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ORIGINAL ARTICLE

Fermentation of sago starch to biobutanol in a batch culture using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564)

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Abstract Acetone–butanol–ethanol (ABE) was produced in *Clostridium saccharoperbutylacetonicum* N1-4 using sago starch as a substrate, and the effects of nutrient supplementation on ABE production were studied. Using sago starch as a substrate led to higher butanol production in *C. saccharoperbutylacetonicum* N1-4 when compared to other starchy materials as substrates. TYA medium supplemented with sago starch led to enhanced ABE and butanol production; however, P2 medium improved ABE production more than TYA medium. The highest ABE and butanol production was achieved with P2 medium supplemented with 70 g/L of sago starch. Using these fermentation conditions, ABE and butanol were produced at 16.65 and 9.83 g/L, respectively, and productivity was 0.15 g/L h for ABE and 0.09 g/L h for butanol. Using P2 medium supplemented with either 50 g/L enzymatically hydrolyzed sago starch or 70 g/L sago starch without hydrolysis, 9.81 and 9.83 g/L butanol were produced, respectively. These results reveal the importance of media formulation in the biobutanol fermentation of sago starch by *C. saccharoperbutylacetonicum* N1-4.

Keywords Biobutanol · Sago starch · *Clostridium saccharoperbutylacetonicum* N1-4 · Nutrient supplementation

Introduction

Biobutanol is a leading liquid biofuel candidate that can be produced via the well-known acetone–-butanol–ethanol (ABE) fermentation process in the solvent-producing organisms Clostridia. Butanol has a lower vapor pressure and higher energy content than ethanol; thus, it safer to use in gasoline blends and offers better fuel economy than ethanol–gasoline blends. Furthermore, butanol has a higher tolerance to water contamination in gasoline blends, making butanol–gasoline blends less susceptible to separation and facilitating the use of existing gasoline supply and distribution channels (Dürre 2007; Al-Shorgani et al. 2011).

Substrate cost is an important factor in butanol fermentation; butanol can be produced from various raw materials or renewable agricultural crops such as sago starch (Madihah et al. 2001), corn (Qureshi and Blaschek 2001), molasses (Syed et al. 2008) and whey permeate (Ennis and Maddox 1985).

Butanol fermentation in solvent-producing Clostridia suffers from many problems, including product toxicity, low productivity and high recovery cost. New techniques have been established to overcome these problems, including the development of higher butanol-tolerant strains and improvement of butanol yield by mutagenesis or continual removal of fermentation products to avoid toxicity (Lee et al. 2008). Recently, the metabolic engineering of *E. coli* for butanol production has been reported. The mutant *E. coli* produced butanol at a concentration of 30 g/L, exceeding production levels obtained by native butanol producers (Shen et al. 2011).

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Sago starch is a carbohydrate staple obtained from the sago palm trunk., and accumulation in the trunk can reach up to 250 kg (dry weight per plant) (Harriman 1998). Sago starch contains 27% amylose and 73% amylopectin (Swinkels 1985). In Malaysia, sago starch is an alternative and cheap carbon source for fermentation processes and has advantages over other carbon sources from economic and geographical perspectives (Suraini 2002). Sago starch is an abundant carbon source that could potentially be used in ABE fermentation (Rosfarizan and Ariff 1998).

The average production of sago starch is 25 tons per hectare per year, the highest productivity among other starchy crops, and annual starch production is estimated at approximately 60 million tons. Sago starch production far exceeds the local demand (Ishizaki 1997).

Solvent-producing Clostridia can utilize sago starch by converting it to maltose and glucose with secreted amylolytic enzymes (α-amylase and glucoamylase); in fact, the presence of amylolytic enzymes during the growth of *Clostridium* species on starch has been reported (Mitchell 1997; Paquet et al. 1991).

Enzymatically hydrolyzed sago starch contains tiny amounts of nitrogenous compounds as well as traces of minerals and nutrient supplements, which enhance fermentation in microorganisms. It has been used as a substrate for several fermentation processes such as lactic acid production (Nolasco et al. 2000), ABE production (Madihah et al. 2001) and butanol production (Hipolito et al. 2008).

Materials and methods

Preparation of microorganisms and inoculum

Clostridium saccharoperbutylacetonicum N1-4 was provided by the Biotechnology Laboratory in the Chemical Engineering and Bioprocess Department at Universiti Kebangsaan Malaysia. It was kept as a stock culture at 4°C as a suspension of spores in a potato glucose medium (PG medium). The inoculum was prepared by transferring the suspension of spores (1 mL) to 10 mL of 15% PG medium and heat shock for 1 min in boiling water, followed by cooling in iced water and incubation under anaerobic conditions for 1–2 days at 30°C. The colony morphology and gram-staining behavior of the inoculum was checked to ensure that the culture was pure. This initial culture was then transferred to tryptone–yeast extract–acetate medium (TYA medium) and incubated for 15–18 h and used as the inoculum.

Preparation of media

The fresh PG medium contained the following per liter of distilled water: 150 g grated fresh potato, 10 g glucose, 0.5 g $(NH_4)_2SO_4$ and 3 g $CaCO_3$. After mixing these ingredients, the medium was incubated in boiling water for 1 h with mixing every 10 min. The medium was filtered through a gauze and then sterilized at 121°C for 15 min.

TYA medium was used for the pre-culture, and per liter of distilled water consisted of 20 g glucose, 2 g yeast extract, 6 g tryptone, 3 g CH_3COONH_4, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.05 g, 0.5 g KH_2PO_4 and 10 mg $FeSO_4{\cdot}7H_2O$. The medium was sterilized at 121°C for 15 min.

The P2 medium consisted of the following: 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}5H_2O$, 1.0 g/L yeast extract, 0.5 g/L cysteine and gelatinized starch. One mL of a solution containing 1 mg/L 4-aminobenzoic acid was added to 1 L of the medium as well as biotin to a concentration of 80 μg/L.

