. Previously, Yüksekdağ, Aslım, Beyatl, and Mercan (2004) reported that complex nitrogen sources (urea, peptone and yeast extract) increased the yield of PHAs in *Bacillus* strains. As in agree with this study, urea showed significantly increasing in both DCW and PHAs production. Since, urea, an uncharged polar and small molecule in contrast to other simple nitrogen sources including NH_4Cl and $(NH_4)_2SO_4$, which are inorganic form, easier uptake into the cells membrane more than that of $(NH_4)_2SO_4$ (Kulpreecha et al., 2009). Moreover, urea is a cheaper nitrogen source than the others. For this reason, this is a one choice for reducing the production cost of PHAs by using urea as nitrogen source supplemented to SSJ.

Table 4.3 PHAs and dry cell weight concentration obtained with different nitrogen sources

Nitrogen sources	Dry cell weight (g/L)	PHAs (g/L)	PHAs content (%)
Control*	1.00	0.40	40.00
Urea	4.0	2.10	52.50
Peptone	3.40	1.80	52.90
Yeast extract	3.50	1.90	54.30
NH_4Cl	3.00	1.20	40.00
$(NH_4)_2SO_4$	2.50	1.00	40.00

* Control, 2 % sweet sorghum stem juice without addition of nitrogen source

4.3.3 Effect of pH on PHAs production

The influence of different initial pH in culture medium on DCW and PHAs production were investigated. The results are summarised in Table 4.4, and showed that the *B. aryabhattai* PKV01 could grow on wide range of initial pH. The maximum DCW of 2.8 g/L was obtained in SSJ medium with initial pH of 7.0. Palleroni and Palleroni (1978) have also reported that pH in range of 6.0-7.0 was the

best for microbial growth. This is in agreement with this case, the maximum PHAs production of 1.1 g/L were obtained in the SSJ medium in both initial pH of 6.5 and 7.0. Considering PHAs content, the highest PHAs accumulation reached at 42.31 % (w/w) at initial pH of 6.5. Where else, Grothe et al. (1999) reported that an initial pH of 6.5 gave the best results in both DCW yield and PHAs production. In addition, previous studies (Nagata, 1963; Kominek & Halvorson, 1965) stated that the initial pH at about 6.4 was suitable for PHAs accumulation by *Bacillus* strain. Hence, initial pH value of 6.5 was chosen throughout in this study.

Table 4.4 PHAs and dry cell weight concentration obtained with different initial pH values

pH	Dry weight (g/L)	PHAs (g/L)	PHAs content (%)
5.0	2.10	0.70	33.33
5.5	1.80	0.60	33.33
6.0	2.20	0.90	40.91
6.5	2.60	1.10	42.31
7.0	2.80	1.10	39.28
7.5	2.70	0.50	18.52
8.0	2.70	0.50	18.52
8.5	2.70	0.40	14.81
9.0	3.00	0.50	16.67

4.3.4 Placklett-Burman design

The six main factors affect on the experiment at two levels, high (+1) and low (−1), are shown in Table 4.5 and all 12 experiments designed by Design Expert software are listed in Table 4.6.

Table 4.5 Range of various factors studied in Plackett-Burman design

Factors	Name	Levels	
		Low (-1)	High (+1)
A (g/L)	Total sugar	10	40
B(g/L)	KH_2PO_4	1	4
C(g/L)	Na_2HPO_4	1	4
D(g/L)	$MgSO_4\,7H_2O$	0.1	0.4
E(g/L)	Urea	2	4
F(mL/L)	Trace element	1	5

