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RESEARCH PAPER

An Alternative Approach to the Fermentation of Sweet Sorghum Juice into Biopolymer of Poly-β-hydroxyalkanoates (PHAs) by Newly Isolated, *Bacillus aryabhattai* PKV01

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Abstract This work revealed for the first time the possible use of a newly isolated *Bacillus aryabhattai* PKV01 for poly-β-hydroxyalkanoates (PHAs) production from fermentative sweet sorghum juice. Its growth and PHA production were investigated under different pH and nitrogen sources. Medium composition was optimized using statistical tools. The highest biomass and PHA content were reached at pH 6.5 with the use of urea. Plackett-Burman design was then applied to test the relative importance of medium components and process variables on cell growth and PHA production. Cell growth and PHAs production were affected by total sugar and urea and were subjected to optimize the sorghum juice medium using response surface methodology (RSM) *via* central composite design (CCD). The predicted optimal culture composition was achieved. Maximum dry cell weight and PHAs were obtained using a flask and almost double the amount was achieved using a bioreactor. After PHA recovery, the structure and thermal properties were characterised and revealed to be similar to the standard of poly-β-hydroxybutyrate (PHB).

Keywords: sweet sorghum juice, *Bacillus aryabhattai*, biopolymer, PHAs, RSM

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1. Introduction

There have been serious concerns on the accumulation of petroleum-based plastic waste in the environment. This increase of non-biodegradable plastic waste does not only bring negative impacts on the quality of human life but also on animals as well as single cell lives because of their persistence in the environment. Several communities are now more confronting to the impact of discarded plastic on the environment and searching for new resources of degradable polymers. Nowadays, a wide variety of bio-based polymers are produced worldwide. Among various types of biodegradable plastic, a class that is drawing considerable attention is poly-β-hydroxyalkanoates (PHAs), intracellular storage compounds that are accumulated as discrete granules in the cytoplasm of cells as carbon and energy reserves in several kinds of microorganisms but mostly in bacteria. PHAs are biodegradable thermoplastic polyesters [1] that are compatible with mammalian cells [2] and have wide physical properties similar to synthetic polypropylene. The application of PHAs as biodegradable plastics have been widely performed for various 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 processes including carbon source, fermentation process as well as downstream processes [3]. Carbon

source alone is reported to cost up to 50% of the overall production costs of PHAs [4]. Thus, inexpensive carbon sources such as agro-industrial raw materials, by-products, wastes and even other cheap renewable sources could contribute to reduce as much as 40 ~ 50% of the overall production costs [5,6].

Previously, various renewable cheaper substrates were used for PHA production such as carbon dioxide [7], molasses [8,9], methanol [10,11], maple sap [12] sucrose [13], and recently, sugar cane [14]. In addition to these, a new type of sugar plant of sweet sorghum appears to be an alternative substrate for PHA production due to several advantages including low price, short growth period of approximately 3-4 months, easy acclimation to sub-tropical and temperate regions of the world, water efficient [15] and high total sugar content in the forms of sucrose and finally presence of glucose and fructose in small amounts. With these properties in hand, this plant would be an attractive alternative carbon source medium for microbial growth to produce added value products such as ethanol [16-18], hydrogen [19], lactic acid [20] and unsaturated fatty acids like docosahexaenic acid (DHA) [21]. However, only our group has paid attention and previously reported the production of PHAs by a Gram-negative bacterium, *Alcaligenes eutrophus* TISTR 1095, from sweet sorghum juice (SSJ), although it gave a low production yield [22]. Since then, we have tried to screen novel bacterial strains that are able to grow in SSJ. A Nile blue A was directly added into sweet sorghum agar medium and their growth was observed under UV light [23]. The results showed that the isolate strain S4 best produced PHAs compared to other strains. This strain was further identified and identified as *Bacillus aryabhattai* [24]. In addition, this strain, screened from sugar cane plantation soil, can produce PHAs and can grow well in SSJ medium. However, the kinetics of production had never been studied. Previous researchers have described the benefit of using Gram-positive bacteria instead of Gram-negative bacteria [24-26]. Gram-positive bacteria are advantages because various agricultural raw materials can be utilized as carbon sources for the production of different metabolites and produced lack of lipopoly-sacharide, an endotoxin pryogenic in humans [26]. This enzyme is undesirable when PHAs are needs for biomedical applications. Hence, the Gram-positive strain *Bacilli* was recommen-ded and used as bacterial culture for PHA production.