The main fermentation medium contained either gelatinized sago starch at various concentrations (from 10 to 70 g/L sago starch) or other starchy materials including tapioca, corn, potato and rice starch as a control. All starches were obtained from a local market in Malaysia.

Gelatinized starches were prepared by heating starch slurry at 80°C for 30 min with interval shaking. The fermentation medium was then autoclaved at 121°C for 15 min. After autoclaving, the fermentation broth was sparged with oxygen-free nitrogen to achieve anaerobic conditions.

Enzymatic hydrolysis of sago starch

Sago starch was hydrolyzed enzymatically to glucose by α-amylase (Termamyl 120L; Novo, Kuala Lumpur, Malaysia) and glucoamylase (AMG 300; Novo). To prepare a glucose hydrolysate, gelatinized sago starch was liquefied and saccharified by α-amylase and glucoamylase. The pH of the gelatinized starch was adjusted to 6.2. Calcium ions were added to stabilize the enzyme using calcium chloride (Ca ≥40 ppm). A heat-stable α-amylase (0.05 mL/100 g) was added to the mixture, and the mixture was heated at 100°C for 10 min. The mixture was cooled to 90°C and held at this temperature for 2 h for more hydrolysis. Saccharification was carried out by adding glucoamylase enzyme (0.1 mL/100 g) at pH 4.5 and incubating at 60°C for 24 h.

Batch fermentation

The anaerobic batch fermentation was conducted in 250-mL Schott (Duran) bottles with a working volume of 150 mL. These bottles have both an inlet and outlet.

The initial pH of the medium was adjusted to 6 by 1 M HCl. The medium was sterilized by autoclaving at 121°C for 15 min. To generate an anaerobic condition, the medium

was sparged with oxygen-free nitrogen, and the vitamin solution was filter-sterilized and added aseptically into the sterilized medium. The batch culture was initiated by inoculation of medium with 10% fresh inoculum that was previously grown on TYA medium for 18 h. The Schott bottles were incubated at 30°C with shaking in an incubator under anaerobic conditions. All fermentations were carried out in duplicate and measurements are average values.

Analytical procedures

Samples were taken at appropriate time intervals and centrifuged at 5,000 rpm for 5 min. The supernatant was analyzed to determine the ABE concentration and sugar and organic acid concentrations. Dry cell weight (DCW) was measured by centrifuging the samples at 10,000 rpm for 10 min in pre-weighed Eppendorf tubes. The supernatant was discharged and the pellet was re-suspended in water and washed twice to release the cells from starchy materials. After removing the supernatant, the cells were dried at 105°C for 4 h to determine the dry cell weight. A fresh starch medium was used as a blank sample.

Concentrations of ABE and acids (acetic and butyric) were measured using a gas chromatograph (7890A GC-System; Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector and a 30-m capillary column (Equity 1; 30 m×0.32 mm×1.0 μm film thickness; Supelco, Bellefonate, PA, USA). The oven temperature was programmed to increase from 40 to 130°C at a rate of 8°C/min. The injector and detector temperatures were set at 250 and 280°C, respectively. Helium, as the carrier gas, was set at a flow rate of 1.5 mL/min.

The glucose concentration in the fermentation broth was determined with a Biochemistry Analyzer (YSI 2700D; YSI Life Sciences, Yellow Springs, OH, USA).

The amount of starch in the culture was determined according to the method of Kitahata and Okada (1974). This method is based on the reaction of starch with iodine that results in a change in color from dark blue to purplish blue. One mL of culture medium was mixed with 4 mL of 0.01 M iodine in 0.25 M potassium iodide and diluted with 15 mL distilled water. The absorbance was measured at 465 nm against a blank of distilled water treated in the same manner as described above. The starch concentration in the culture filtrate was quantified according to the standard curve obtained for pure starch.

Results

Effect of different starchy materials on ABE fermentation

The effect of various starchy materials on ABE fermentation using *C. saccharoperbutylacetonicum* N1-4 is shown in Table 1. The starch concentration was fixed initially at 30 g/L. The starchy materials used in this experiment for fermentation media were: sago, tapioca, corn, potato and rice starch. These materials were added separately to P2 media and anaerobic batch culture fermentation was done at an incubation temperature of 30°C, inoculum size of 10%, initial pH of 6.0, and agitation speed of 80 rpm.

The highest concentration of butanol (5.24 g/L) was produced from fermentation of sago starch. In corn and rice starch fermentations, butanol was produced at nearly the same level, 4.1 and 4.2 g/L, respectively, which was about 20% lower than butanol production from sago starch fermentation. The lowest butanol production was observed when potato starch was used as a carbon source. The total concentrations of ABE and butanol observed in fermentation with 30 g/L glucose were comparable to that produced by fermentation of corn and rice starches. Although the starches used were different, the same dry cell weight concentration (approx. 2.0 g/L) of *C. saccharoperbutylacetonicum* N1-4 was observed for each.

Previously, Madihah et al. (2001) reported that *C. acetobutylicum* P262 showed the highest growth (2.5 g/L dry cell weight) in medium with sago starch, followed by corn starch (2.0 g/L), potato starch (1.9 g/L), and cassava

Table 1 Direct fermentation of different starchy materials to ABE (30 g/L starch)

Type of starch (30 g/L)	Final pH	DCW (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	$Y_{ABE/Starch}$ (g/g)	$Y_{Butanol/starch}$ (g/g)
Sago starch	4.62	2.09	1.58	5.24	0.89	7.71	0.257	0.175
Tapioca starch	4.44	2.08	1.30	3.11	0.77	5.18	0.173	0.104
Rice starch	4.52	1.96	1.80	4.17	0.78	6.75	0.225	0.139
Corn starch	4.62	2.20	1.53	4.08	0.78	6.40	0.213	0.136
Potato starch	4.41	1.94	0.90	2.51	0.76	4.17	0.139	0.089
TYA(glucose 30 g/L)	4.63	2.16	1.30	4.33	0.90	6.53	0.218	0.144

Table 2 The effect of sago starch concentration on ABE fermentation (without supplementation)

