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Production of Acetone, Butanol, and Ethanol as Bioenergy Source Materials by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) using Different Substrates

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In the effort of developing bioenergy source materials, a laboratorium study was performed to investigate the effects of substrate types and concentrations on the production of acetone-butanol-ethanol (ABE) from various substrates by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) in defined TYA (Tryptone-Yeast extract-Acetate) media. The results demonstrated that the ABE ratios produced by the strain N1-4 were greatly influenced by the types of substrate and the concentrations being used. It was also shown that fermentation of disaccharides (cellobiose and dextrin) by the strain N1-4 could give relatively as high butanol and ABE yields as glucose fermentation after 24 h fermentation time. The strain N1-4 was also found to be able to ferment xylans from birchwood sources producing a relatively high ABE concentration, especially at a prolonged incubation time (after 48 h incubation period). A subsequent experiment using several different concentrations of glucose or cellobiose revealed that the higher the concentration of the substrate being used, then the higher the total ABE concentration or productivity could be obtained but not necessarily in the same increasing ratio. To the best of our knowledge, there was no other previous study about the effect of substrate type and concentration on the ABE ratio by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using various substrates in defined TYA media, specifically by using xylans and dextrin substrates.

Key words: ABE ratio, acetone-butanol-ethanol fermentation, biomass substrate effect, *Clostridium saccharoperbutylacetonicum* N1-4

Dalam rangka mendapatkan bahan baku bioenergi, sebuah studi laboratorium dilakukan untuk mengetahui pengaruh dari jenis dan konsentrasi substrat biomasa terhadap proses fermentasi langsung yang menghasilkan aseton, butanol, dan etanol oleh bakteri *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) dalam media TYA (*Tryptone-Yeast extract-Acetate*) yang terdefinisi. Hasil penelitian menunjukkan bahwa rasio dari aceton:butanol:etanol (rasio ABE) ternyata sangat dipengaruhi oleh jenis substrat biomass dan konsentrasi yang dipergunakan dalam proses fermentasinya. Data menunjukkan bahwa dengan menggunakan substrat disakarida (yang diwakili oleh dextrin dan cellobiose), strain N1-4 mampu menghasilkan ABE dengan yield yang relatif sama tinggi dengan menggunakan glukosa setelah masa fermentasi 24 jam. Strain N1-4 ternyata juga mampu memproduksi ABE dengan konsentrasi yang cukup tinggi dengan menggunakan substrat xylan yang diekstraksi dari kayu, walaupun memerlukan masa fermentasi lebih panjang, yaitu 48 jam. Percobaan dengan menggunakan konsentrasi yang berbeda dari substrat biomasa tersebut menunjukkan lebih lanjut bahwa semakin tinggi konsentrasi substrat biomasa yang digunakan, maka produksi ABE akan semakin tinggi pula walaupun tidak selalu dalam rasio yang sama. Sepanjang pengetahuan kami, belum ada studi yang sama dengan penelitian kami ini tentang efek dari jenis dan konsentrasi substrat biomasa terhadap rasio ABE pada proses fermentasi aseton, butanol, dan etanol secara langsung oleh bakteri *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) dalam media TYA yang terdefinisi.

Kata kunci: biomasa, *Clostridium saccharoperbutylacetonicum* N1-4, efek substrat, fermentasi aseton-butanol-etanol, rasio ABE

The cost of raw materials has been known to be the major limitation on the economic feasibility of acetone-butanol-ethanol (ABE) production. About 60-70% of the total ABE production cost is the cost for substrates (Madihah *et al.* 2001). Therefore, many researches have been conducted to find the most economical substrates for ABE production, for examples those who used simple sugars such as glucose, lactose, galactose, and xylose (Shinto *et al.*2008; Keis *et al.* 2001; Bahl *et al.* 1986), or more

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complex substrates such as sago starch (Madihah *et al*. 2001), maltodextrin (Formanek *et al.*1997), packing peanuts (Jesse *et al.*2002), corn starch (McNeil and Kristiansen 1986), maize, and potato starch (Nicole *et al.*1993), as well as xylans from different sources of hemicellulosic residues (Qureshi *et al.* 2006; Lemmel *et al.* 1985), wheat straws (Qureshi *et al.* 2008) and other residues which were used either directly in the fermentation or with different types of pre-treatments before being used in the ABE fermentation process. Most of such experiments employed bacterial strains of *C. acetobutylicum* or *C. beijerinckii*, consequently there are a lot of information regarding the ABE production performed by such bacteria.

On the other hand, the investigations employing strains of *C. saccharoperbutylacetonicum*, in particular the strain N1-4, were still limited. This strain was chosen in our ABE production study because it is hyper-butanol producing strain which can produce a high concentration of butanol (Ogata *et al.* 1982). This strain was first isolated by Hongo *et al*. in 1960 and then patented under US Patent No. 2945786 (Hongo 1960). This strain showed unusually large clostridial forms (spore-containing cells) and differences in substrate utilization (Keis *et al.* 2001). It was also distinguished by a higher butanol/acetone ratio than with other industrial strains, i.e. 4:1 vs. 2:1 (Biebl 1999). Several reports on ABE production employing this strain used only glucose or xylose as the carbon source (Shinto *et al.* 2007; Shinto *et al.* 2008; Tashiro *et al.* 2007) or a limited number of complex substrates such as maize and molasses fermentation media (Shaheen *et al.* 2000), palm oil waste (Lee *et al.* 1995) and excess sludge from a local sewage disposal facility (Kobayashi *et al*. 2005). A previous study by Shinto *et al.* (2008) using a kinetic modeling method with glucose or xylose as the substrate in the TYA media suggested that the strain N1-4 was substrate-dependent. Nevertheless, there was no sufficient information about how the substrate type and concentration could affect the ABE ratio of acetone:butanol:ethanol directly produced by the strain N1-4. Therefore, the objective of this experiment was to investigate the performance of the strain *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) in production of ABE from fermentation process using several carbon sources that have not been investigated previously using this strain, such as cellobiose, dextrin, as well as more complex carbohydrates such as xylan. In particular, the effect of such various substrate types and concentrations on the ABE ratio will also be studied.