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

2. Material and Methods

2.1. Microbial strain

The PHA producing strain *Bacillus* sp. was isolated from sugar cane plantation soil in the north eastern area of Thailand. The strain used throughout for this study was recently identified and closely related to *B. aryabhattai* (99.7% similarly) [24]. Stock cultures maintained their growth at 30°C innutrient agar (NA) containing beef extract (0.3%), peptone (0.5%) and agar (15%) and were kept at 4°C prior to use.

2.2. Raw material

Sweet sorghum KKU 40 (Sorghum bicolor L. Moench), a 3 harvesting annual crop and yields large of sugar in the stems, was used as a carbon source. Typically, it mainly consists of sucrose (up to 55% of dry weight biomass), fructose and glucose. Sweet sorghum KKU 40 used in this study 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 a cotton sheet. The SSJ was then kept at -20°C to prevent microbial contamination.

2.3. Media and culture condition

2.3.1. Effect of different nitrogen sources on growth and PHAs production

Ammonium chloride, ammonium sulphate, urea, peptone and yeast extract as different nitrogen sources were investigated by adding into mineral salt medium which consisted of 20 g/L of total sugars (sucrose, fructose andglucose) in SSJ. Other supplements were prepared bythe method of Grothe *et al.* [27] using 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) as different nitrogen sources (1 g/L), respectively. After incubation for 24 h at 30°C and 200 rpm, the culture was withdrawn and biomass and PHA content were estimated.

2.3.2. Effect of different initial pH on cell growth and PHAs production

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 SSJ medium were investigated on cell growth and PHA production. The culture was maintained at a desired value by adding 6 N NaOH or HCl. After fermentation, Dry cell weight (DCW) and PHA concentration were determined and the optimal pH obtained was used for further studies.

Statistical analysis data are presented as the mean of three independent repeats and were analyzed by ANOVA, using $p \leq 0.05$ as the level of significant difference.

2.4. Plackett–burman optimization design

In this study, the statistical tool of Plackett-Burman's design was preliminary used to screen medium culture used in fermentation. Six main factors, total sugar (A), KH_2PO_4 (B), Na_2HPO_4 (C), $MgSO_4$ 7 H_2O (D), urea (E), and trace element solution (F), were screened earlier. A design of 12 experiments was formulated for six factors using 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 on PHA production [8,9,27,28]. Experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of media at 30°C, 200 rpm for 72 h in duplicate. Responses were measured in terms of DCW (G) and PHA(H) production. DCW and PHAs obtained from all experiments were subjected to compatible analysis. Components that gave a highly significant ($p \leq 0.05$) effect on the responses were taken into consideration by Response Surface Methodology (RSM).

2.4.1. Response surface methodology (RSM)

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

2.4.2. Batch fermentation in 3 L bioreactor

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

2.5. Analytical techniques

2.5.1. Determination of sugar concentration and other components

The SSJ was characterised in terms of glucose, fructose and sucrose using High Performance Liquid Chromatography (HPLC) (Shimadzu, Japan) equipped with a refractive index detector (RID) using a Vertical GES-NH_2 HPLC column (4.6 × 250 mm, 5 μm) and 70% acetonitrile in double distilled water as the mobile phase. The flow rate and the injection volume were constantly controlled at 1 mL/min and 20 μL. Sugarconcentrations including glucose, fructose, and sucrose werecalculated from calibration curves. Otherminerals (Na, K, Mg, Ca, and Zn) were estimated using a Perklin Elmer atomic absorption spectrophotometer (Model 2380, England) as described in the AOAC (1998). In addition, organic nitrogen concentration was measured using the total Kjeldahl nitrogen method (TKN).

2.6. DCW (biomass) measurement

After fermentation, the culture broth was centrifuged at 15,000 × *g* for 10 min and the supernatant was discarded. The pellets were washed with distilled water twice and dried in a hot air oven at 60°C until constant weight and biomass were obtained.