Starch concentration (g/L)	Final pH	Production (g/L)				Yield (g/g)	
		Acetone	Butanol	Ethanol	ABE	ABE	Butanol
Sago starch 10	3.20	0.26	0.77	0.00	1.03	0.103	0.077
Sago starch 20	3.22	0.45	1.38	0.00	1.83	0.092	0.069
Sago starch 30	3.23	0.71	2.57	0.00	3.28	0.109	0.086
Soluble starch 30	3.29	0.76	2.49	0.00	3.25	0.108	0.083
Sago starch 40	3.39	0.80	2.36	0.00	3.16	0.079	0.059
Sago starch 50	3.35	1.03	3.16	0.00	4.19	0.084	0.063
Soluble starch 50	3.30	0.98	3.50	0.75	5.23	0.105	0.070
Sago starch 60	3.28	1.29	3.96	0.77	6.02	0.100	0.066
Sago starch 70	3.37	0.81	2.67	0.77	4.25	0.061	0.038

starch (which gave the lowest at 1.1 g/L). Furthermore, in a different study, Thang et al. (2010) found that the maximum dry cell weight of *C. saccharoperbutylacetonicum* N1-4 from fermentation of 60 g/L cassava starch and 50 g/L of corn starch was 2.6 g/L, but this value decreased to 2.5 g/L with fermentation using 60 g/L of sago starch.

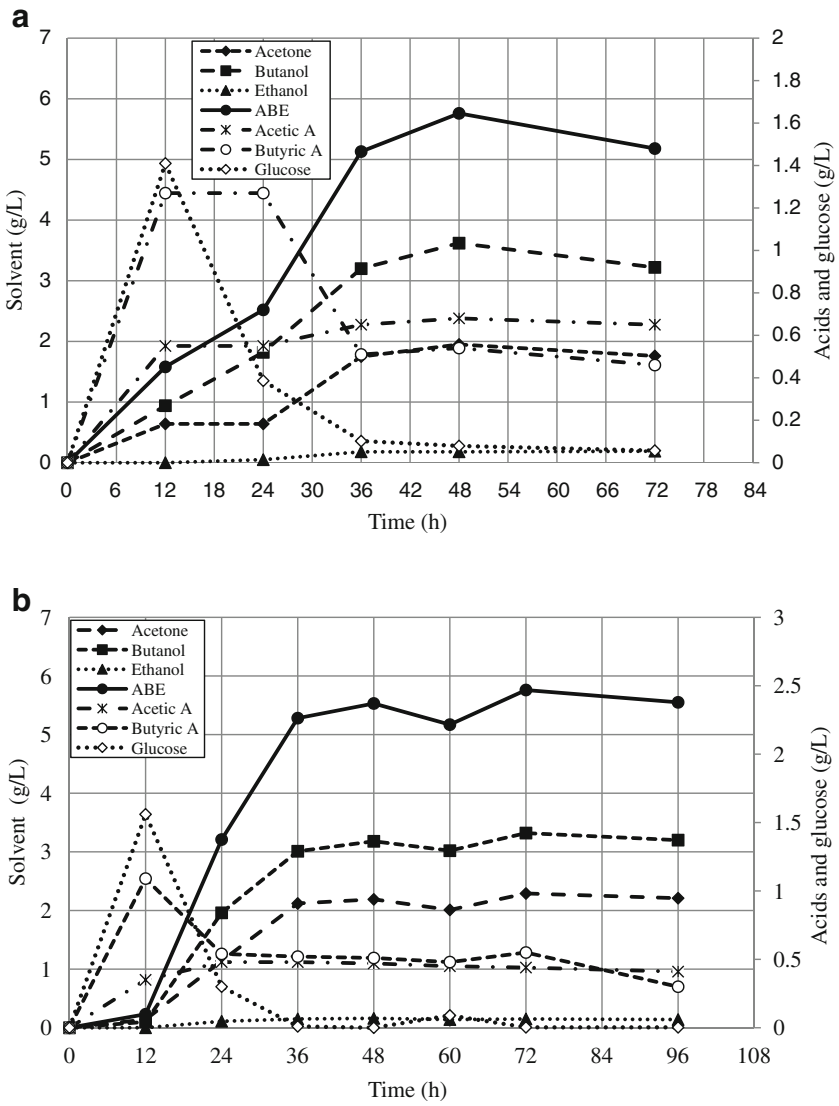

Fig. 1 Profile of ABE fermentation from 30 g/L sago starch by *C. saccharoperbutylacetonicum* N1-4; **a** with P2 medium and **b** with TYA medium

Effect of initial sago starch concentration on ABE production without supplementation

The potential for sago starch as a renewable and cheap carbon resource has stimulated research into its use in ABE fermentation using *C. saccharoperbutylacetonicum* N1-4, which is known to be a hyperamylolytic and solvent-producing strain (Thang et al. 2010).

The direct fermentation of sago starch into butanol with a solvent-producing bacterium, *C. saccharoperbutylacetonicum* N1-4, was investigated under anaerobic conditions in gelatinized sago starch media containing different initial concentrations of sago starch ranging from 10 to 70 g/L without any nutrient addition. Table 2 shows the solvent production values and yields obtained with this fermentation.

In this study, the ABE concentration and productivity increased with increasing sago starch concentrations up to 60 g/L. The maximum ABE production was obtained with 60 g/L sago starch, giving a yield of 0.1 g/g, whereas the highest ABE and butanol yields were achieved with 30 g/L sago starch and were 0.109 and 0.86 g/g, respectively.

Table 2 shows that the butanol concentration increased from 0.77 to 3.9 g/L as the sago starch concentration increased from 10 to 60 g/L sago. However, 70 g/L sago starch produced only 2.6 g/L butanol. There was no significant difference in the concentration of butanol when 30 and 50 g/L soluble starches were used compared to the same concentration of sago starch. No ethanol was produced in fermentations with lower concentrations of sago starch (less than 50 g/L).

