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Effectiveness enhancement of sugar cane juice fermentation for polyhydroxyalkanoates (PHAs) production

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Abstract

Sugar cane juice (SCJ) was used as a carbon source for polyhydroxyalkanoates (PHAs) production by pure bacterium strain of *Azotobactor vinelandii*. Its growth and PHAs production were investigated via batch fermentation. Three main types of sugars as glucose, fructose and sucrose were found in the SCJ and then was preliminary tested by *A. vinelandii* to evaluate its capability for utilizing in each sugar. It was found that bacterium strain could grow in all type of sugars at properly initial concentration of 20 g/l. Subsequently, when the initial total sugar was increased to 40 g/l, the higher dry cell weight (DCW) and PHAs concentration were achieved. Then, effect of air supply on cell growth and PHAs production was further investigated in 5 l fermenter. The maximum PHAs content reached at about 72% when the process was maintained the air flow at 2 vvm without controlled the dissolved oxygen (DO). However, the highest productivity (0.062 g/l/h) was obtained in the controlled condition of air flow (2 vvm) and the DO at 5%. To improve the process, repeated batch fermentation was performed; the PHAs productivity in final batch was reached at about 0.142 g/l/h that was 2.09 fold when compared to the first batch of the process (0.068 g/l/h).

Keywords: batch, repeated batch, polyhydroxyalkanoates, *Azotobactor vinelandii*, sugar cane juice

1. Introduction

Nowadays, synthetic plastics derived from petrochemical industry are very important in many applications for examples in packaging industry, food products, medical, pharmaceutical products and etc. The amounts of synthetic plastic wastes have increased every year because they cannot degrade in the environment. Unlike bioplastic produced from various microorganisms such as yeast, fungi and especially from more than 150 bacterial species. Typically, there are categorized into two main kinds of biopolymer of polyhydroxyalkanoates (PHAs) and polylactic acid (PLA) (1). They can be completely degraded in the environment.

PHAs is a group of aliphatic polyesters which has properties similar to synthetic plastics in a type of polyethylene (PET) and can be used to produce biodegradable products for several applications such as medical equipment, pharmaceutical, functional foods and etc. PHAs are produced in various microorganisms such as yeast, fungi and especially bacteria for example in the strain of *Alcaligines latus* (2-4), *Cupriavidus necator* (formerly called *Alcaligenes eutrophus*, *Wautersia eutropha*, or *Ralstonia eutropha* (5) and recombinant

Escherichia coli (6-8) are preferred produced PHAs under the nutrients limitation such as nitrogen, sulfur, phosphorous and magnesium coupling with the presence of an excess carbon source (9).

In this research, the SCJ was preliminary used as a carbon source for production of PHAs. It was characterized and found that the SCJ mainly consisted of sucrose, glucose and fructose, respectively. However, sucrose was mainly contained in quite high concentration. We also have chosen to use a bacterium strain of *Azotobactor vinelandii* as a PHAs producer strain because of its capacity to utilize sucrose directly as carbon source.

Naturally, *A. vinelandii* is a Gram negative, obligate aerobe capable of fixing nitrogen. It produces PHAs during growth and is not any required nutrient limiting for the production of biopolymer (10). It perhaps can accumulate PHAs in quite high (≥ 85%) of DCW under glucose medium with and without 0.1% fish peptone (11). Previous work, (12) reported the production of PHAs by *A. vinelandii* using sugar beet molasses. The results showed that PHAs concentration and the productivity were reached to 22 g/l and 0.55 g/l/h, respectively.

In this present research, we aim to study some effects of batch fermentation on cell growth and PHAs production by *A. vinelandii* when SCJ was used as a carbon source. The batch fermentation was performed along with the repeated batch fermentation. Effectiveness of the processes was evaluated in terms of biomass, PHAs content and its productivity. In addition, some physicochemical properties of PHAs were also considered.

2. Materials and methods

2.1 Microorganism

A pure bacterium strain of *A. vinelandii* UWD was purchased from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. The strain was maintained on Burk's agar at 4°C and then was transferred monthly on fresh Burk's agar.

2.2 Sugar cane juice

Some stalks of sugar cane were collected from sugar cane plantation area of Khon Kaen province of Thailand. It was squeezed for the juice by roller mills and the SCJ was obtained after filtering by cotton sheet. It was kept at -20°C prior to use.