2.7. PHAs recovery and quantification

PHAs were then extracted directly from wet cell pellets. In brief, wet cells were disrupted by 6% sodium hypochlorite (commercial grade bleach) at 37°C for 1 h. White pellets were collected after centrifugation and then washed with distilled water and a mixture of acetone: ethanol (1:1). When a soxlhet apparatus was built, the pellets was transferred and 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 was achieved. PHAconcentration was determined by the method of Law and Slepeky [29].

2.8. PHAs characterisation

The extracted PHAs were characterised for their thermal properties through differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) while their structures were measured using the Nuclear Magnetic Resonance (NMR) technique. Following this, they were compared to the polymer standard in terms of poly-β-hydroxybutyrate (PHB) (Sigma-Aldrich, USA). It was noted that PHAs are mostly found in the form of PHB. For DSC and TGA measurements, we followed the method by Tanamool *et al.* [13].

3. Results and Discussion

3.1. Sweet sorghum juice (SSJ) composition

Composition of SSJ is shown in Table 1. SSJ contained a high total sugar content in the form of sucrose and in small amounts, glucose and fructose. In the present study, SSJ contained 175.97, 12.32, and 5.75 g/L of sucrose, glucose

Table 1. Comparison of SSJ composition from various crop locations

Crop location	Harvest month	Sugar compositions (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	[21]
Thailand	NA	124.10	20.90	16.80	161.80	[18]
Hungary	September	75.10	25.00	18.10	118.20	[17]
Thailand	December	124.05	14.22	11.46	149.73	[22]
Greece	November	211.90	20.10	-	232.00	[16]

Table 2. The various elements content in sweet sorghum stem juice

Component	Concentration (mg/L)		
	This study	Laopaiboon *et al*. [18]	Massoud and Abd El-Razek [43]
N	410*	21.4	N.D.
K	5,175	1,790	3,028
Ca	639.3	166	66.5
Mg	396	194	N.D.
Na	200	170	2,710
Zn	0.31	1.4	N.D.

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

Table 3. DCW and PHAs concentration obtained from different nitrogen sources

Nitrogen sources	DCW (g/L)	PHAs (g/L)	PHAs content (%)
Control*	1.00^{a}	0.40^{a}	40.00^{a}
Urea	4.0^{e}	2.10^{f}	52.50^{b}
Peptone	3.40^{d}	1.80^{d}	52.90^{b}
Yeast extract	3.50^{d}	1.90^{e}	54.30^{b}
NH_4Cl	3.00^{c}	1.20^{c}	40.00^{a}
$(NH_4)_2SO_4$	2.50^{b}	1.00^{b}	40.00^{a}

Data are the mean derived from three independent repeats. Means with different superscript letters (a – f) within the same column are significantly different at the $p \leq 0.05$ level (ANOVA Test).
*Control, 2% sweet sorghum stem juice without addition of nitrogen source.

and fructose, respectively. The results are in agreement and are comparable to previous studies [16-18,22] thatreported varying sugar contents in SSJ. The order of sugar contents found in SSJ wassucrose glucose fructose, which is consistent with the results reported by Liang *et al.* [21]. Difference in observation may be found due to variety, location, and harvest time. In addition, the first two factors are clearly understandable; however, harvest time has also been shown to be a critical factor for sugar composition [21]. Other trace elements found in SSJ are also shown in Table 2. Minerals such aspotassium (P) and calcium (Ca) were dominant followed by nitrogen (N), magnesium (Mg), sodium (Na) and zinc (Zn), respectively.

According to the composition of SSJ shown in Tables 1 and 2, we made an assumption that SSJ may contain suitable components for microbial growth and such microbes could directly utilize the sugars in SSJ without requiring pretreatment processes such as hydrolysis. High total sugar concentration is one of the factors for increasing efficient fermentation. However, some elements still needed to be added into the SSJ based medium (*e.g.* nitrogen) since a lower concentration appeared when the SSJ was diluted to the desired condition.