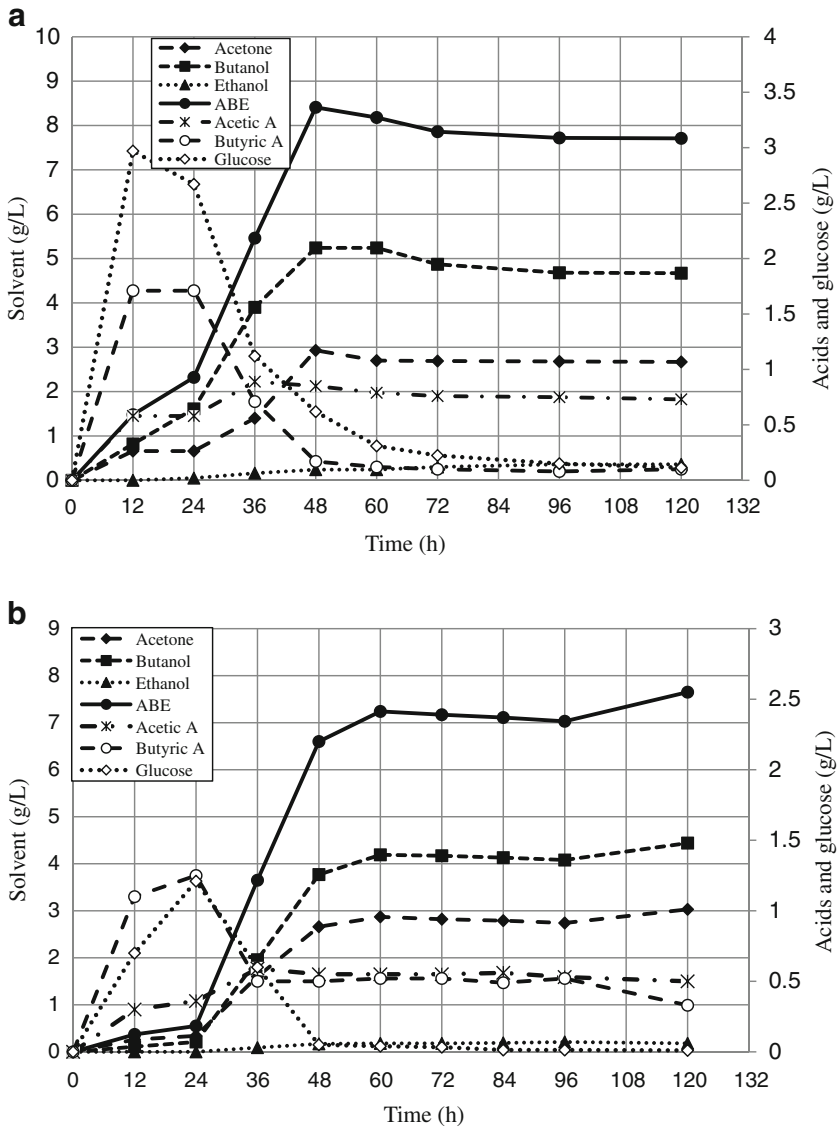

Fig. 2 Profile of ABE fermentation from 40 g/L sago starch by *C. saccharoperbutylacetonicum* N1-4; **a** with P2 medium and **b** with TYA medium

The effect of sago starch supplementation on ABE fermentation

A typical time course of ABE fermentation using a batch culture of *C. saccharoperbutylacetonicum* N1-4 with different concentration of sago starch (30–70 g/L) in P2 and TYA media is shown in Figs. 1, 2, 3, 4 and 5. It is well known that ABE fermentation of glucose in Clostridium can be divided into two different phases: the acidogenesis phase and solventogenesis phase (Jones and Woods 1986; Welsh et al. 1986). However, the direct fermentation of sago starch to solvent can be divided into three phases: (1) the hydrolysis of sago starch to glucose and maltose, (2) the acidogenesis phase and (3) the solventogenesis phase (Liew et al. 2006; Madihah et al. 2001).

The properties of this fermentation are summarized in Table 4. Glucose and maltose were detected during the first 12 h of fermentation at all sago starch concentrations, indicating the activity of amylase enzymes produced by *C. saccharoperbutylacetonicum* N1-4 (Table 3). It has been reported previously that *C. saccharoperbutylacetonicum* N1-4 is a hyper amylolytic bacteria (Thang et al. 2010), and increased sago starch concentrations resulted in a steady increase in ABE and butanol production.

In P2 medium supplemented with 30 and 40 g/L sago starch, the fermentation time was similar (48 h), and the total solvent concentrations were 5.75 and 8.41 g/L, respectively. The highest ABE and butanol productivity was obtained from medium with 40 g/L sago starch (Table 4). Fermentation of 30 g/L sago starch showed a similar production of ABE and butanol when either TYA or P2 medium was used. However, when 40 g/L sago starch was used with P2 medium, higher amounts of ABE and butanol were observed compared to the same amount of sago starch in TYA (Table 4).

Table 4 shows that the highest ABE concentration (16.65 g/L) was achieved with the highest starch concen-