Table 4.6 Experimental design and response of Plackett-Burman study

Expt. no.	A (g/L)	B (g/L)	C (g/L)	D (g/L)	E (g/L)	F (g/L)	Responses	
							DCW (g/L)	PHAs (g/L)
1	10	0.5	4	0.1	3	5	0.96	0.28
2	40	3	0.5	0.1	1	5	3	1.02
3	40	3	0.5	0.5	3	5	1.92	0.56
4	10	0.5	0.5	0.1	1	1	2.62	0.94
5	10	3	4	0.1	3	5	1.2	0.16
6	40	0.5	4	0.5	1	5	3.02	0.98
7	40	0.5	4	0.5	3	1	3.14	1.04
8	10	3	0.5	0.5	3	1	2.48	0.92
9	10	3	4	0.5	1	1	3.16	1.22
10	40	3	4	0.1	1	1	2.98	1.04
11	10	0.5	0.5	0.5	1	5	1.9	0.7
12	40	0.5	0.5	0.1	3	1	2.6	0.98

It was found that Factors (A-F) were screened-out the few important main effects that were affected on both responses of DCW and PHAs. A range obtained for DCW and PHAs production were 0.96-3.16 and 0.16-1.22 g/L. Regression coefficient, F-values and p-values were analysed from 6 factors (See Table 4.7). It was found that total sugar, KH_2PO_4, $MgSO_4\,7H_2O$ and Urea had a

positive effect on DCW production. This is reasonable because these factors are served for energy and co-factors of cell mechanisms. The strain produced PHAs under excess carbon source and nitrogen sufficient that could consider to be a growth associated product. On the other hand, Na_2HPO_4 and trace element showed negative results. It may be due to the SSJ contained of high Na (See Table 4.2) and enough for their growth. A solution of Na_2HPO_4 could be acted as a buffer that is very important for PHAs accumulation (Mokhtari-Hosseini et al., 2009). Whereas the negative effects of KH_2PO_4, $MgSO_47H_2O$, and trace element on PHAs production were also found in this case. These could be explained as a similar way as above that both of K and Mg in the SSJ contained in a high concentration (See Table 4.2).

Table 4.7 Analysis of Plackett-Burman design results for the growth media of *B. aryabhattai*

Factors	DCW			PHAs		
	F-values	Coefficient	p-values Prob > F	F-values	Coefficient	p-values Prob > F
A-Total sugar	42.25	1.26	0.0013	19.06	0.47	0.0072
B-KH_2PO_4	0.10	0.06	0.7668	1.94	-0.15	0.2222
C-Na_2HPO_4	0.32	-0.11	0.5984	0.15	0.04	0.7146
D-$MgSO_4$ $7H_2O$	2.19	0.29	0.1989	0.10	-0.03	0.7693
E-Urea	10.77	0.64	0.0219	12.03	0.37	0.0179
F-Trace element	2.93	-0.33	0.1476	0.44	-0.07	0.5350

In Table 4.7, high F-values and confidence levels that were higher than 95% ($p \leq 0.05$) of total sugar and urea are determined as significant factors. Then, they were taken to optimise media for improving both in DCW and PHAs production of *B. aryabhattai*. The insignificant factors including KH_2PO_4, Na_2HPO_4, $MgSO_4$ $7H_2O$, and trace element were maintained at constant levels.

4.3.5 Media optimisation by response surface methodology (RSM)

Based on Plackett-Burman design, insignificant factors of KH_2PO_4, Na_2HPO_4, $MgSO_4$ $7H_2O$ and trace element were maintained at 1 g/L, 4 g/L, 0.1 g/L

and 1mL/L combined with different concentrations of influent factors designated from two affected factors of total sugar and urea (Table 4.8). Five levels CCD with 13 runs are shown in Table 4.9. The results were analysed and predicted by Design Expert software. Quadratic regression equations which were derived in term of DCW and PHAs production were obtained as follows:

$$DCW = +6.36 - 1.19A - 0.95B + 0.38AB - 2.39A2 - 2.21B2 \quad (1)$$

$$PHAs = +2.21 - 0.44A - 0.45B + 0.53AB - 0.86A2 - 0.81B2 \quad (2)$$

The statistical significance of the model equations were determined by the F-test for analysis of variance (ANOVA) as showed in Table 4.10. Eq. (1) of DCW revealed the regression was statistically significant at 95% ($p \leq 0.05$) confidence level and Eq. (2) showed that the p-value was less than 0.0001 and indicated that model term was highly significant (Pandian, Deepak, Kalishwaralal, Rameshkumar, Jeyaraj, & Gurunathan, 2010). The coefficient of determination (R^2) in both DCW and PHAs production model were 0.9916 and 0.9814. They showed in a good adjustment of the model to the experimental data. The R^2 almost reach to 1.0, the stronger the model and the better predicted responses. Furthermore, the lack-of-fit test of both models was insignificant, suggesting that the model represented experimental data adequately. The three-dimensional response surfaces drawn based on the graphical representations of the regression equation are shown in Figure 4.1. The 3D response surface plot illustrated that as the DCW increased (4.1a), increasing urea or total sugar concentration areas were increased and trended to its peak, unless decreased with a further increase in urea or total sugar concentration. In the same way, total sugar and urea concentration have a positive influence on PHAs production (4.1b) as well.

Table 4.8 Factor levels for central composite design of the growth media for *B. aryabhattai*

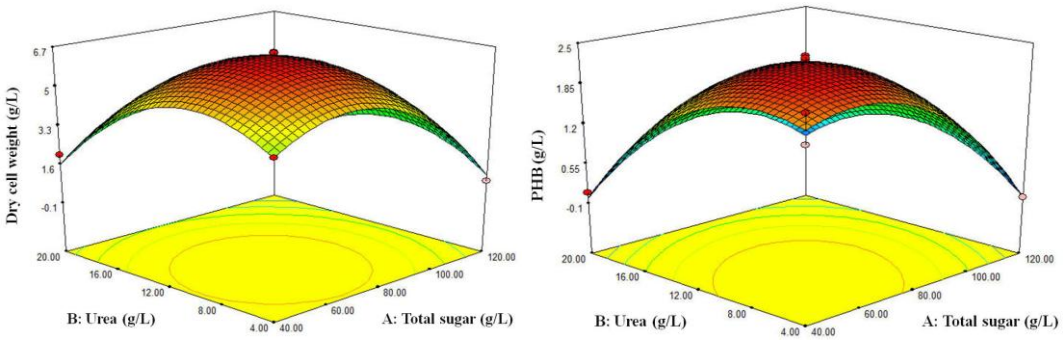
Factors (g/L)	Code levels				
	-α(-1.414)	-1	0	+1	+ α(+1.414)
Total sugar	23.43	40	80	120	136.57
Urea	0.69	4	12	20	23.31

Table 4.9 The 13 runs of central composite design for dry cell weight and PHAs production

Runs	Total sugar (g/L)	Urea (g/L)	Dry cell weight (g/L)	PHAs (g/L)
1	40	20	2.05	0.074
2	23.43	12	2.98	0.88
3	120	4	0.89	-
4	80	12	6.46	2.27
5	80	12	6.48	2.30
6	80	0.69	3.48	1.07
7	80	12	6.44	2.22
8	80	23.31	0.22	0.012
9	80	12	6.43	2.24
10	120	20	0.14	-
11	80	12	5.98	2.01
12	40	4	4.3	2.20
13	136.57	12	-	-

Table 4.10 Analysis of variance (ANOVA) for response surface quadratic models

Sources	DCW		PHAs	
	F-values	p-values	F-values	p-values
Models	164.536	< 0.0001	73.693	< 0.0001
A-Total sugar	110.738	< 0.0001	43.873	0.0003
B-Urea	70.554	< 0.0001	46.498	0.0002
AB	5.482	0.0517	32.036	0.0008
A^2	387.33	< 0.0001	147.398	< 0.0001
B^2	331.19	< 0.0001	130.68	< 0.0001
Lack of Fit	3.985	0.1075	4.916	0.079



(a) (b)

Figure 4.1 Response surface plot for the effect of total sugar of sweet sorghum juice and urea on dry cell weigh (1a) and PHAs production (1b)