3.2. Influence of nitrogen sources on PHAs production

Nitrogen plays a major role in the metabolism of microorganisms. Cells need nitrogen for formation of its structures. With regard to PHA production, the type of nitrogen sources also had an effect on PHA accumulation in the cells. Table 3 shows the DCW, PHA concentration and PHA content obtained from 5 tested nitrogen sources. The highest DCW (4.0 g/L) and PHA concentration (2.10 g/L) were obtained with the use of urea was. Considerable, maximum PHAs content of 54.3% (w/w) was achieved when yeast extract was used as nitrogen source. Previously, Yüksekdag *et al*. [30] reported that complex nitrogen sources (protease peptone) increased the yield of PHAs in *Bacillus megaterium*. Kulpreecha *et al.* [9] reported that urea was found to be a better nitrogen source than $(NH_4)_2SO_4$ for both cell growth and PHB content in the cells of *Bacillus megaterium*. In agreement with these studies, the maximum production of DCW and PHAs were obtained when urea was used as a nitrogen source. Because urea is an uncharged polar and small molecule in contrast to other simple inorganic nitrogen sources including NH_4Cl and $(NH_4)_2SO_4$, easier uptake into the cell membrane was possible compared to $(NH_4)_2SO_4$ [9]. Moreover, urea is a cheaper nitrogen source than others sources of interest. For this reason, urea is a possible choice as a nitrogen source for reducing the production cost of PHAs.

3.3. Effect of pH on PHAs production

The influence of different initial pH of culture medium on

Table 4. PHAs and DCW obtained with different initial pH

pH	DCW (g/L)	PHAs (g/L)	PHAs content (%)
5.0	2.10^{b}	0.70^{d}	33.33^{c}
5.5	1.80^{a}	0.60^{c}	33.33^{c}
6.0	2.20^{b}	0.90^{e}	40.91^{d}
6.5	2.60^{c}	1.10^{f}	42.31^{d}
7.0	2.80^{d}	1.10^{f}	39.28^{d}
7.5	2.70^{cd}	0.50^{b}	18.52^{b}
8.0	2.70^{cd}	0.50^{b}	18.52^{b}
8.5	2.70^{cd}	0.40^{a}	14.81^{a}
9.0	3.00^{e}	0.50^{b}	16.67^{ab}

Data are the mean derived from three independent repeats. Means with different superscript letters (a – f) within the same column are significantly different at the $p \leq 0.05$ level (ANOVA Test).

Table 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

DCW and PHA production were investigated. The results are summarized in Table 4, which showed that *B. aryabhattai* PKV01 could grow at a wide range of initial pH. The maximum DCW of 2.8 g/L was obtained in SSJ medium with an initial pH of 7.0. Palleroni and Palleroni [31] have also reported that pH in the range of 6.0 ~ 7.0 was best for microbial growth. This is in agreement with the present case; the maximum PHA production of 1.1 g/L was obtained in the SSJ medium at both initial pH of 6.5 and 7.0. Considering the PHA content, the highest PHA accumulation reached 42.31% (w/w) at an initial pH of 6.5. On the other hand, Grothe *et al.* [27], reported that an initial pH of 6.5 produced the best results in both DCW yield and PHB production. In addition, previous studies [32,33] stated that an initial pH of about 6.4 was suitable for PHA accumulation with the *Bacillus* strain. Hence, an initial pH value of 6.5 was chosen for this study.

3.4. Placklett-burman design

The six main factors that affected the experiment at two levels, high (+1) and low (1), are shown in Table 5. All 12 experiments designed by Design Expert software are listed in Table 6.

It was found that factors (A-F) were screened and found that affected on both response of DCW and PHAs. A range obtained for DCW and PHA production was 1.09 ~ 5.88 and 0.07 ~ 2.20 g/L, respectively. Regression coefficient, *F*-values and *P*-values were analysed from 6 factors (see Table 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 part of energy and co-factors of cell mechanisms. The strain produced PHAs in the exponential phase under excess carbon source and sufficient nitrogen that could be considered to be a growth associated product. On the other hand, Na_2HPO_4 and trace elements showed negative results. It may be due to the SSJ containing high Na (see Table 2) which was enough for their growth. A solution of Na_2HPO_4 could act as a buffer which is very important for PHAs accumulation [11]. Whereas, the negative effects of KH_2PO_4, $MgSO_4 7H_2O$, and trace elements on PHA production were also found in this case. This could be explained in a similar way as above which showed that both of K and Mg were contained in