2.3 Inoculums preparation

A. vinelandii was grown in Burk's medium free nitrogen containing (g/l): K_2HPO_4, 0.64; KH_2PO_4, 0.20; $MgSO_4.7H_2O$, 0.2; NaCl, 0.2; $CaSO_4.2H_2O$, 0.05; mannitol, 20; Na_2MoO_4, 0.001 and $FeSO_4$, 0.003. The pH was adjusted to 7.0 and incubated at 30°C, 200 rpm for 48 h. After that 10% (v/v) of inoculums was transferred into fresh Burk's medium for 24-30 h. The culture was used as initial inoculums for next experiments.

2.4 Batch fermentation

2.4.1 The effect of different sugars on cell growth and PHAs production

Glucose, fructose and sucrose (initial sugar of 20 g/l) were separately used as a pure carbon source for testing the ability of growth and PHAs production by *A. vinelandii*. The experiment was carried out in 500 ml Erlenmeyer flaks containing 250 ml of Burk's medium with ammonium acetate (1.2 g/l) as a nitrogen source. These flasks were inoculated with 10% (v/v) of initial inoculums and incubated at 30°C on shaker (200 rpm). Samples were taken for analyzing the DCW, PHAs and residual total sugar concentrations.

2.4.2 Influence of initial sugar concentrations on cell growth and PHAs production

Influence of initial sugar concentrations was studied by using SCJ as a carbon source. Initial total sugar concentrations of 20, 40 and 60 g/l were prepared by dilution SCJ with Burk's medium (initial total sugar of SCJ about 200 g/l). The condition was carried out as batch fermentation that was mentioned as previous. Samples

were withdrawn for analyzing the DCW, PHAs and residual total sugar concentrations.

2.4.3 The effect of air supply on cell growth and PHAs production

The effect of air supply was investigated via batch fermentation using 5 l fermenter with a working volume of 2.5 l and SCJ was used as a carbon source. Initial inoculums (10% v/v) were inoculated into the productive medium with ammonium acetate as a nitrogen source (1.2 g/l). Temperature and pH were maintained at 30°C and 7 constant throughout fermentation. The air supply into the system was fed continuously at a rate of 2 vvm via air pump and compared between controlled DO of 5% and without controlled DO. The best condition of aeration giving the maximum DCW, PHAs content and productivity was used for further repeated batch experiments.

2.5 Repeated batch fermentation

For performing the repeated batch fermentation, the procedure was similar to the batch fermentation but at the end of each cycle was remove fermentation broth and remained 10% of broth for using as a seed culture in next cycle and addition of fresh productive medium (total sugar in SCJ of 20 g/l).

2.6 Analytical technique

Cell growth was monitored by measuring optical density at 600 nm (OD_{600}) (PG Instruments Limited, T60, UK). Samples were taken and centrifuged (10000 rpm, 10 min). The supernatant was analyzed of residue sugar using high performance liquid chromatography (HPLC) (Shimadzu, Japan).

The pellet cells were washed twice with deionized water, and then were dried to a constant weight (80°C). The dried pellets were used for analysis of DCW.

The presence of PHAs as intracellular product was confirmed by Sudan black B staining method. PHAs were extracted from cells and determined as dry weight according to the method recommended by Grothe et al. (13).

The physicochemical properties of PHAs film was analyzed by Fourier Transform Infrared Spectroscopy (FTIR). It was scanned at spectrogram between 4000-600 cm^{-1} and then compared to the polymer standard of polyhydroxybutyrate (PHB). (Sigma-Aldrich, USA).

3. Results and discussions

3.1 Batch fermentation

3.1.1 The effect of different sugars on cell growth and PHAs production

As our previous study, sugars contained in the SCJ were characterized. It was found that the concentration of sucrose (187.63 g/l) showed in higher than that of glucose (8.69 g/l) and fructose (5.32 g/l). Therefore, *A. vinelandii* was investigated its ability on growth and PHAs production in Burk's medium with sucrose sugar compared to other sugars. The results are shown in Figure 1; *A. vinelandii* could grow in all type of sugars and also could grow properly on sucrose. The maximum DCW and PHAs concentration of 2.55g/l and 1.51g/l were obtained during 126 h on sucrose as a carbon source thus *A. vinelandii* has suitable to be used as a producer strain.