MATERIAL AND METHODS

Microorganism Preparation. For a long-term stock culture, *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was previously kept as spores in sterilized sand, while for short-term storage, it was maintained in 15% PG (potato glucose) medium containing substances mentioned in previous reports (Hipolito *et al.* 2008; Tashiro *et al.* 2007). For refreshing the stock culture, per 1 mL of this stock was transferred into 9 mL of fresh PG medium, heat-shocked in boiling water for 1 min, cooled in iced water for several min and anaerobically incubated at 30 °C for 28 h without agitation or pH control. This refresh culture was then transferred into TYA (Tryptone-Yeast extract-Acetate) fresh medium (10% v/v) to pre-culture the bacteria at 30 °C for 15 h without agitation. The TYA medium components per liter of distilled water were 6 g of bactotryptone (Difco), 2 g of yeast extract (Difco), 3 g of CH_3COONH_4, 0.5 g of KH_2PO_4, 0.3 g of $MgSO_4.7H_2O$, and 10 mg of $FeSO_4.7H_2O$. The initial pH of this TYA pre-culture medium was adjusted to 6.5 with 1 N NaOH or 1 N HCl, and glucose was then added into the medium to constitute a 20 g L^{-1} gLucose concentration before it was sterilized at 115 °C for 15 min.

Main Culture Fermentation. Main culture media for the fermentation experiment were prepared similarly to the pre-culture medium except the use of various substrates as the carbon resources, in addition to glucose as the control substrate. During the experiment dextrin, cellobiose, and xylan were used in addition to glucose as the control substrate. Each of such carbon sources was a laboratory graded chemical and was added as a concentrate and diluted with distilled water to a final total reducing sugar concentration of 10 or 20 g L^{-1}. A separate experiment was also performed to investigate if the multiplication of increasing substrate concentration will also affect the multiplication of the increasing ABE ratio by applying different concentrations of glucose and cellobiose (i.e. 10, 20, and 40 g L^{-1} of glucose; 10, 20, and 40 g L^{-1} of cellobiose). The main culture medium for each carbon substrate was prepared in a 500 mL flask with the working volume of 250 mL and the initial pH of 6.5 adjusted by adding 1 N NaOH or 1 N HCl, after which was sterilized at 115 °C for 15 min. The inoculum was 10% (v/v) of the culture volume. Following inoculation, the broth was sparged with filtered oxygen-free nitrogen gas for 20 min to maintain strict anaerobic conditions. All cultivations

were static batch fermentations conducted anaerobically at 30 °C without pH control and agitation for a total fermentation period of 24 h or 48 h.

Sampling and Analysis. Sampling was performed periodically at every 6 h. One set of the samples was centrifuged with 15 000 rpm (or 20 400 G) at 4 °C for 10 min using a high speed refrigerated micro centrifuge (TOMY MX-300; TOMY TECH, U.S.A. Inc., Tokyo, Japan) and supernatants were obtained. The supernatants were analyzed for the acids and ABE concentrations using a gas chromatograph (6890A; Agilent Technologies, Palo Alto, Ca, USA) equipped with a flame ionization detector and a 15-m capillary column (Innowax; i.d. 0.53 mm; 19095N-121; Agilent Technologies) using isobutanol as the internal standard with 1 M HCl (Tashiro *et al.* 2004). The glucose concentration in the supernatant was determined with a glucose analyzer (Biosensor BF-5; Oji Scientific Instrument, Osaka, Japan). Another set of the samples was directly analyzed for the pH, the bacterial density and the total sugar concentration. The pH values were measured using the pH-meter. The bacterial growth was monitored over time as the culture turbidity (OD 562 nm) with a spectrophotometer (V-530; JASCO, Tokyo, Japan). The residual total sugar was measured by a spectrophotometer (V-530) applying the phenol-sulfuric-acid method described in detail elsewhere (Dubois *et al.* 1956).

Calculation. As mentioned previously (Jesse *et al.* 2002), the ABE concentration (g L^{-1}) was defined as the difference between the ABE concentration at the indicated fermentation time and that at the beginning of fermentation period. The ABE yield was calculated as the ABE (g L^{-1}) produced at the indicated fermentation time divided by the total sugar (g L^{-1}) being utilized at the same period (Formanek *et al.* 1997). These definitions were also used to calculate the concentration of fermentation products (acetone, butanol, or ethanol) and the butanol yield. The ABE productivity was defined as the ABE concentration (g L^{-1}) produced per hour. The ABE ratio was defined as the ratio of acetone:butanol:ethanol by using butanol as the standard of calculation.

RESULTS

Effect of Substrate Type and Concentration on Direct ABE Fermentation by the Strain N1-4. The data clearly show that the ABE ratio obtained in the fermentation by the strain N1-4 using different substrate types but the same concentrations, as well as by using the same substrate type but different concentrations, generally could be varied (Table 1). Particularly between glucose and cellobiose, the ABE ratio of 0.22:1:0 using 10 g L^{-1} glucose could be changed into 0.29:1:0.05 ratio using 20 g L^{-1} glucose, further which could be changed into 0.30:1:0.06 ratio by using 10 g L^{-1} cellobiose or into 0.41:1:0.07 ratio by using 20 g L^{-1} cellobiose. The difference of ABE ratio between glucose and dextrin fermentation was not relatively significant, that was 0.22:1:0 with 10 g L^{-1} of either glucose or dextrin and 0.29:1:0.05 with 20 g L^{-1} of glucose compared to 0.24:1:0.04 with 20 g L^{-1} of dextrin. On the other hand, the ABE ratio between glucose fermentation and xylans fermentation was significantly varied, that was 0.26:1:0 or 0.44:1:0 using 10 g L^{-1} or 20 g L^{-1} of xylans, respectively, compared to those of glucose fermentation, that was 0.22:1:0 or 0.29:1:0.05 using 10 g L^{-1} or 20 g L^{-1} of glucose, respectively.