Table 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	1	4	0.1	4	5	1.34	0.48
2	10	1	1	0.4	2	5	1.09	0.27
3	40	1	4	0.4	2	5	3.93	1.01
4	40	1	4	0.4	4	1	5.88	2.10
5	40	1	1	0.1	4	1	5.55	2.20
6	10	4	4	0.1	4	5	2.69	1.11
7	40	4	1	0.1	2	5	3.59	0.76
8	40	4	4	0.1	2	1	3.26	0.81
9	10	4	4	0.4	2	1	2.46	0.07
10	10	4	1	0.4	4	1	3.00	0.41
11	10	1	1	0.1	2	1	2.06	0.17
12	40	4	1	0.4	4	5	5.58	1.27

Table 7. Analysis of Plackett-Burman design results for the growth media of *B. aryabhattai* PKV01

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

Table 8. Factor levels for central composite design (CCD) of the growth media for *B. aryabhattai* PKV01

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 9. The 13 runs of CCD for DCW and PHAs production

Run	Total sugar (g/L)	Urea (g/L)	DCW (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	-	-

high concentrations in SSJ (See Table 2).

In Table 7, high *F*-values and confidence levels higher than 95% ($p \leq 0.05$) of total sugar and urea were determined as significant factors. These factors were then taken to optimize the media for improving both DCW and PHA production of *B. aryabhattai*. Insignificant factors including KH_2PO_4, Na_2HPO_4, $MgSO_4$ $7H_2O$, and trace element were maintained at constant levels throughout the experiment.

3.5. Media optimization by response surface methodology (RSM)

Based on the Plackett-Burman design, the insignificant factors KH_2PO_4, Na_2HPO_4, $MgSO_4$ $7H_2O$ and trace elements were maintained at 1, 4, and 0.1 g/L and 1 mL/L combined with different concentrations of influencing factors designated from two affected factors of total sugar and urea (Table 8). Five levels of CCD with 13 runs are shown in Table 9. The results were analysed and predicted by Design Expert software. Quadratic regression equations which were derived in terms of DCW and PHA production were obtained as follows:

$$DCW = +6.36-1.19A-0.95B+0.38AB-2.39A^2-2.21B^2 \quad (1)$$

$$PHA = +2.21-0.44A-0.45B+0.53AB-0.86A^2-0.81B^2 \quad (2)$$

The statistical significance of the model equations were determined by the *F*-test for analysis of variance (ANOVA) as shown in Table 10. Eq. (1) of DCW revealed that the regression was statistically significant at the 95% ($p < 0.05$) confidence level, and Eq. (2) showed that the *p*-value was less than 0.0001, indicating that the model term was highly significant [34]. The coefficient of determination (R^2) in both DCW and PHA production models were 0.9916 and 0.9814, respectively. This showed in good adjustment of the model to the experimental data. The R^2 almost reached 1.0, demonstrating a stronger model and 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 Fig. 1. The 3D response surface plot illustrated that as DCW increased (1A), urea or total sugar concentration areas were