3.1.2 Influence of initial sugar concentrations on cell growth and PHAs production

Initial total sugar concentrations in SCJ at 20, 40 and 60 g/l were prepared by diluting with Burk's medium and ammonium acetate as a nitrogen source for investigation of the effects on cell growth and PHAs production. The results obtained are shown in Figure 2.

Initial total sugar concentration of 40 g/l could increase the DCW and PHAs concentration when compared to the concentrations of 20 and 60 g/l. The highest of DCW and PHAs concentration were obtained at 6.50 g/l and 5.00 g/l. Although the bacterium showed in a well growth and PHAs production with concentration of

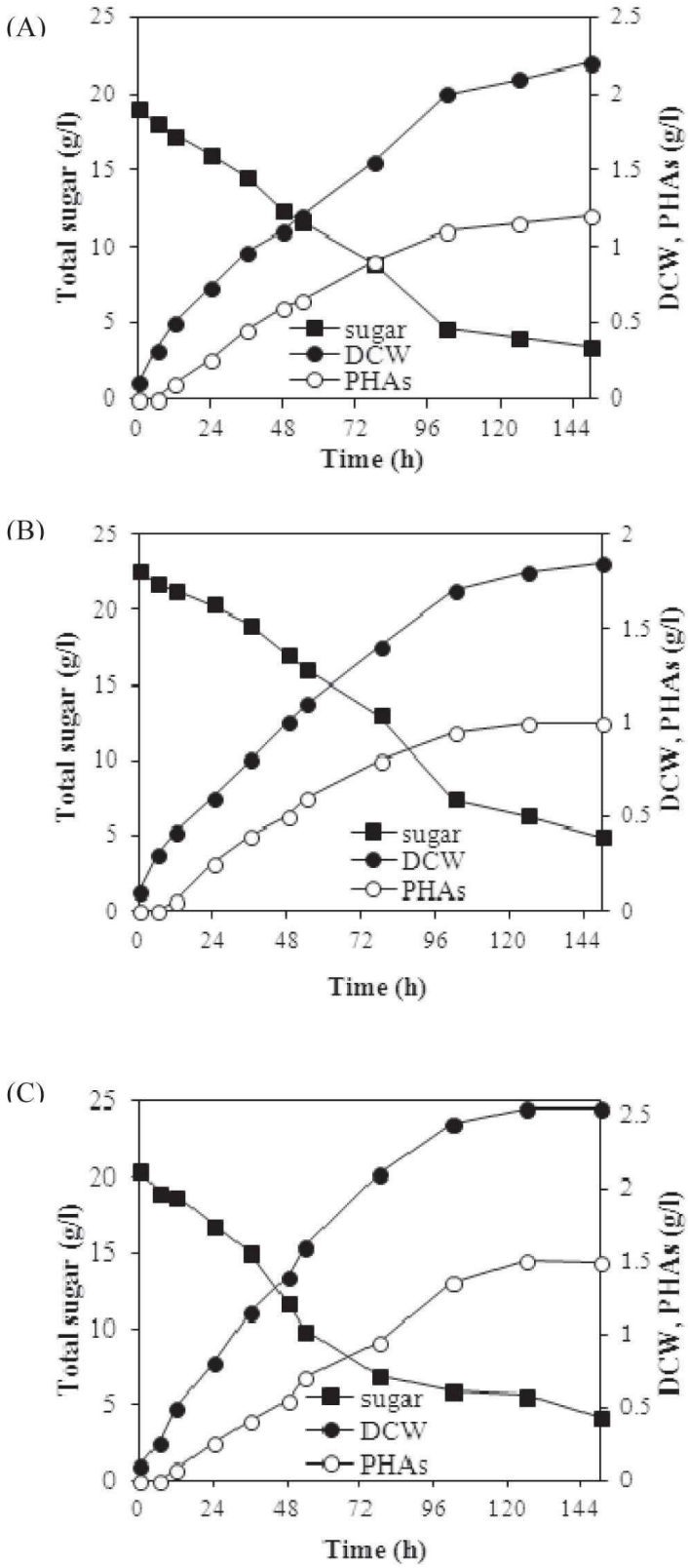

Figure 1. Growth profile and PHAs production by *A. vinelandii* in different sugar type under batch fermentation using shake flasks technique. (A) glucose (B) fructose (C) sucrose.