There was also a difference in the product concentrations of the strain N1-4 between glucose and cellobiose fermentation (Fig 1 and Table 1). The ABE concentration produced by the strain N1-4 using both concentrations of cellobiose (10 g L^{-1} or 20 g L^{-1}) as the substrate was higher (4.1 g L^{-1} or 9.5 g L^{-1}) than those using the same concentrations of gLucose (3.5 gL^{-1} or 8.9 g L^{-1}). All concentrations of acetone, butanol, and ethanol produced in cellobiose fermentation were higher than those in glucose fermentation, except the butanol concentration in the fermentation of 20 g L^{-1} glucose (6.6 g L^{-1}) which was slightly higher than that in the fermentation of 20 g L^{-1} cellobiose (6.4 g L^{-1}). In addition, the yields of butanol and total ABE as well as the ABE productivity at 24 h incubation period were all relatively higher in cellobiose fermentation than those in glucose fermentation.

The results of this experiment also confirmed that the strain N1-4 could utilize the xylans from birchwood. The xylans fermentation by this strain N1-4 was producing lower concentrations of acetone, butanol, and ethanol than the glucose fermentation after 24 h incubation period (Table 1 and Fig 1). Interestingly, there was a significant increase in the fermentation products between 24 and 48 h incubation period using the xylans by the strain N1-4. After 24 h incubation period, the strain N1-4 could only produce 0.8 g L^{-1} and 1.4 g L^{-1} total ABE concentrations by using 10 g L^{-1} and 20 g L^{-1} xylans, respectively (Table 1). However, after 48 h incubation period, the total ABE concentrations increased up to 1.1 and 3.2 g L^{-1} (or increase up to 36% and 124%) by using 10 and 20 g L^{-1} xylans, respectively (data not shown).

Table 1 Effect of substrate type on ABE production and ABE ratio by *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 after 24 h (substrate concentration 10 or 20 g L^{-1}; working volume 250 mL)

C-Sources	Acetone (g L^{-1})	Butanol (g L^{-1})	Ethanol (g L^{-1})	ABE (g L^{-1})	Consumed substrate (g L^{-1})	A:B:E ratio	Butanol yield (ggT S^{-1})	ABE yield (ggT S^{-1})	ABE productivity (g L^{-1} h^{-1})
Glucose10	0.6	2.9	0	3.5	10	0.22:1:0	0.28	0.34	0.15
Glucose20	1.9	6.6	0.4	8.9	22	0.29:1:0.05	0.31	0.41	0.37
Cellobiose10	0.9	3.0	0.2	4.1	10	0.30:1:0.06	0.29	0.39	0.17
Cellobiose20	2.6	6.4	0.4	9.5	20	0.41:1:0.07	0.32	0.47	0.40
Dextrin10	0.5	2.2	0	2.6	7.6	0.22:1:0	0.28	0.35	0.11
Dextrin20	1.3	5.6	0.2	7.1	22	0.24:1:0.04	0.26	0.33	0.30
Xylan10	0.2	0.6	0	0.8	4.8	0.26:1:0	0.13	0.16	0.03
Xylan20	0.4	1.0	0	1.4	7.8	0.44:1:0	0.13	0.18	0.06

Note: number following the substrate indicate the concentration of the substrate

Table 2 Effect of glucose or cellobiose concentrations on the ABE fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 after 24 h (working volume 250 mL)

C-sources	Acetone (g L^{-1})	Butanol (g L^{-1})	Ethanol (g L^{-1})	ABE (g L^{-1})	Consumed substrate (g L^{-1})	A:B:E ratio	Butanol yield (ggT S^{-1})	ABE yield (ggT S^{-1})	ABE productivity (g L^{-1} h^{-1})
glucose10	0.6	2.9	0	3.5	10	0.22:1:0	0.28	0.34	0.15
glucose20	1.7	6.1	0.3	8.1	20	0.28:1:0	0.30	0.40	0.34
glucose40	2.8	9.0	0.6	12.4	31	0.31:1:0.07	0.29	0.40	0.52
cellobiose10	0.9	3.0	0.2	4.1	10	0.30:1:0.06	0.29	0.40	0.17
cellobiose20	1.9	6.0	0.4	8.3	20	0.31:1:0.06	0.300	0.41	0.34
cellobiose40	3.0	8.8	0.5	12.3	37	0.35:1:0.06	0.240	0.34	0.51

Note: number following the substrate indicate the concentration of the substrate in g L^{-1}

There was different pattern of total sugar consumption and the bacterial density (OD_{562}) in xylans fermentation by the strain N1-4 compared to those of the other substrates (Fig 2A,B). There was a long lag period up to 12 h before the strain started to grow exponentially until 24 h incubation period and then entered the stationary phase. In contrast, by using the other substrates the strain N1-4 could directly grow exponentially until 18 h incubation period before the bacterial density declined.

The pH change in the xylans medium was also different from those in the other media (Fig 2C). The pH in xylans medium was relatively constant up to 12 h incubation period and start decreasing until 24 h incubation period before increasing again, while the pH in the other substrates media showed the similar decrease up to 6 h of incubation period which was then followed by the similar increase until the end of incubation period.