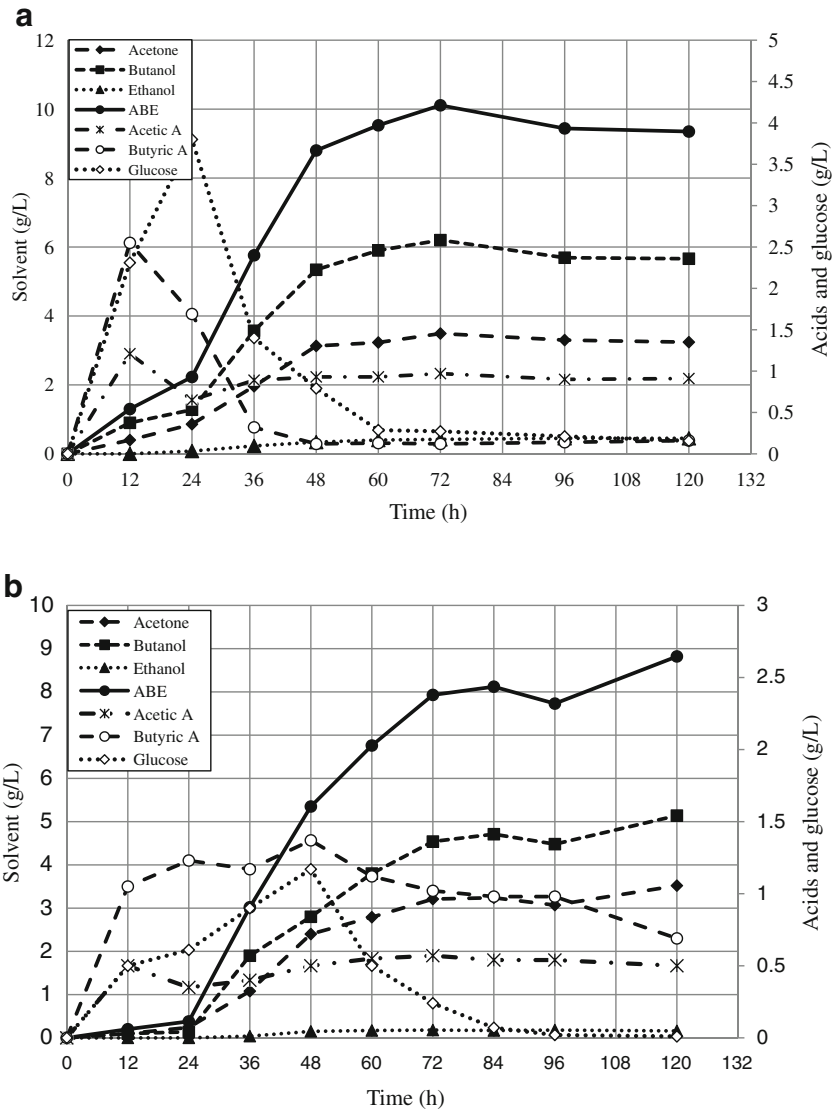

Fig. 3 Profile of ABE fermentation from 50 g/L sago starch by *C. saccharoperbutylacetonicum* N1-4; **a** with P2 medium and **b** with TYA medium

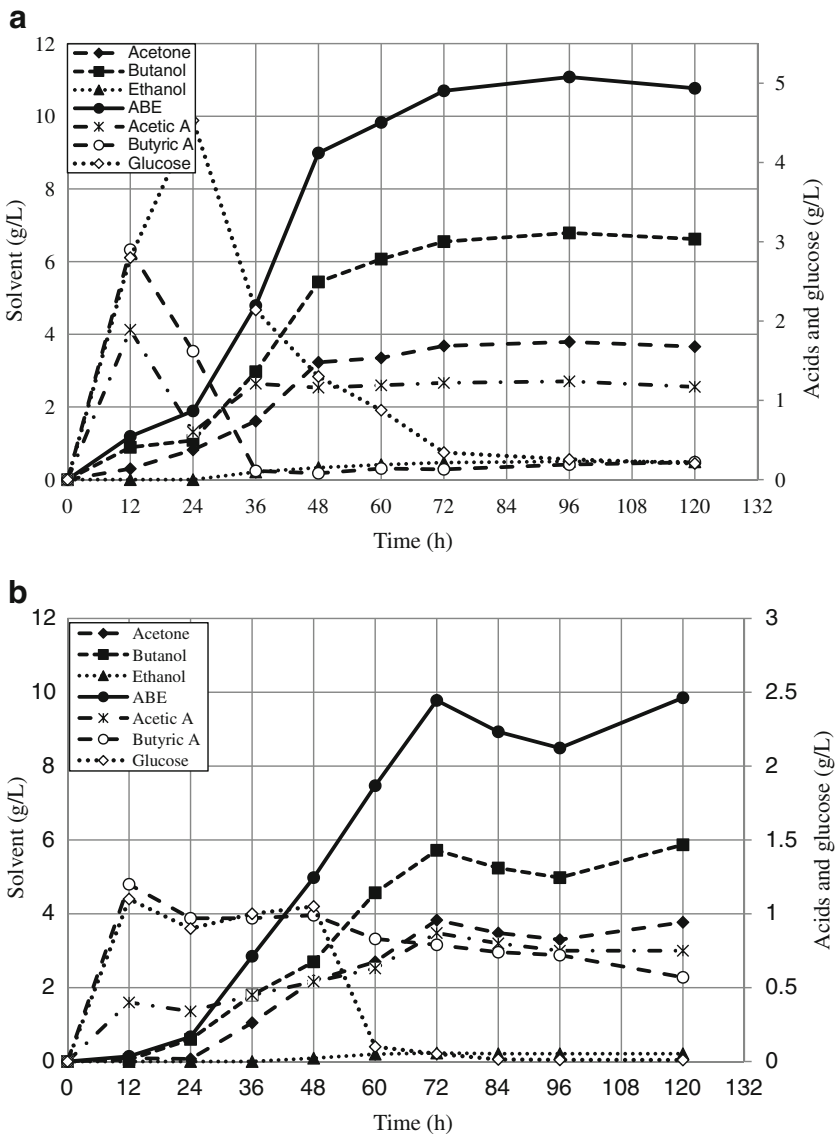

Fig. 4 Profile of ABE fermentation from 60 g/L sago starch by *C. saccharoperbutylacetonicum* N1-4; **a** with P2 medium and **b** with TYA medium

tration (70 g/L sago starch with P2 medium), and the lowest total solvent production was obtained with 30 g/L sago starch. P2 medium supplemented with sago starch at 30 and 60 g/L showed similar ABE productivity (0.12 g/L h), while the same butanol productivity (0.09 g/L h) was noticed with 50 and 70 g/L sago starch.

Using lower initial concentrations of sago starch (30 and 40 g/L) in P2 medium allowed for a faster fermentation time (48 h), which improved ABE productivity. However, butanol and total solvent concentrations were lower than those obtained with higher starch concentrations. This discrepancy may be due to the difference in substrate concentration.