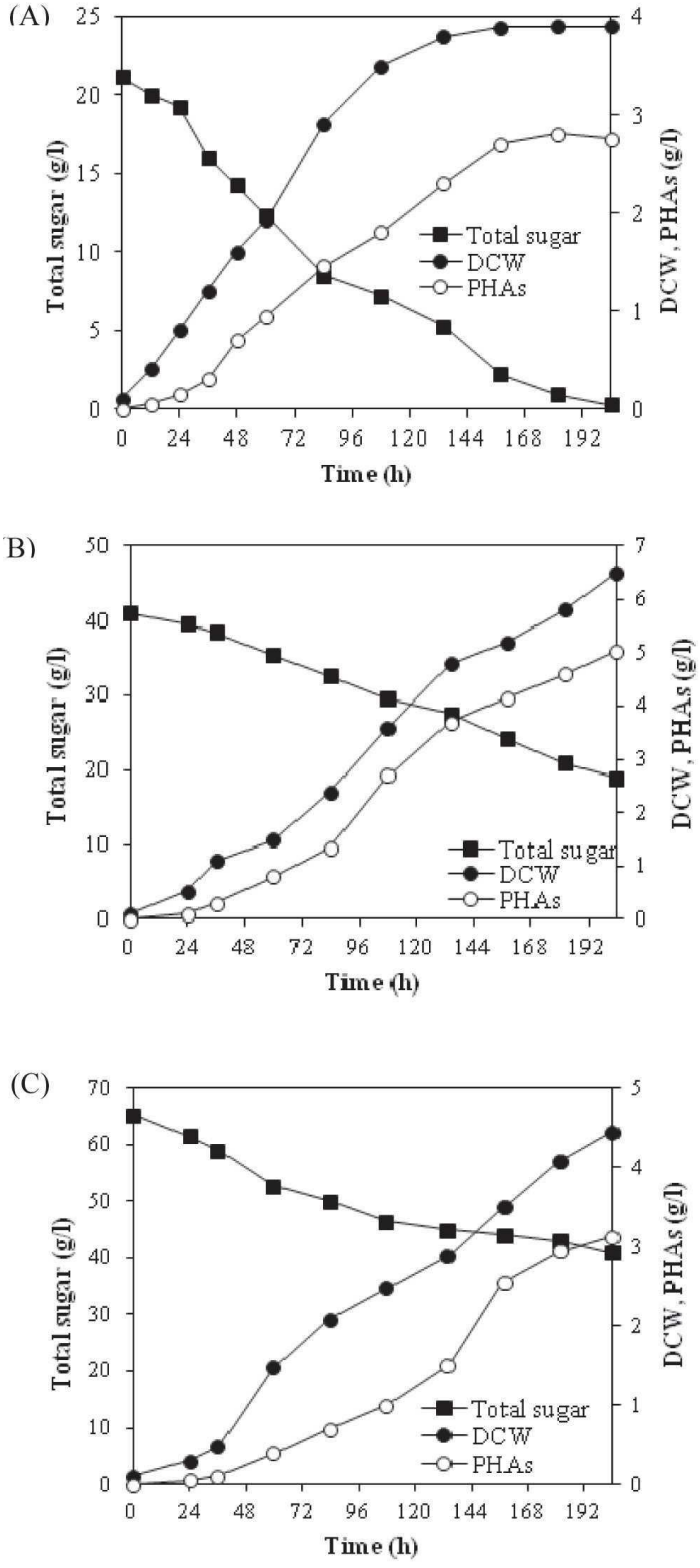

Figure 2. Growth profile and PHAs production by *A. vinelandii* on different initial total sugar concentrations in SCJ under batch fermentation; (A), concentration of 20 g/l, (B), concentration of 40 g/l, (C), concentration of 60 g/l.

40 g/l but the growth profile showed in slower than that the concentration of 20 g/l. Therefore, the concentration of 20 g/l was further used in both batch and repeated batch fermentation.

3.1.3 The effect of air supply on cell growth and PHAs production

To study the effect of air feed in the system on both cell growth and PHAs production, the air rate was maintained at 2 vvm via air pump. While the DO at 0% and 5% were controlled via DO probe. The results show that at 5% DO, *A. vinelandii* began to grow and produce PHAs faster than in case of using 2 vvm without controlled DO (after about 12 h and about 18 h).