There was different performance of strain N1-4 in the utilization or production of acids and total organic acids using different substrates (Fig 3). The acetate reutilization in xylans fermentation was much lower than those of the other substrates (Fig 3A). On the other hand, the butyrate production was much higher in xylans fermentation than in the fermentation of other substrates being used in this experiment (Fig 3B), especially up to 24 h, after which the butyrate started to being used. Nevertheless, the acetate and butyrate were still present in the media until the end of fermentation time. Fig 3C shows the reutilization of total organic acids using different substrates.

Effect of Substrate Increasing Concentrations on ABE Production by the Strain N1-4. The results show that the increasing substrate concentrations could significantly increase the total ABE concentration, but not necessarily in the same increasing ratio. In glucose fermentation, the total ABE concentration could increase from 3.5 g L^{-1} to 8.1 and 12.4 g L^{-1} (131% and 254% increase) by increasing the initial substrate from 10 g L^{-1} to 20 and 40 g L^{-1}, respectively (Table 2). Similarly, in cellobiose fermentation using the

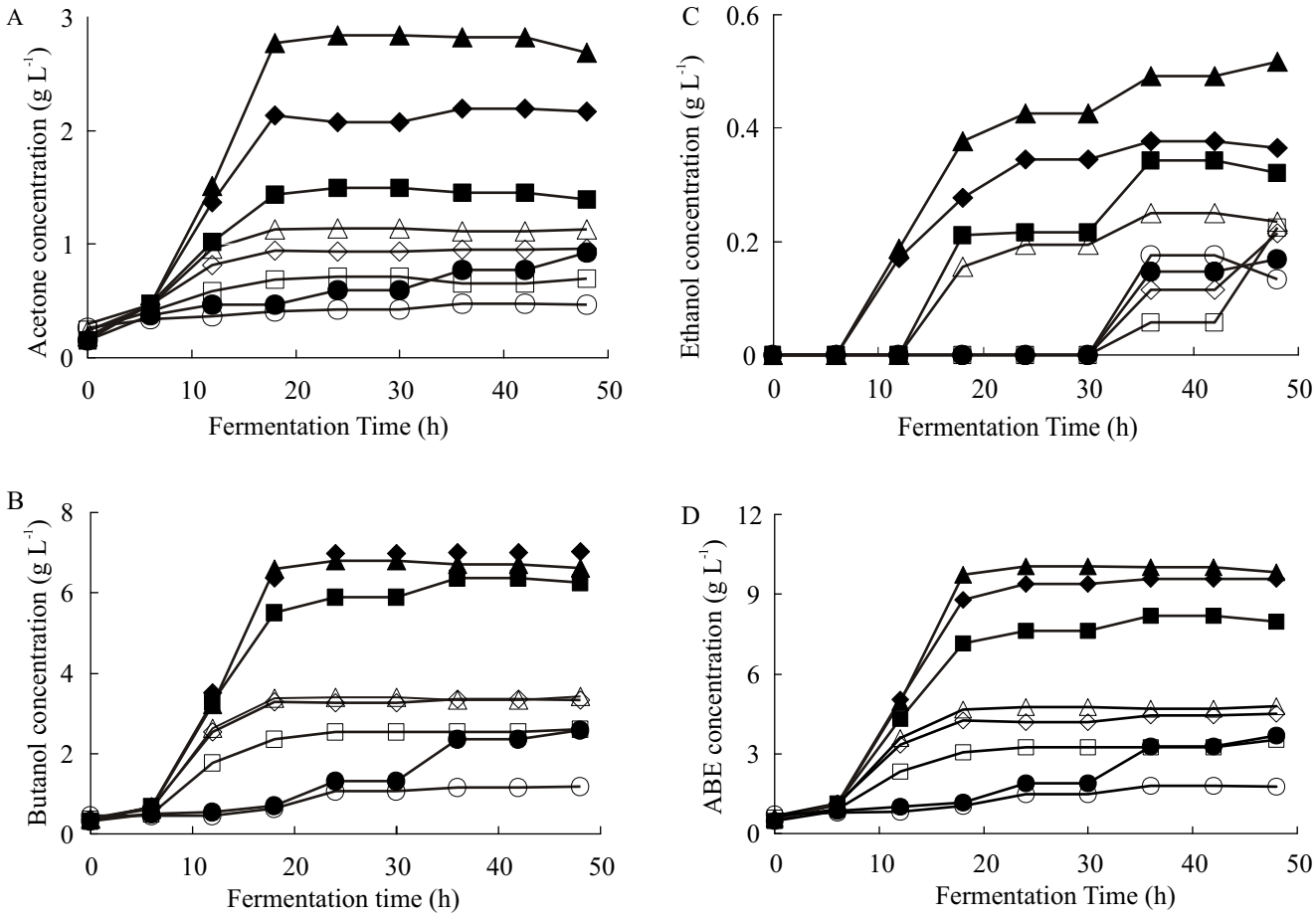

Fig 1 Concentrations of acetone (A), butanol (B), ethanol (C), and total ABE (D) directly produced by *C. saccharoperbutylacetonicum* N1-4 ATCC 13564 using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 mL; static batch culture without pH control. Symbols: (◇) glucose 10 g L^{-1}; (◆) glucose 20 g L^{-1}; (△) cellobiose 10 g L^{-1}; (▲) cellobiose 20 g L^{-1}; (□) dextrin 10 g L^{-1}; (■) dextrin 20 g L^{-1}; (○) xylan 10 g L^{-1}; (●) xylan 20 g L^{-1}.

increasing initial substrate concentrations from 10 g L^{-1} to 20 and 40 g L^{-1}, the total ABE concentrations could be enhanced from 4.1 g L^{-1} to 8.3 and 12.3 g L^{-1}, or there was an increase of 102% and 200%, respectively. The ABE ratio in glucose fermentation was also significantly affected by the increasing initial substrate concentration, but not in the case of cellobiose fermentation. The yields of butanol and total ABE were also not affected by the increasing initial substrate concentrations, but more likely to be influenced by the total sugar (substrate) being consumed by the strain N1-4. By using 40 g L^{-1} of substrate, the total sugar utilization by the strain seemed to be incomplete, as there was some residual substrate left in the glucose or cellobiose media.