Design Expert software was predicted as follows: total sugar 70.57 g/L and urea 9.37 g/L combined with rest factor of KH_2PO_4 1 g/L, Na_2HPO_4 4 g/L, $MgSO_4$ $7H_2O$ 0.1 g/L and Trace element 1mL/L. For the actual DCW and PHAs concentration reached at 6.22 and 2.43 g/L. While, the optimised DCW and PHAs production were predicted at about 6.61 g/L of 2.36 g/L. Thus, the validation of the predicted models was achieved at 94% and 103 % for DCW and PHAs production. It was acceptable agreement on experimental and implies that the mathematical models are very reliable for both cell growth and PHAs production by *B. aryabhattai* PKV01.

4.3.6 PHAs production in 3 L bioreactor

The bacterial growth and sugars consumption in 3 L batch fermentation of the SSJ by *B. aryabhattai* PKV01 during the time course of 72 h are represented in Figure 4.2. The PHAs production by this strain showed highly significant difference between flask scale and 3 L bioreactor. The results indicated that DCW (10.38 g/L), PHAs concentration (4.36 g/L) and PHAs (42 % (w/w)) were increased significantly after 72 h fermentation. In this period, sucrose was fully consumed while glucose and fructose concentration were slightly consumed by this strain within 24 h. Interestingly, the increasing of glucose and fructose in fermentation broth indicated that the strains could rapidly convert sucrose to glucose and fructose for growth and synthesized PHAs in their cells. There is no one reported that the *B. aryabhattai* PKV01 to be a natural PHAs producer. However, other *Bacillus* strains had been reported to be PHAs producers. The PHAs yields varied from 11 to 69 % (w/w) (Singh et al, 2009). Vallapi et al. (2007) reported that *Bacillus cereu*s SPV was found to produce PHB at a concentration of 38% of its dry cell weight. Yilmaz, Soran, and Beyatli (2005) also reported that 29 strains of the genus *Bacillus*, isolated from different soil samples**,** produced PHB ranged from 1.06–41.67% (w/v) depending on the dry cell weight. Wu, Huang, Hu, Chen, Ho, and Chen (2001) reported that a strain of *Bacillus* sp. JMa5, isolated from molasses contaminated soil, accumulated 25-35% (w/w) PHB. In addition, the higher PHB contents were obtained from works of Thirumala, Reddy, and Mahmood (2010) (70.04% w/w) and Yüksekdag et al. (2004) (78.69% w/w).