In Figure 3, it can demonstrate that, when the process was fulfilled with a high O_2 (DO 5%) *A. vinelandii* could grow and produce PHAs properly. The highest DCW, PHAs concentration and productivity of 4.36 g/l, 2.55 g/l and 0.062 g/l/h, respectively were obtained from the process that the DO was controlled at 5%. On the other hand, bacterium can accumulate PHAs up to 72 % in case of the process has a low O_2 (air flow continuously at a rate of 2 vvm). This is corresponded to previous study reported by Chen et al. (14) who studied PHB production by *A. vinelandii*. The strain was suppressed by high aeration of beet molasses medium. They found that higher O_2 increased protein but decreased PHB formation. The parameters effect of aeration was summarized in Tables 1 and 2.

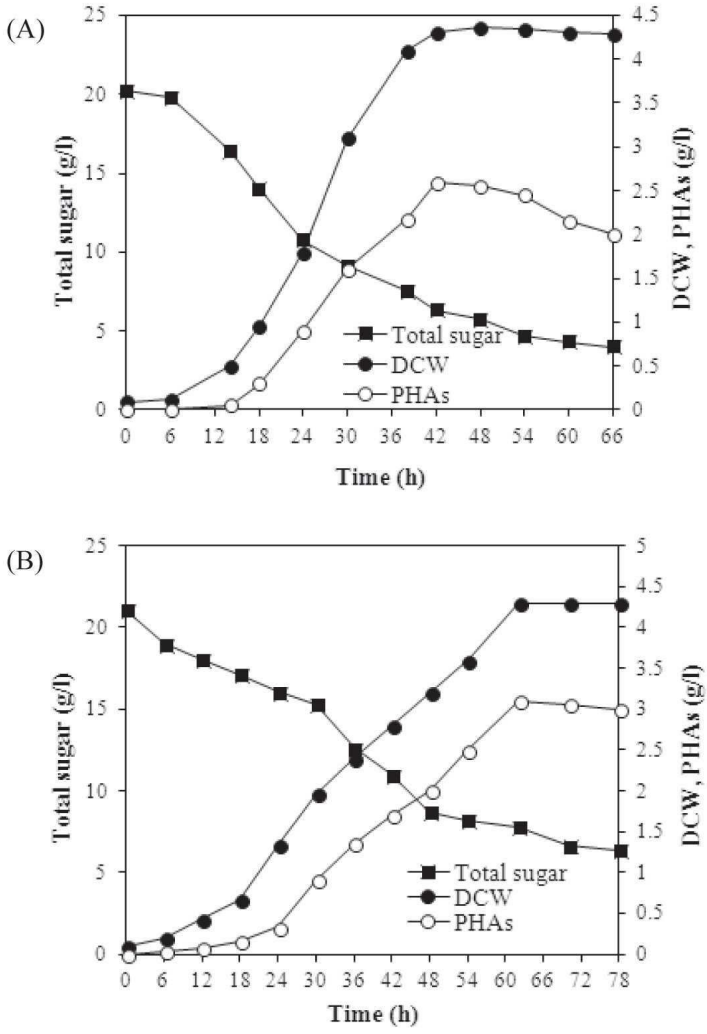

Figure 3. Growth and PHAs production of *A. vinelandii* during batch fermentation with the air in the system flow continuously at a rate of 2 vvm (using SCJ as a carbon source) and (A), controlled DO at 5%, (B) without controlled DO.

Table 1. Kinetic parameters during batch fermentation with air flow and Do control (using SCJ as a carbon source).

Time (h)	DCW (g/l)	PHAs (g/l)	Residual total sugar (g/l)	Consumed total sugar (g/l)	$Y_{X/S}$	$Y_{P/S}$	PHAs content (%)	Productivity (g/l/h)
0	0.09	0.00	20.20	0.00	0.000	0.000	0.00	0.000
6	0.12	0.00	19.78	0.42	0.286	0.000	0.00	0.000
14	0.50	0.05	16.41	3.79	0.132	0.013	10.00	0.004
18	0.95	0.30	14.00	6.20	0.153	0.048	31.58	0.017
24	1.80	0.90	10.77	9.43	0.191	0.095	50.00	0.038
30	3.10	1.60	9.09	11.11	0.279	0.144	51.61	0.053
38	4.10	2.17	7.53	12.67	0.324	0.171	52.93	0.057
42	4.31	2.59	6.31	13.89	0.310	0.186	60.09	0.062
48	4.36	2.55	5.74	14.46	0.302	0.176	58.49	0.053
54	4.35	2.45	4.65	15.55	0.280	0.158	56.32	0.045
60	4.30	2.15	4.30	15.90	0.270	0.135	50.00	0.036
66	4.29	2.00	4.00	16.20	0.265	0.123	46.62	0.030