DISCUSSION

There have been a number of previous studies about the effect of nutrient, including the carbon substrate, on the onset and maintenance of solvent production, in both batch- and continuous-culture systems (Jones and Woods 1986; Amador-Noguez *et al*. 2010; Heluane *et al*. 2011; Ambarsari and Sonomoto 2012). In batch culture, it was reported in several previous studies employing *C. acetobutylicum* strains that only acids were produced when the concentration of the carbon source was limited (Long *et al.* 1984; Monot *et al.* 1982). In particular, when glucose was present below the threshold level of about 10 g L^{-1}, then no shift to solvent production was obtained since all the glucose was consumed during the acidogenic phase and cell yield was reduced (Long *et al.* 1984). Similar results were also obtained in continuous cultures, such that it is now generally accepted that, under conditions of carbon source limitation, there is insufficient amount of acid end products which can be generated to reach the threshold concentration needed to induce the solvent production (Jones and Woods 1986). Nevertheless, as far as we know, there were no other studies previously done to investigate the effect of substrate type and

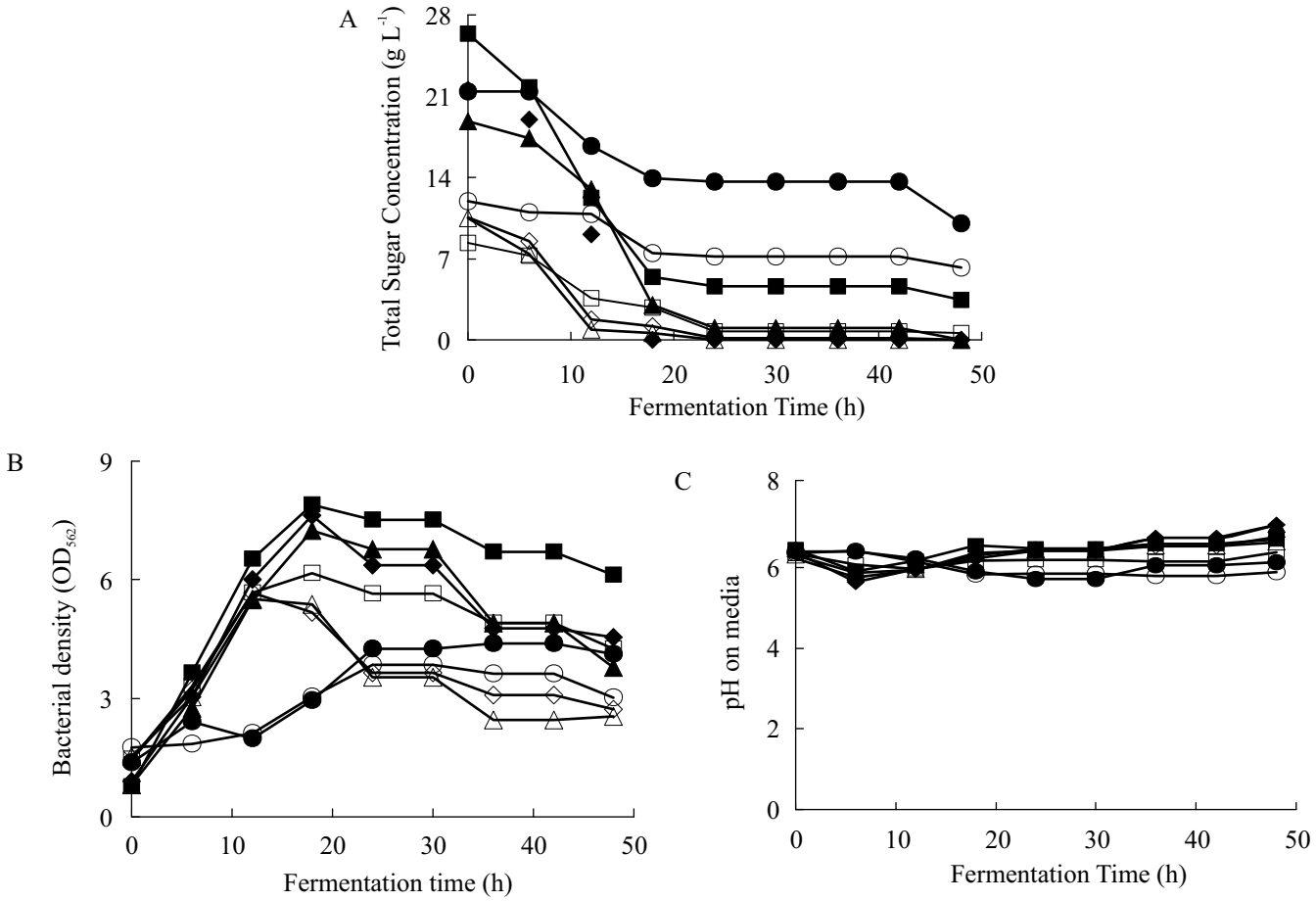

Fig 2 Total sugar consumption (A), bacterial density (A), and pH (C) during direct ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 ATCC 13564 using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 mL; static batch culture without pH control. Symbols: (◇) glucose 10 g L^{-1}; (◆) glucose 20 g L^{-1}; (△) cellobiose 10 g L^{-1}; (▲) cellobiose 20 g L^{-1}; (□) dextrin 10 g L^{-1}; (■) dextrin 20 g L^{-1}; (○) xylan 10 g L^{-1}; (●) xylan 20 g L^{-1}

concentration using dextrin and xylans on the ABE ratio from the fermentation process by the strain *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564).