Table 10. Analysis of variance (ANOVA) for response surface quadratic model

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

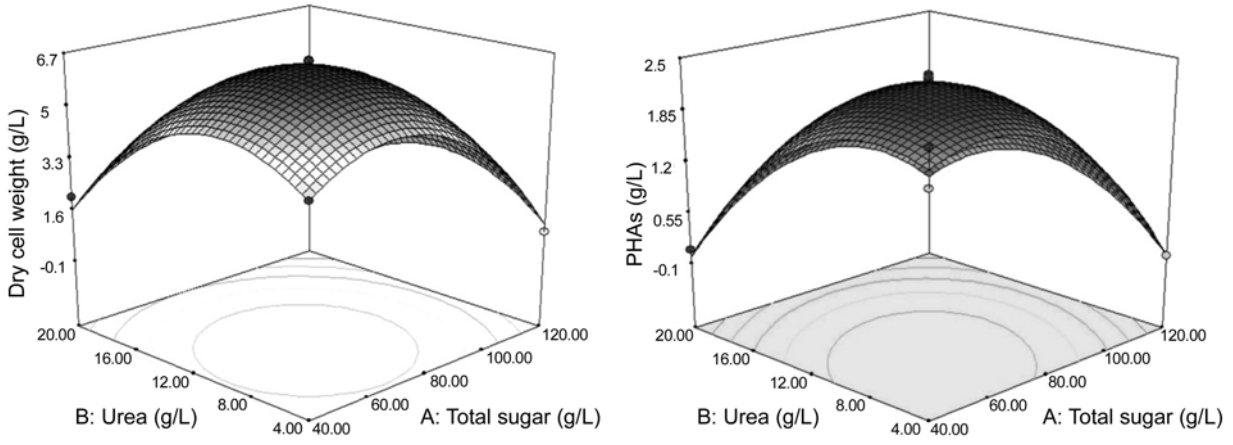

Fig. 1. Response surface plot for effect of total sugar of SSJ and urea on dry cell weight (1A) and PHAs production (1B).

increased and trended to its peak, unless DCW was decreased with further increase in urea or total sugar concentration. In the same way, total sugar and urea concentration had a positive influence on PHA production (1B). A significant increase of DCW and PHA production could be achieved when total sugar and urea were increased from 23.43 to 70.57 g/L and 0.69 to 9.37 g/L, then slightly decreased when both total sugar and urea concentrations were higher than 70.57 and 9.37 g/L, respectively, which indicated occurrence of substrate and nitrogen inhibition.

Maximum production was predicted in the medium as follows: total sugar 70.57 g/L and urea 9.37 g/L combined with the remaining factors of KH_2PO_4 1 g/L, Na_2HPO_4 4 g/L, $MgSO_4$ $7H_2O$ 0.1 g/L and trace element 1 mL/L. For the experimental values of DCW and PHAs concentration reached at 6.22 and 2.43 g/L. Whereas, optimized DCW and PHA production was 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 PHA production, respectively. These values are within acceptable experimental data and imply that the mathematical models are very reliable for both cell growth and PHA production using *B. aryabhattai* PKV01.

3.6. PHAs production in 3 L bioreactor

Bacterial growth and sugar consumption during 3 L batch fermentation of SSJ by *B. aryabhattai* PKV01forthe time course of 72 h are represented in Fig. 2. PHA production by this strain showed a highly significant difference between the flask scale and the 3 L bioreactor. The results indicated that DCW (10.38 g/L), PHAs concentration (4.36 g/L) and PHAs (42% (w/w)) were significantly increased after 72 h of fermentation. In this period, sucrose was fully consumed while glucose and frutose concentrations were slightly consumed by this strain within 24 h. Interestingly, the increase of glucose and fructose in the fermentation broth

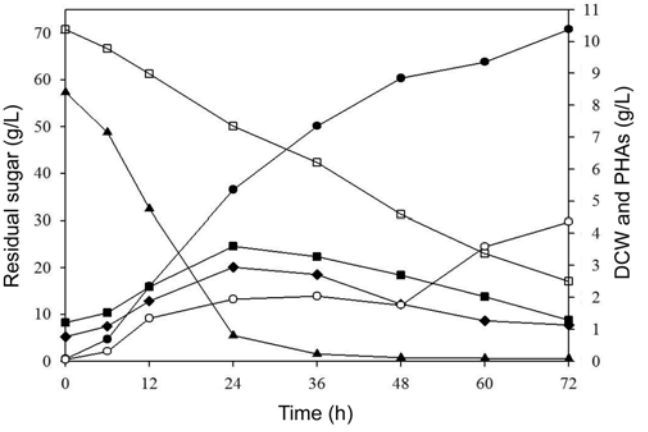

Fig. 2. Batch kinetic of *B. aryabhattai* PKV01 in 3 L bioreactor using SSJ as a sole carbon source (total sugar (□), sucrose (↖), glucose (⇦), fructose (■), DCW (↓), and PHAs (↓)).