$Y_{X/S}$ = Biomass yield (DCW per consumed total sugar)

$Y_{P/S}$ = PHAs yield relative substrate (PHAs produced per consumed total sugar)

Productivity = (PHAs produced per time)

Table 2. Kinetic parameters during batch fermentation and without air flow and DO control (using SCJ as a carbon source).

Time (h)	DCW (g/l)	PHAs (g/l)	Residual total sugar (g/l)	Consumed total sugar (g/l)	$Y_{X/S}$	$Y_{P/S}$	PHAs content (%)	Productivity (g/l/h)
0	0.10	0.00	21.10	0.00	0.000	0.000	0.00	0.000
6	0.20	0.03	18.99	2.11	0.095	0.014	15.00	0.005
12	0.43	0.07	18.04	3.06	0.140	0.023	16.28	0.006
18	0.67	0.15	17.15	3.95	0.169	0.038	22.39	0.008
24	1.33	0.33	16.05	5.05	0.263	0.065	24.81	0.014
30	1.97	0.90	15.28	5.82	0.038	0.155	45.69	0.030
36	2.40	1.35	12.61	8.49	0.282	0.159	56.25	0.038
42	2.80	1.70	10.97	10.13	0.276	0.168	60.71	0.040
48	3.20	2.00	8.73	12.37	0.259	0.162	62.50	0.042
54	3.60	2.50	8.17	12.93	0.278	0.193	69.44	0.046
62	4.30	3.10	7.78	13.32	0.323	0.233	72.09	0.050
70	4.30	3.05	6.63	14.47	0.297	0.211	70.93	0.044
78	4.29	3.00	6.40	14.70	0.292	0.204	69.93	0.038

$Y_{X/S}$ = Biomass yield (DCW per consumed total sugar)

$Y_{P/S}$ = PHAs yield relative substrate (PHAs produced per consumed total sugar)

Productivity = (PHAs produced per time)

3.2 Repeated batch fermentation

Repeated batch fermentation was carried out in 5 L fermenter. A working volume of 10% in the first batch was remained for using as a seed culture in a next batch step. The same amount of the fresh productive medium was immediately replaced for maintaining the working volume at 2.5 l. The results of repeated batch fermentation on DCW, PHAs production and total sugar are given in Figure 4 (A-B) and Table 3.