It was found in our experiment that the ABE ratio produced by the strain N1-4 was very dependent on the type and concentration of substrates being used. These results were in accordance with other previous studies which suggested that depending on the nature of the carbohydrate and the culture conditions, the ratio of conversion to solvents can vary (Monot *et al.* 1982; Taha *et al.* 1973; Bahl *et al.* 1986). Our results were also in agreement with those of Awang *et al.* (1992) employing *C. acetobutylicum* P262. They found that butanol concentration was more related to the type and amount of carbohydrate utilized. A previous report from our laboratory by applying a sensitivity analysis using glucose and xylose as the substrates also revealed that the strain N1-4 could produce higher butanol in a substrate-dependent pathway (Shinto *et al.* 2008; Shinto *et al.* 2007).

Moreover, although it was reported previously that in the fermentation broth employing *C. acetobutylicum* or *C. beijerinckii*, the typical ABE ratio was 3:6:1 (Qureshi *et al.* 2008; Qureshi *et al.* 2006) or 0.5:1:0.17 with butanol as the major product, however it can be noted in our experiment that the strain N1-4 could vary the ABE ratio depending on the type and concentration of the substrates being utilized. Therefore, it can be hypothesized that the ABE ratio obtained in an ABE fermentation by a certain bacterial strain could be changed by changing the substrate type and concentration. Our hypothesis was supported by the results from our experiments employing *C. saccharoperbutylacetonicum* N1-4 ATCC 13564, also from previous works which suggested that according to the culture conditions, butanol and acetone production from *C. acetobutylicum* (Matta-El-Ammouri *et al.* 1987) or *C. thermocellum* (Brener and Johnson 1984) could be different.

It was also confirmed in our experiment that the

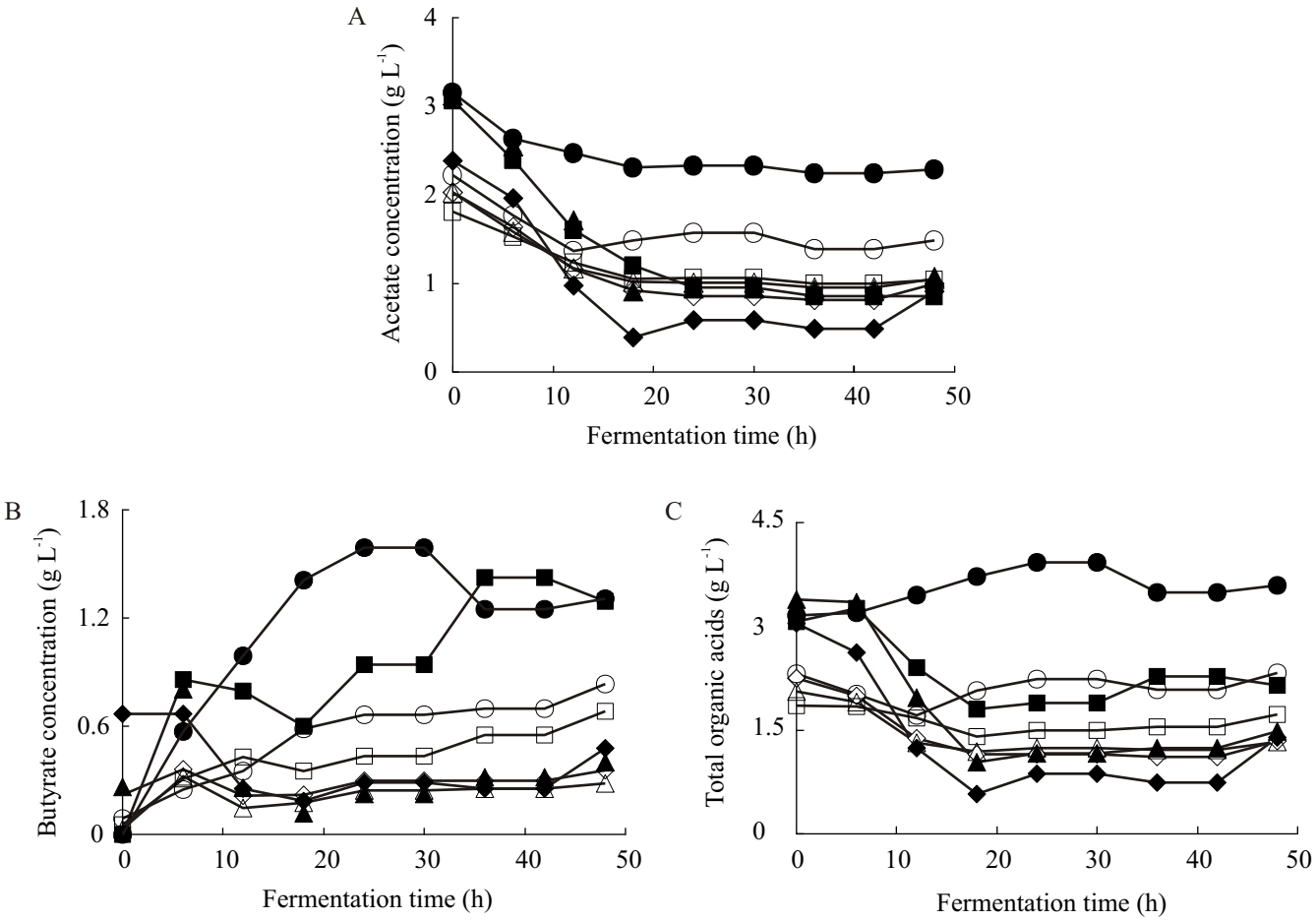

Fig 3 Acetate concentration (A), butyrate concentration (B), and consumption of total sugar (C) during ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 ml; static batch culture without pH control. Symbols: (◇) glucose 10 g L^{-1}; (◆) glucose 20 g L^{-1}; (△) cellobiose 10 g L^{-1}; (▲) cellobiose 20 g L^{-1}; (□) dextrin 10 g L^{-1}; (■) dextrin 20 g L^{-1}; (○) xylans 10 g L^{-1}; (●) xylans 20 g L^{-1}.