indicated that the strain can be rapidly utilized sucrose to glucose and fructose for growth and synthesis of PHAs in their cells. No study has been conducted on *B. aryabhattai* PKV01 as a natural PHAs producer. However, other *Bacillus* strains had been reported as PHAs producers. PHAs yields varied from 11 to 69% (w/w) [26]. Vallapil *et al.* [25] reported that *Bacillus cereu*s SPV was found to produce PHB at a concentration of 38% of its dry cell weight. Yilmaz *et al.* [35] also reported that 29 strains of the genus *Bacillus*, isolated from different soil samples**,** produced PHB rangingfrom 1.06 ~ 41.67% (w/v) depending on the dry cell weight. Wu *et al.* [36] reported that a strain of *Bacillus* sp. JMa5, isolated from molasses contaminated soil, accumulated 25 ~ 35% (w/w) PHB. In addition, higher PHB contents were obtained byThirumala *et al*. [37] (70.04% w/w) and Yüksekdag *et al*. (78.69% w/w) [30].

The results from the shaking flask and the bioreactor showed that DCW and PHA production were significantly increased in the fermenter and was greater than that in the

shaking flask at about 1.8 fold. These results were similar to Khanna and Srivastava [28] who reported that a maximum DCW of 13.39 g/L with PHB 6.75 g/L was obtained in a shaking flask. 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. The results collectively suggest that the mass and oxygen transfer in the bioreactor were better than in the flask and allowed cell growth and PHA production at the same time. Thus, PHA production was observed at a higher level. Although previous studies [34,35,38] who reported that biopolymer production by *Bacillus megaterium* in a large scale bioreactor was decreased when the production was extended to a bioreactor. In contrast, the results obtained in this study show that the production of PHAs in a large scale is not an affected by this strain.

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. The spectrum of ^{1}H-NMR obtained is shown in Fig. 3A. Three groups of signal peak at 1.25, 2.52, and 5.23 ppm were found which corresponded to C-H_3, C-H_2, and C-H, respectively. The chemical shift signals of the ^{13}C-NMR spectrum of PHAs were at 19.72 (C-H_3), 40.75 (C-H_2), 67.58 (C-H), and 169.10 (C=O), respectively. (See Fig. 3B). This is in agreement with previous studies (See Table 11). The results obtained through both techniques corresponded to the different types of carbon atoms presented in the PHB structure, [-O-CH-(CH_3)-CH_2-(C=O)-]$_n$. It is not surprising that PHAs produced from the SSJ was mainly dominant in the form of PHB. Previous reports stated that even production of PHAs from such a kind of sugars as a carbon source, the products obtained were dominant in the form homopolymer of PHB by various bacteria [28].

The DSC results show that melting temperature (T_m) and glass transition temperature (T_g) of 167.30 and 1.11ºC, respectively (See in Fig. 4). These showed in the lower values than the temperatures measured from the standard of PHB (176.29 and 2.81ºC). Fig. 5 shows TGA measurements. Bacterial PHB gave a rapid thermal degradation between 230 and 400ºC while standard PHB was represented between 255 and 293ºC. It should be noted that some small peaks were found in Fig. 4 while the two steps degradation curve still existed (Fig. 5). Perhaps bacterial PHB was not pure according to the recovery process.