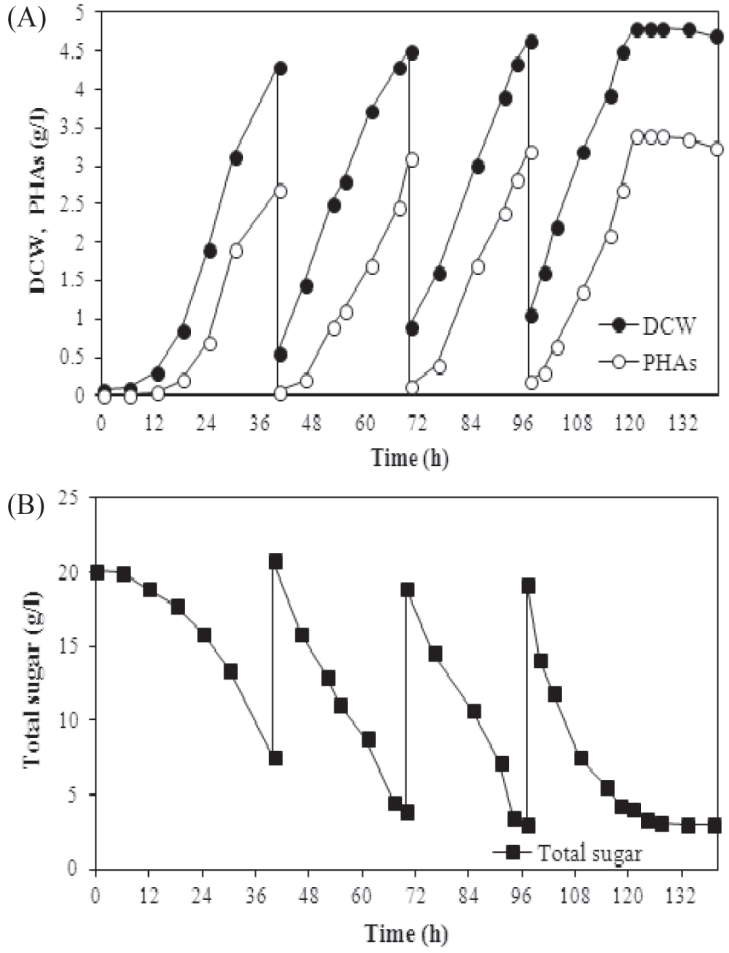

Figure 4. Fermentation kinetics during PHAs production in repeated batch fermentation by *A. vinelandii* (using SCJ as a carbon source): (A), DCW and PHAs concentration (B), total sugar.

Table 3. Comparison of some parameters monitored during in repeated batch fermentation (using SCJ as a carbon source).

Batch Number	Time (h)	DCW (g/l)	PHAs (g/l)	Residual total sugar (g/l)	Consumed total sugar (g/l)	$Y_{X/S}$	$Y_{P/S}$	PHAs content (%)	Productivity (g/l/h)
1	40	4.30	2.70	7.61	12.52	0.343	0.216	62.79	0.068
2	30	4.50	3.00	3.96	16.84	0.267	0.178	66.67	0.100
3	27	4.65	3.20	3.04	15.89	0.293	0.201	68.82	0.119
4	24	4.80	3.40	4.00	15.16	0.317	0.224	70.83	0.142

$Y_{X/S}$ = Biomass yield (DCW per consumed total sugar)

$Y_{P/S}$ = PHAs yield relative substrate (PHAs produced per consumed total sugar)

Productivity = (PHAs produced per time)

In Table 3, some parameters related to cell growth and PHAs production were monitored such as DCW, PHAs in g/l and in percentage and its productivity. They were all increased when compared to the first batch. The PHAs productivity was obtained at about 0.142 g/l/h in the fourth batch cycle that was 2.09 fold when compared to the first batch fermentation (0.068 g/l/h). In addition, duration time in each batch operated during repeated batch fermentation was shorter than that found in the batch fermentation. These results are similar with those of Huang et al. (15) who reported that repeated batch fermentation improved the volumetric hyaluronic acid productivity at approximately 2.5 fold of the batch fermentation. Yang et al. (16) study lipase productivity by repeated batch fermentation. They were found that lipase productivity increased from 3.1 U/ml in batch fermentation to 17.6 U/ml in repeated batch fermentation.

The molecule was identified on wavelength by FTIR spectrophotometer which was scanned between 4000-600 cm^{-1}. The peak at 1721 $cm^{-1}$and 1277 cm^{-1}corresponds to C=O and –CH group, respectively (see in Figure 5).

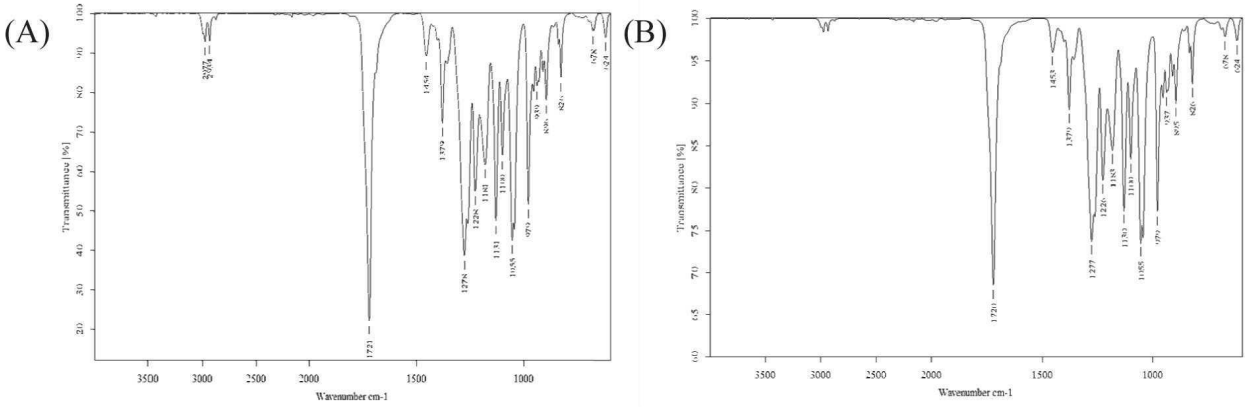

Figure 5. FTIR spectrum obtained from commercial grade of PHB (A) and PHAs produced by *A. vinelandii* (B).