In P2 medium, the glucose concentration in the first 24 h increased as the starch concentration increased up to 60 g/L (i.e., from 0.39 to 4.53 g/L) but then declined to 3.08 g/L when 70 g/L sago starch was used (Table 3).

At the end of the fermentations, the butanol concentration increased from 3.62 to 9.83 g/L as the initial concentration of sago starch added to the P2 medium increased from 30 to 70 g/L. However, the butanol yield based on the initial substrate amount was not enhanced significantly and only varied from 0.11 to 0.14 g/g. The maximum solvent yield observed was 0.24 g/g when P2 medium was supplemented with 70 g/L sago starch; whereas a maximum solvent productivity of 0.18 g/L h was obtained with 40 g/L sago starch was used as the sole carbon source in the P2 medium.

The final solvent and butanol concentrations obtained increased with increasing starch concentration (Table 4). When TYA medium was used, the maximum butanol production (8.11 g/L), maximum butanol productivity (0.07 g/L h), and maximum butanol yield (0.12 g butanol/g starch) were obtained when the sago starch concentration was 70 g/L.

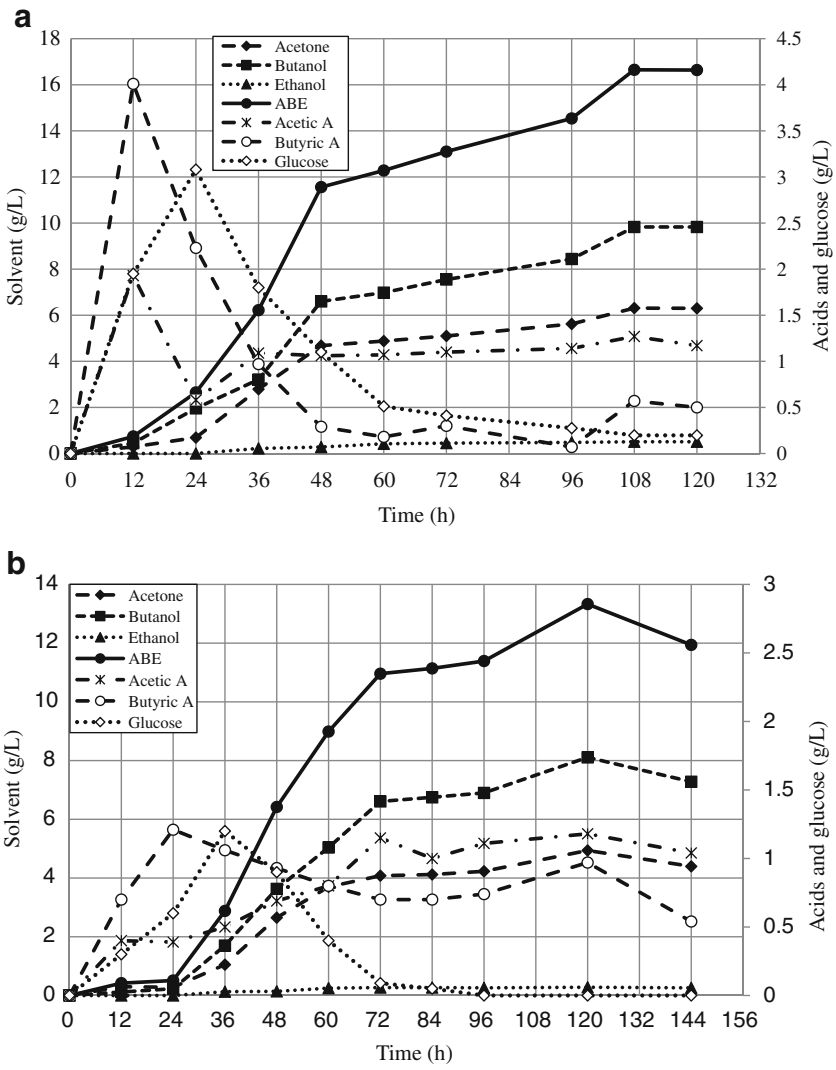

Fig. 5 Profile of ABE fermentation from 70 g/L sago starch by *C. saccharoperbutylacetonicum* N1-4; **a** with P2 medium and **b** with TYA medium

During the fermentation of sago starch in TYA medium, there was an increase and then decrease in solvent production, and the final solvent and butanol productivity were low. In TYA medium, the highest ABE productivity was measured as 0.12 g/L h when 30 and 40 g/L of sago starch were fermented for 48 and 60 h, respectively. In

Table 3 Concentrations of glucose and maltose measured in the medium during ABE fermentation of sago starch by *C. saccharoperbutylacetonicum* N1-4

Sago starch concentration (g/L)	12 h		24 h		Final	
	Glucose (g/L)	Maltose (g/L)	Glucose (g/L)	Maltose (g/L)	Glucose (g/L)	Maltose (g/L)
30+P2	1.41	1.32	0.39	0.95	0.06	0.30
40+P2	2.97	3.82	2.67	2.71	0.11	0.86
50+P2	2.31	3.21	3.80	4.32	0.16	1.74
60+P2	2.80	4.34	4.53	5.72	0.20	2.30
70+P2	1.95	3.20	3.08	6.01	0.20	2.13
30+TYA	1.56	1.79	0.03	0.14	0.003	0.26
40+TYA	1.21	3.66	0.05	0.08	0.013	5.07
50+TYA	0.61	5.09	1.17	6.40	0.002	0.52
60+TYA	0.90	3.71	1.05	3.82	0.011	0.68
70+TYA	0.60	3.97	1.09	4.30	0.0	1.87

Table 4 ABE fermentation of sago starch supplemented with P2 and TYA media by *Clostridium saccharoperbutylacetonicum* N1-4