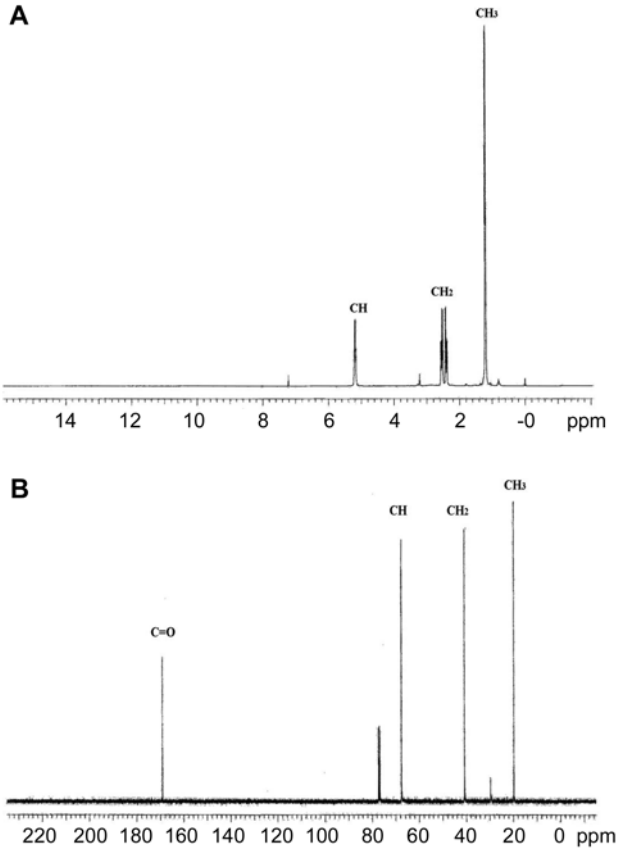

Fig. 3. ^{1}H NMR (3A) and ^{13}C NMR (3B) spectrum of PHB extracted from *B. aryabhattai* PKV01 using the SSJ as a sole carbon source.

4. Conclusion

We successfully demonstrated the use of SSJ as a carbon source for a newly isolated strain *B. aryabhattai* PKV01. There are no reports on the production of PHAs from SSJ

Table 11. The chemical shift signal obtained the ^{13}C NMR spectra for PHB extracted from *B. aryabhattai* PKV01 compared to the PHB from the other works

C atom	Chemical shift (ppm)					
	PHB_{SSJ} (This study)	PHB [39]	PHB [13]	PHB [40]	PHB [41]	PHB [42]
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

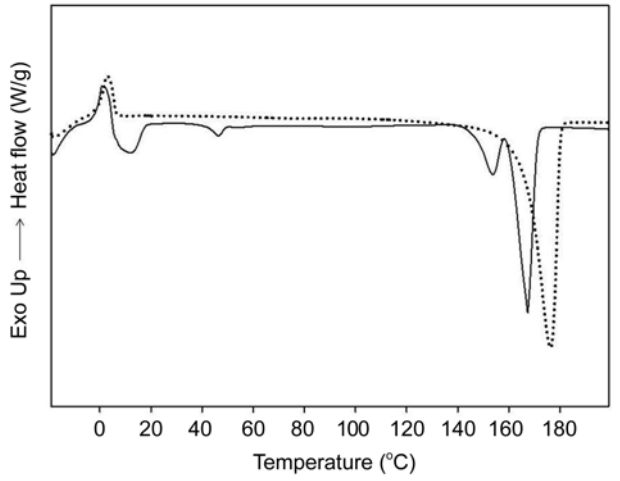

Fig. 4. DSC thermograms of standard PHB (····) and PHB produced by *B. aryabhattai* PKV01 (—).

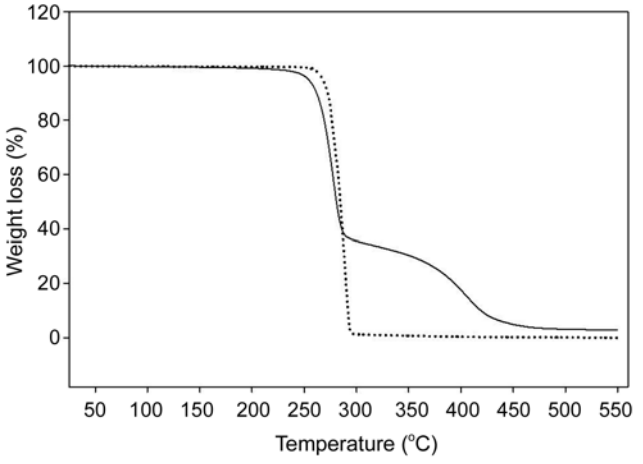

Fig. 5. TGA curves of standard PHB (····) and PHB produced by *B. aryabhattai* PKV01 (—).

by *B. aryabhattai*. Considering the cost of PHAs production, cheap carbon and nitrogen sources from the SSJ and urea could replace other more expensive carbon and nitrogen sources. This would lead to the reduction in the cost of production. The statistical methodology was obviously improved for the process optimization of PHA production from SSJ. Apart from this work, we believed that SSJ has a great potential as a sole carbon source for PHA production in an industrial scale in the future.

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