The FTIR spectrum of PHAS obtained from SCJ fermentation which was in full agreement with those obtained from a standard sample of polyhydroxybutyrate (PHB). The result indicated that PHAs from sugar cane juice is purely PHB. The FTIR spectrogram showed similar to the results obtained from previous study reported by Kumalaningsih et al. (17).

4. Conclusion

Successfully, the SCJ was rich of both sugars and some elements. Thus, it can be directly used as a raw material for cell growth coupling with PHAs production. The repeated batch fermentation also has been successfully used to improve production yield and it productivity. The advantage of non-productive downtime for cleaning and sterilization can be eliminated, leading to reduction in the cost of fermentation and can be employed successfully for PHAs production. Number of batch cycles can be withdrawn and fresh medium addition until the batch is contaminated.

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References

(1) Basnett P, Roy I. Microbial production of biodegradable polymers and their role in cardiac stent development. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 2010; 1405-415.

(2) Braunegg G, Bogensberger B. Zur kinetic des wachstums und der speicherung von poly-D (-)-3-hydroxybuttersäure bui *Alcaligenes latus*. Acta Biotechnol. 1985; 5: 339-45.

(3) Hanggi UJ. Pilot scale production of PHB with *Alcaligenes latus*. In: Dawes E D, ed. Novel Biodegradable Microbial Polymers. Kluwer Academic Publishers, Netherlands. 1990: 65-70.

(4) Hrabak O. Industrial production poly-β-hydroxybutyrate. FEMS Microbiol Rev. 1992; 103: 251-56.

(5) Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo SI. Production of poly (hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol Bioeng. 1994; 43: 892-98.

(6) Lee SY. *E. coli* moves into plastic age. Nature Biotechnol. 1997; 15: 17-8.

(7) Lee SY, Chang HN. Production of poly (3-hydroxybutyric acid) by recombinant *Escherichia coli* strains: genetic and fermentation studies. Can J Microbiol. 1995; 41: 207-15.

(8) Anderson A, Dawes E. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev. 1990; 54: 450-72.

(9) Lee SY, Yim KS, Chang HN, Chang YK. Construction of plasmids, estimation of plasmid stability, and use of stable plasmids for the production of poly (3-hydroxybutyric acid) in *Escherichia coli*. J Biotechnol. 1994; 32: 203-11.

(10) Page WJ, Knosp O. Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl Environ Microbiol. 1989; 58: 2866-73.

(11) Page WJ, Cornish A. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of poly-β-hydroxybutyrate. Appl Environ Microbiol. 1993; 59: 4236-44.

(12) Page WJ. Production of polyhydroxyalkanoates by *Azotobacter vinelandii* UWD in beet molasses culture. FEMS Microbiol Rev. 1992; 103: 149-57.

(13) Grothe E, Moo-Young M, Chisti Y. Fermentation optimization for the production of (hydroxybutyric acid) microbial thermoplastic. Enzym Microb Tech. 1999; 25: 132-41.

(14) Chen GQ, Page WJ. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. Biotechnol Tech. 1997; 5: 347-50.

(15) Huang WC, Chen SJ, Chen TL. Production of hyaluronic acid by repeated batch fermentation. Biochem Eng. 2008; 40: 460-64.

(16) Yang X, Wang B, Cui F, Tan T. Production of lipase by repeated batch fermentation with immobilized *Rhizopus arrhizus*. Process Biochem. 2005; 40: 2095-103.

(17) Kumalaningsih S, Nur H, Nur A. Optimization of polyhydroxyalkanoates (PHA) production from liquid bean curd waste by *Alcaligenes latus* bacteria. J Agric Food Tech. 2011; 1: 63-7.