concentrations of acetone, butanol, and ethanol as well as the butanol yield, total ABE yield and productivity in cellobiose fermentation by the strain N1-4 were indeed relatively higher than those in glucose fermentation. On the other hand, by using dextrin or xylan, the strain N1-4 could produce such products in lower values than by using glucose. The higher production in cellobiose fermentation than in glucose fermentation by the strain N1-4 was similar to the results obtained in a previous study (Awang *et al.* 1992) employing *C. acetobutylicum* P262. In that study, they demonstrated that fermentation of cellobiose by *C. acetobutylicum* P262 led to conditions resulting in complete acid reutilization and the highest butanol concentration, while glucose had a greater enhancing effect on the sporulation process than starch and cellobiose which in turn could lower the butanol production.

The better performance of our strain in cellobiose fermentation than in glucose fermentation was also in accordance with the results from other previous studies employing *C. thermocellum* (Ng and Zeikus 1982) and a cellulolytic mesophilic *Clostridium* sp. Strain H10 (Giallo *et al.* 1983). In those studies, it was demonstrated that by *C. thermocellum* the substrate uptake rates in cellobiose- and glucose-grown cell suspensions were 18 and 17 nmol min^{-1} mg^{-1} (dry weight), respectively and the molar yields were 38 on cellobiose and 20 on glucose, while the cellulolytic mesophilic *Clostridium* sp. strain H10 presented a better adaptation to cellobiose than to glucose, producing higher fermentation products in cellobiose than in glucose fermentation. It was suggested from those studies that the better yield obtained on cellobiose may be the result of a higher level of phosphorylation if a cellobiose phosphorylase occurs. We suggested that a further experiment was then needed to investigate the type of enzyme involved in the cellobiose direct utilization by the strain N1-4, whether it is cellobiase as detected previously in *C. acetobutylicum* (Lee *et al.* 1985) or cellobiose phosphorylase as detected in *C. thermocellum* or in the

cellulolytic mesophilic *Clostridium* sp. Strain H10.

Reutilization of acetate by the strain N1-4 using glucose, cellobiose, and dextrin was relatively in a similar rate (Fig 3A), even though the production of acetone using such substrates varied significantly (Fig 1A). These suggested that the acetate reutilization in this strain was partly contributing the production of acetone by using such substrates. In other words, it was suggested that the strain N1-4 could produce acetone by not only reutilizing the acetate in the media, but also by consuming the total sugars still available during the same period of fermentation (Fig 2A). This suggestion was supported by early workers who had observed that once the solventogenesis occurred, the acids produced during the acidogenesis phase were reassimilated, however, the uptake of acetate and butyrate only happened when sugars were metabolized concomitantly (Jones and Woods, 1986).

Butyrate uptake in cellobiose fermentation until 24 h incubation period was higher than that in glucose and dextrin fermentation (Fig 3B), which could be the cause of the higher production of acetone and butanol in cellobiose fermentation than in glucose and dextrin fermentation (Fig 1A, Fig 1B, and Fig 2A). These results supported the conclusion made in previous study (Hartmanis *et al.* 1984) stating that the uptake of acids and the formation of acetone are coupled and that there could not be any uptake of acetate or butyrate without the formation of an equivalent amount of acetone. Also it was suggested before that in contrast to acetic acid (acetate), which specifically increases acetone formation, butyric acid (butyrate) increases both acetone and butanol formations (Matta-El-Ammouri *et al.* 1987). The previous report from our laboratory using the same strain with glucose as the substrate in a pH-stat continuous culture system also demonstrated that the presence of butyrate in the broth could induce and promote the butanol production (Tashiro *et al.* 2004). Another recent study also indicated that addition of 4 g L^{-1} butyric acid to TYA medium could strongly improve the butanol and total ABE production, suggesting that butyric acid could be potentially used as co-substrate for butanol production without contributing any remarkable inhibitory effects on cell growth (Al-Shorgani *et al.* 2012).

Ethanol concentrations in ABE fermentation by the strain N1-4 using all utilized substrates in these experiments were lower than acetone concentrations (Table 1 and 2) and required longer time than acetone and butanol production (Fig 1C). These results were similar with the results from other previous experiments employing various clostridial strains, in which the ethanol was always the lowest amount produced during the ABE fermentation (Jones and Woods 1986). Our previous report also indicated that the ethanol production in the presence of butyric acid was lower than that in its absence (Tashiro *et al.* 2004).

The data in Table 2 clearly indicated the effect of increasing substrate concentrations on the ABE production during fermentation process by the strain N1-4. Our results show that the higher the substrate concentration, the higher the total ABE concentration could be produced, but not necessarily in the same increasing ratio. However, the yields of butanol and total ABE were not affected by the increasing substrate concentrations, especially in cellobiose fermentation. As indicated in our another separated experiment, even by using the excessive cellobiose (80 g L^{-1}), the yields of butanol and total ABE were the lowest, whilst on the other hand, by using 5 g L^{-1} (or 0.5%) the yields of butanol and total ABE were the highest, which was similar with the result from another previous study by Brener and Johnson (1984) employing *C. thermocellum* to ferment a broad range of cellobiose concentrations. In that study, it was found that the bacteria did not grow at 5.0% cellobiose and the ethanol accumulation was maximal (38.3 mol/10^9 cells) in cultures using an initial cellobiose concentration of 0.8%, but only 17.3 mol using 2.0%.