Sago starch (g/L)	Fermentation time (h)	Production (g/L)				Acetic acid (g/L)	Butyric acid (g/L)	Yield (g/g)		Productivity (g/L·h)	
		Acetone	Butanol	Ethanol	ABE			ABE	Butanol	ABE	Butanol
30+P2	48	1.95	3.62	0.18	5.75	0.68	0.54	0.19	0.12	0.12	0.08
40+P2	48	2.93	5.24	0.24	8.41	0.85	0.17	0.21	0.13	0.18	0.11
50+P2	72	3.49	6.20	0.42	10.11	0.97	0.12	0.20	0.12	0.14	0.09
60+P2	96	3.79	6.79	0.50	11.08	1.24	0.19	0.18	0.11	0.12	0.07
70+P2	108	6.31	9.83	0.51	16.65	1.27	0.57	0.24	0.14	0.15	0.09
30+TYA	72	2.29	3.32	0.15	5.76	0.44	0.55	0.19	0.11	0.08	0.05
40+TYA	120	3.03	4.44	0.18	7.65	0.50	0.33	0.19	0.11	0.06	0.04
50+TYA	120	3.52	5.14	0.16	8.82	0.50	0.69	0.18	0.10	0.07	0.04
60+TYA	120	3.77	5.87	0.21	9.85	0.75	0.57	0.16	0.10	0.08	0.05
70+TYA	120	4.94	8.11	0.28	13.33	1.18	0.97	0.19	0.12	0.11	0.07

addition, fermentation with 60 g/L sago starch gave higher ABE productivity (0.14 g/L h) at 72 h compared to 70 g/L sago starch (0.11 g/L h). The highest concentration of ABE was obtained using 70 g/L sago starch medium.

In comparison to fermentations with 30, 40 and 50 g/L sago starch without medium supplementation, the total solvent production was about two times higher when TYA medium was used. However, P2 medium improved the production more than TYA medium, suggesting that supplementation with nutrients is a key factor in the enhancement of butanol or solvent fermentation from sago starch using *C. saccharoperbutylacetonicum* N1-4.

ABE fermentation from sago starch hydrolysate

Sago starch was hydrolyzed enzymatically and supplemented with P2 medium as described in the "Materials and methods".

The parameters of this fermentation are summarized in Table 5, and Fig. 6 shows a time course of fermentation with 30 g/L sago starch hydrolysate. The total solvent and butanol concentrations produced using sago starch hydrolysates were more than that produced from direct fermentation of sago starch at the same concentrations, whereas direct fermentation of 30 g/L sago starch resulted in a shorter fermentation time compared to fermentation with 30 g/L sago starch hydrolysate. Moreover, sago starch hydrolysates gave higher ABE yield and productivity.

In a control experiment using 30 g/L glucose, the total concentration of ABE obtained was 6.53 g/L with a yield of 0.21 g/g. However, fermentation with 30 g/L sago starch hydrolysate produced more solvent (8.4 g/L) and resulted in a higher yield (0.28 g/g), and 50 g/L sago starch hydrolysate produced approximately 50% more butanol than the butanol produced from 50 g/L sago starch (9.81 vs. 6.2 g/L).

Discussion

In this study, among all the starches fermented by *C. saccharoperbutylacetonicum* N1-4, sago starch gave the highest ABE production. In a different study, Madihah et al. (2001) found that corn starch produced more ABE than sago starch when *C. acetobutylicum* P262 was used. In a study with *C. saccharoperbutylacetonicum* N1-4, more solvent was produced using cassava starch as compared to sago starch (Thang et al. 2010).

Different starch sources showed different fermentation behavior, although all fermentations were done under the same conditions. These differences were attributed to the physico-chemical characteristics of the different starches. The starch properties that play an important role in fermentation are factors such as the granule size, amylose and amylopectin content, crystalline structure, and the presence of enzyme inhibitors (Shariffa et al. 2009; Wang et al. 1996).

The average granule size and amylose content of sago starch is 30 μm and 24–31%, respectively (Ahmad et al.

Table 5 ABE fermentation of sago starch hydrolysates supplemented with P2 medium in batch culture of *C. saccharoperbutylacetonicum* N1-4

Sago starch hydrolysate (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE (g/L)	ABE yield (g/g)	ABE productivity (g/L·h)	Butanol yield (g/g)	Butanol productivity (g/L·h)
30	2.38	5.14	0.88	8.40	0.28	0.127	0.17	0.078
50	2.10	9.81	0.80	12.71	0.25	0.177	0.2	0.136

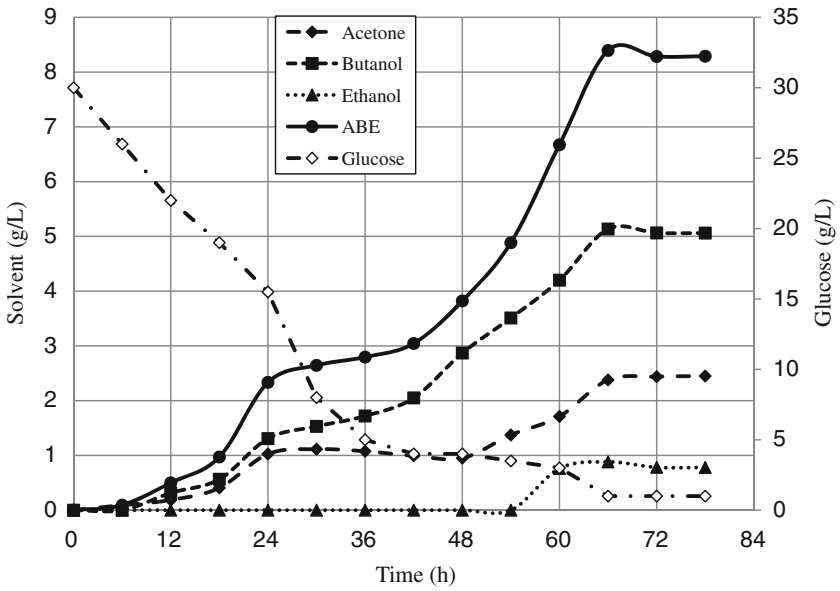

Fig. 6 Fermentation of 30 g/L sago starch hydrolysate to butanol by *C. saccharoperbutylacetonicum* N1-4

1999), while corn starch has a smaller granule size of 15 μm and a amylose content of 28% (Ma et al. 2006). Cassava starch has an even smaller granule size at 14 μm and an amylose content of 20.5% (Thang et al. 2010), whereas tapioca and potato starch have granule sizes of 14.7 and 30.5 μm and amylose contents of 23.7 and 29.3%, respectively (Yuan et al. 2007). Smaller granules have greater surface area which allows more amylase enzymes to bind, and hence allows for more rapidly hydrolysis. This relationship between particle size and hydrolysis has been investigated previously (Cone and Wolters 1990).