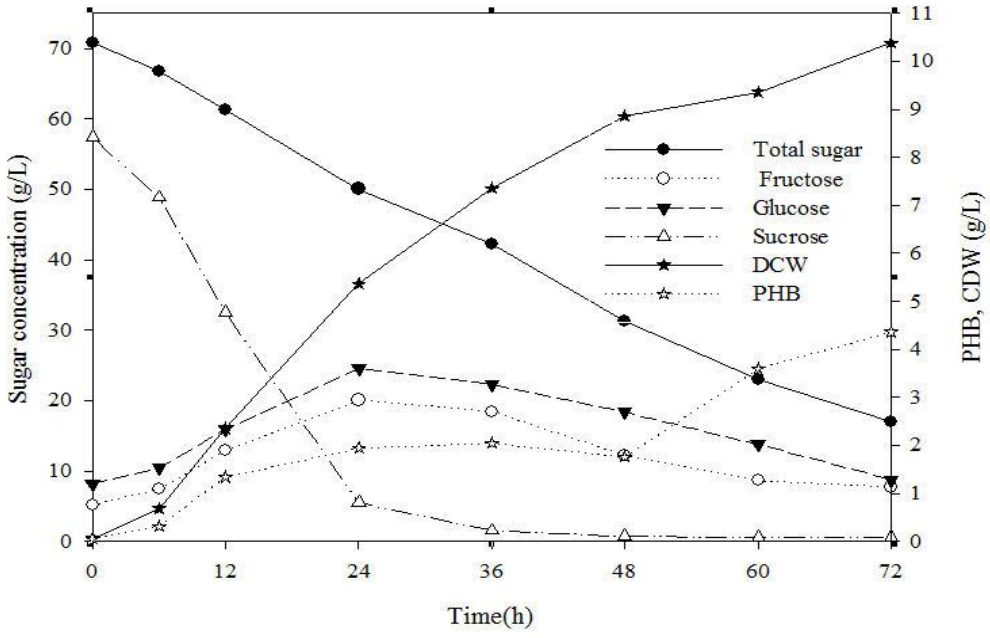


Figure 4.2 Batch kinetic of *B. aryabhattai* in 3 L bioreactor using sweet sorghum juice as a sole carbon source

Table 4.11 Comparison of PHAs production between predicted by software, shake flasks and fermenter by *B. aryabhattai* PKV01

Scales	Biomass (g/L)	PHAs (g/L)	PHAs content (%)
Prediction	6.61	2.36	35.70
Flask	6.22	2.43	39.07
3 L fermenter	10.38	4.36	42.00

Comparing, the results of shaking flask and bioreactor (Table 4.11), the DCW and PHAs production were significantly increased in the fermenter and greater than that as found in shaking flask at about 1.8 folds. That was similar to Khanna and Srivastava (2005) who reported that a maximum DCW of 13.39 g/L with PHB 6.75 g/L was obtained in shaking flask. A significantly increased DCW of 20.73 g/L with a PHB content of 9.35 g/L was obtained in a 7 L lab scale bioreactor. It would conclude that the mass and oxygen transfer in bioreactor were better than those in the flask and allowed in parallel of cell growth and PHAs production. Thus, PHAs production was

observed at a higher level. Although previous studies (Faccin, Martins, Cardozo, Rech, Ayub, & Alves, 2009; Pandian et al., 2010) who reported that biopolymer productions by *Bacillus megaterium* in large scale bioreactor were decreased when the production was extended to bioreactor. In contrast, the result obtained in this study, it is stated that the production of PHAs in a large scale would not an effect by this strain.

4.3.7 Characterisation of PHAs production from SSJ

The PHAs film was further characterised using 400 MHz of ^{1}H-NMR and ^{13}C-NMR techniques. The spectrum of ^{1}H-NMR obtained is shown in Figure 4.3. Three groups of signals peak at 1.25, 2.52 and 5.23 ppm were found and corresponding to C-H_3, C-H_2 and C-H, respectively. The chemical shift signals of ^{13}C-NMR spectrum of PHAs produced were at 19.72 (C-H_3), 40.75 (C-H_2), 67.58 (C-H), and 169.10 (C=O), respectively (See Figure 4.4). This in agreed with previous works (See Table 4.12). The results obtained in both techniques are corresponded to the different types of carbon atoms presented in the PHB structure, [-O-CH-(CH_3)-CH_2-(C=O)-]$_n$. There is no surprise that PHAs produced from the SSJ was mainly dominant in form of PHB. Previous reports stated that even the PHAs production from such a kinds of sugar as a carbon source, the products obtained were dominant in form of homopolymer of PHB by various bacteria (Khanna & Srivastava, 2005).

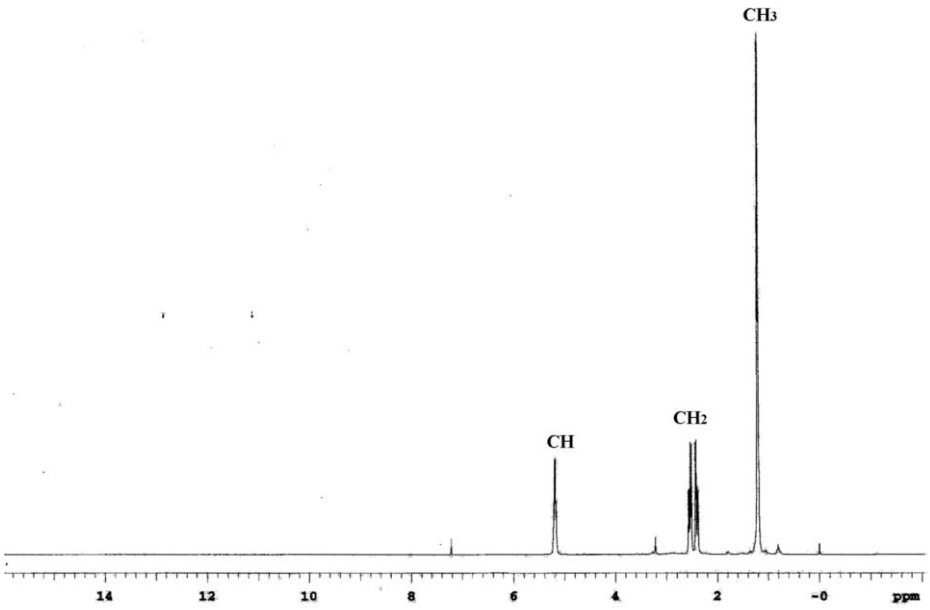


Figure 4.3 400 MHz ^{1}H NMR spectrum of PHB extracted from *B. aryabhattai* PKV01 using the SSJ as a sole carbon source

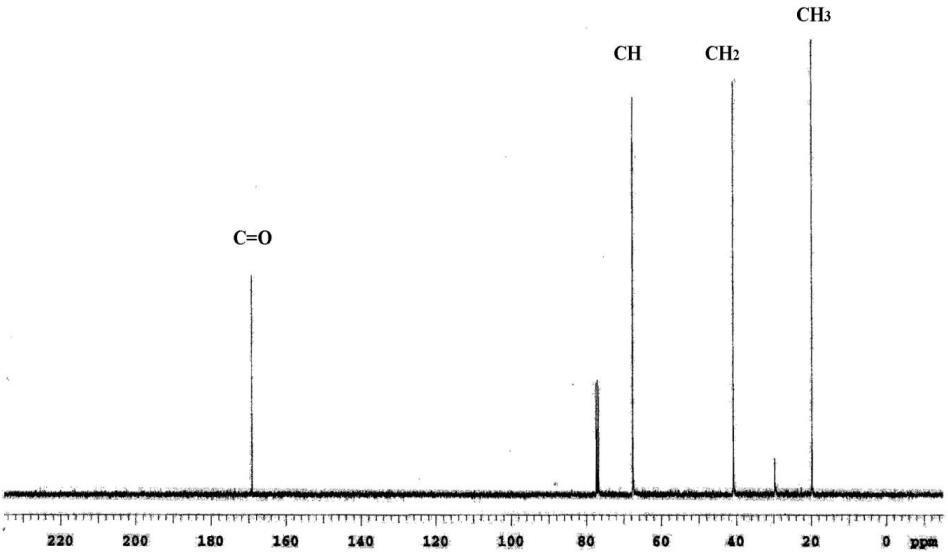


Figure 4.4 ^{13}C NMR spectrum of PHB extracted from *B. aryabhattai* PKV01 using SSJ as a sole carbon source

Table 4.12 The ^{13}C NMR spectra for PHB extracted from *B. aryabhattai* PKV01 compared to the PHB from other works

C atom	Chemical shift (ppm)					
	PHB_{SSJ} (This study)	PHB (Sindhu et al. 2011)	PHB (Tanamool et al. 2011)	PHB (Jiang et al. 2008)	PHB (Oliveira et al. 2007)	PHB (Doi et al. 1989)
CH_3	19.73	19.74	19.72	19.77	19.66	19.76
CH_2	40.75	40.76	40.75	40.80	40.68	40.77
CH	67.58	67.63	67.58	67.62	67.49	67.40
C=O	169.12	169.21	169.10	169.14	169.01	169.14