The poor production of ABE and butanol from sago starch without nutrient supplementation can be attributed to the lack of trace minerals and a nitrogen source in the starch, which are required for ABE fermentation (Table 2). It is well known that sago starch has a only small amount of nitrogen content (Swinkels 1985: Hipolito et al. 2008). In this study, solvent production was increased with an increase in sago starch concentration up to 60 g/L. Higher concentrations of sago starch decreased the ABE production. This may be due to the high viscosity of gelatinized sago starch, which hinders mass transfer. This problem can be solved by shaking or stirring a tank reactor. The final pH of all concentrations of starches were acidic, which indicated the inability of the *C. saccharoperbutylacetonicum* N1-4 strain to convert or re-assimilate acids to produce solvent due to the lack of nutrients. Accumulation of organic acids during the fermentation of starch has been found to inhibit the cell growth of *C. acetobutylicum*, and therefore sugar utilization results in decreased ABE production (Madihah et al. 2001).

The results indicate that the hydrolysis rate of starch into glucose was larger than the conversion rate of glucose into acids in the first stage of fermentation, and that the increase of glucose with an increase in starch concentration can be attributed to the availability of starch in the medium. The highest concentration of sago starch produced less glucose, which can be attributed to the viscosity which hindered mass transfer of the amylase enzymes in the medium. To counteract this, an agitation speed of 150 rpm was used to increase mass transfer during the fermentation.

In this study, increasing the starch concentration resulted in a prolonged fermentation time. The use of 70 g/L sago starch produced the maximum ABE concentration of 16.65 g/L when supplemented with P2 medium, and this can be explained by the accumulation of solvent. After 108 h, ABE production ceased due to the effect of solvent on *C. saccharoperbutylacetonicum* N1-4 (Fig. 5a). We reported the effect of solvent or butanol on solvent-producing clostridia in a previous study (data will be published elsewhere). We found that at a concentration of 13 g/L butanol, the growth of *C. saccharoperbutylacetonicum* N1-4 was inhibited. These results are in agreement with those reported by Soni et al. (1987).

Notably, ABE fermentation in the TYA medium with various sago starch supplements was much slower than fermentation in P2 medium, which gave higher total ABE and butanol concentrations. TYA medium is known as a typical medium for *C. saccharoperbutylacetonicum* N1-4 (Ishizaki et al. 1999); these results show that P2 is a good medium for butanol production using sago starch by *C. saccharoperbutylacetonicum* N1-4.

The P2 medium is a semisynthetic medium containing buffer, minerals, vitamins, yeast extract and sugar and was actually designed for saccharolytic clostridia (Annous and Blaschek 1990). Supplementation with P2 medium has

been found to enhance ABE production using different substrates such as cassava starch (Thang et al. 2010), maltodextrins (Formanek et al. 1997), and starch packing peanuts (Ezeji et al. 2003) as well as carbohydrates. The results indicate that addition of other nutrients besides sago starch to the fermentation medium improves solvent production. We found that P2 was the best medium for solvent and butanol production from sago starch when the concentration of sago starch was higher than 30 g/L.

Fermentation of 30 g/L glucose produced 6.53 g/L of ABE, while 30 g/L of sago starch produced more ABE (7.71 g/L) (Table 1). These results are in agreement with an earlier study conducted by Madihah et al. (2001). The study stated that fermentation of 30 g/L sago starch produced 11.2 g/L ABE at 42 h when *C. acetobutylicum* was used, while 30 g/L glucose produced 8.2 g/L ABE at 89 h (Madihah et al. 2001). This study also showed that enzymatic hydrolysis of sago starch enhanced solvent production, yield and productivity. However, the *C. saccharoperbutylacetonicum* N1-4 strain can hydrolyze sago starch efficiently and produces ABE. It appears sago starch is a good carbon source for ABE fermentation using solvent-producing *C. saccharoperbutylacetonicum* N1-4, but sago starch needs supplementation with a nitrogen source and other minerals which are required to enhance fermentation.

The main problem with direct fermentation of sago starch using *C. saccharoperbutylacetonicum* N1-4 is that the optimum pH for the amylolytic enzymes to hydrolyze starch to maltose and glucose differs from the optimum pH for solvent production..

This study revealed that manipulation of the medium is an important factor in improving ABE production via direct fermentation of sago starch. Improvement in ABE production via the direct fermentation of sago starch has been reported when a mixture of yeast extract and NH_4NO_3 was used as opposed to yeast extract alone (Madihah et al. 2001). Another possible approach to improve solvent production from sago starch is the development of a pH control strategy (Madihah et al. 2001).

Conclusion

In this study, among the different starchy materials fermented to solvent, sago starch produced the highest amounts of ABE and butanol. The results obtained in this study showed that the solvent production from sago starch fermentation using *C. saccharoperbutylacetonicum* appears to depend on the initial concentration of sago starch. However, this fermentation was limited to 7% sago starch concentration due to deleterious effects from high medium viscosity. Using P2 medium for additional nutrients for sago starch fermentation improved the solvent productivity and yield compared to TYA medium and no medium supplementation. P2 medium with 7% sago starch gave the highest ABE concentration, productivity and yield: 16.65 g/L, 0.15 g/L h and 0.24 g/g respectively. Fermentation of sago starch hydrolyzed by amylolytic enzymes from *C. saccharoperbutylacetonicum* N1-4 resulted in higher solvent productivity compared to fermentations with non-hydrolyzed sago starch.

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