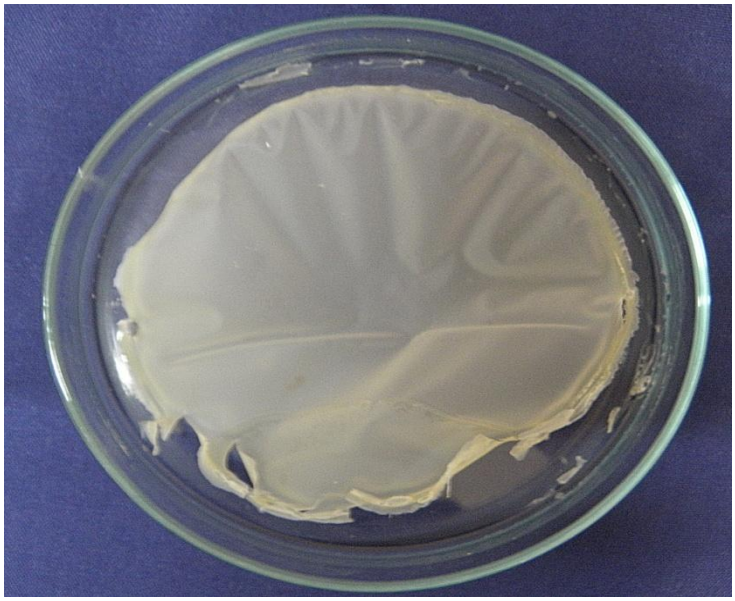


Figure 4.5 Solution cast PHB film produce by *B. aryabhattai* using sweet sorghum juice as a sole carbon source

A thin bacterial PHB film was obtained and illustrated in Figure 4.5. The DSC results showed that, melting temperature (T_m) and glass transition temperature (T_g) were obtained at 167.30 and 1.11°C (See in Figure 4.6). They showed in lower than the standard PHB (176.29°C and 2.81°C). For TGA

measurement are shown in Figure 4.7. The bacterial PHB gave a rapid thermal degradation between 230 and 400°C while the standard PHB represented at between 255 and 293°C. It should be remarked that there were some small peak found in Figure 4.6 while the two steps degradation curve was also existed (Figure 4.7). It perhaps the bacterial PHB was not purity according to the recovery process.

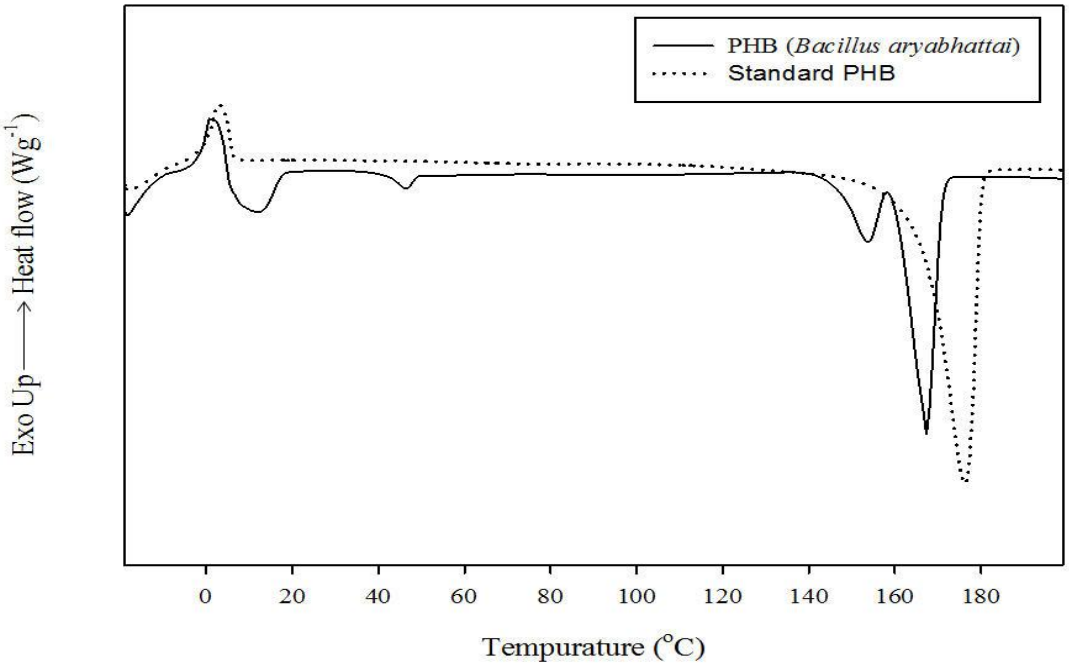


Figure 4.6 DSC thermograms of pure PHB and PHB produce by *B. aryabhattai*

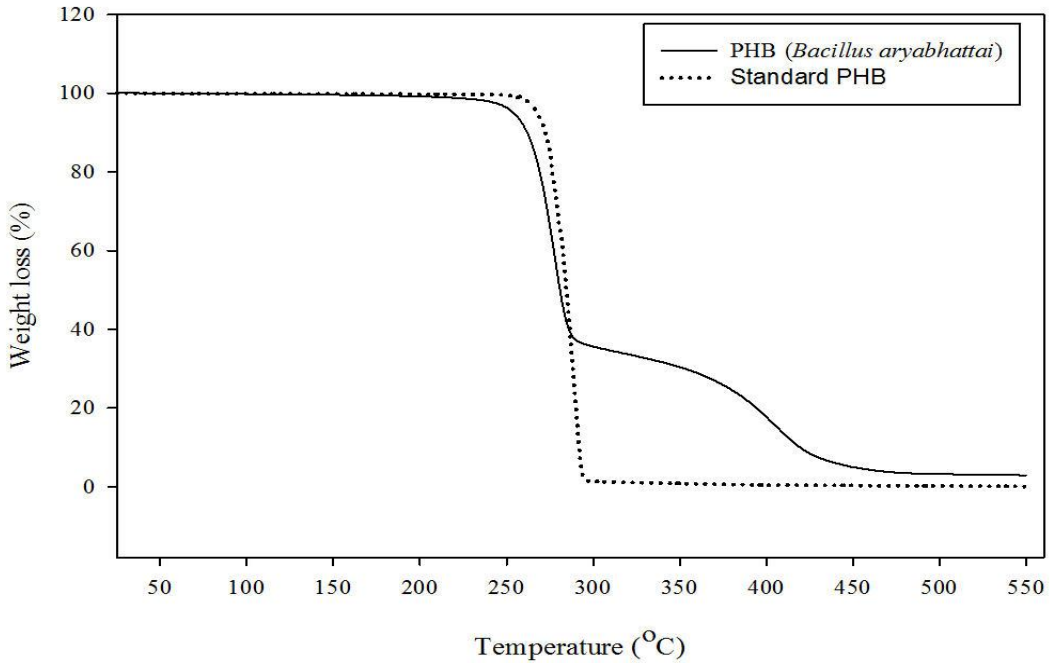


Figure 4.7 TGA curves of pure PHB and PHB produce by *B. aryabhattai*

4.4 Conclusion

Successfully demonstrating use of the SSJ as a carbon source for a newly isolated strain *B. aryabhattai* PKV01 is possible. There are no reports for the production of PHAs from the SSJ by *B. aryabhattai*, which in turn the bioconversion of C4 plant into PHAs production. Considering the cost of PHAs production, cheap carbon and nitrogen sources from the SSJ and urea could replace others carbon and nitrogen sources that are more expensive. This leads to reduce the cost of production. The statistical methodology was obviously improved for the process optimisation of PHAs production from the SSJ. Apart from this work, we believed that the SSJ showed a great potential to use as the sole carbon source for PHAs production in an industrial scale in the future.

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