Our results obtained by using the increasing glucose concentrations were similar to the results obtained by Shaheen *et al.* (2000), in which the increasing glucose concentrations caused the increasing solvent concentration and yield produced by *C. saccharoperbutylacetonicum* N1-4, and the glucose concentration of 4% (or 40 g L^{-1}) was suggested to be the optimum concentration for glucose fermentation by this strain. Cellobiose was chosen as the carbon source in this separate experiment because it is a hydrolysis product of cellulose which mostly contained in the abundant lignocellulosic materials, and the use of this carbohydrate could illustrate the potential to ferment sugars derived from hydrolysates of agricultural residues to butanol (Qureshi and Ezeji 2008).

In contrast, the concentrations of acetone, butanol, and ethanol (Fig 1) as well as the total sugar consumption (Fig 2A) in xylan fermentation by the strain N1-4 were much lower than those in glucose, cellobiose, and dextrin fermentations. The butyrate production was much higher even though needed a longer period in xylan fermentation than that in the other substrates fermentation (data not shown). These

results were compliant with those of a previous study which pointed out that the accumulation of organic acids (acetate and butyrate) greatly influenced the solvent production, in which the higher amounts of such acids appear to inhibit cell growth, sugar consumption and hence, reduce the total solvent production (Madihah *et al.* 2001). Our results in xylan fermentation were also similar those obtained previously by other researchers employing *C. acetobutylicum* (ATCC 39236), in which the organic acids were mainly produced with only traces of acetone, butanol, or ethanol if xylans were directly used (Lemmel *et al.* 1986). They also found that xylans fermentations were characterized by long lag times, slow fermentation rates (i.e. 190-240 h total fermentation time compared to 48-72 h when using starch as the carbohydrate source), and low xylanase activities. In addition, they never observed the complete consumption of xylans even though different types of xylans were used in their experiment, indicating that the limited amount of xylans fermented is probably due to the nature of the xylanase activity produced, not the growth conditions or media being used.

Interestingly, the bacterial curve obtained in the fermentation of 20 g L^{-1} xylans showed a diauxie growth with much lower bacterial density than the others (Fig 2B). This phenomenon was maybe due to the effect of the slower total sugar consumption (Fig 2A) in xylans fermentation. The first phase of bacterial growth (during the 6 h fermentation period) in xylans fermentation might use the little amount of solubilized xylans available in the beginning of fermentation period due to the autoclaved auto hydrolysis, such in the same way as the xylans solubilization due to the hot-liquid-water (LHW) pretreatment previously mentioned in a review on lignocellulosic pretreatments, which stated that due to the high water input in LHW pretreatment, the yield of solubilized (monomeric) xylans is generally also high (Hendriks and Zeeman 2009). This assumption was also supported by the data obtained in our separated experiment, which show that by using the very small working volume (10 mL) the consumed xylans after 24 h fermentation was much lower than that in the experiment using a higher working volume (250 mL) (Ambarsari *et al.* 2010). The second phase of bacterial growth was then suggested to be caused by the concurrent consumption of total sugar and acids in xylans fermentation as happened previously in another study (Monot *et al.* 1984).

Based on the results of our experiment, it could be suggested that the different performances on ABE production during fermentation process by the strain N1-4 using various types and concentrations of the substrates, in particular between the glucose and cellobiose fermentation, might be related to the different substrate uptake systems in the strain N1-4 as previously demonstrated in *C. thermocellum*. Previous kinetic studies on *C. thermocellum* supported our hypothesis, which indicated that cellobiose and larger cellodextrin were taken up by a common uptake system, while glucose entered via a separate mechanism (Strobel *et al.* 1995). They proposed a revised model for carbohydrate transport and utilization which incorporated the elements of ATP-driven transport and internal phosphorylytic cleavage of intact-linked carbohydrates. Their model could predict higher yields on oligosaccharides compared with that on the monosaccharide, if one assumes that an ATP molecule is required for uptake of either glucose or other larger carbohydrates. It was also proposed in another study that phosphorylytic cleavage of cellobiose partially conserves the energy of the glycosidic bond as glucose-1-phosphate, and only one ATP per cellobiose is required to form two activated glucose molecules. In contrast, hydrolytic cleavage of cellobiose leads to the consumption of two ATP for the activation of the resulting glucose. Thus, 0.5 ATP per activated hexose is theoretically conserved through phosphorylytic cleavage, and this comparison predicts that bacterial yields on cellobiose would be greater than on glucose (Strobel 1995).

Past work with *C. thermocellum* did indeed demonstrated that higher yields (Ng and Zeikus 1982) and lower maintenance energy coefficients were seen in cells grown on cellobiose versus glucose (Strobel 1995). Additionally, it was previously suggested that it was more efficient to take up an intact oligomer rather than cleave it extracellularly and transport the monomer sugar. It was then proved in *C. thermocellum* that the cellobiose and cellodextrin phosphorylase activities were detected in the cytosol and were not associated with cell membranes, meaning that the phosphorylation of carbohydrates occurred intracellularly (Strobel *et al.* 1995). A recent study performed by Servinsky *et al.* (2010) reported that *C. acetobutylicum* ATCC 824 can uptake cellobiose directly by the phosphotransferase systems (PTSs) and hydrolyzes cellobiose to glucose by the intracellular β-glucosidases for further metabolism (Noguchi *et al.* 2013). Finally, a further experiment needs to be performed in *C. saccharoperbutylacetonicum* N1-4

ATCC 13564 to investigate what type of enzyme involved in the direct utilization of cellobiose by the strain, whether it is cellobiase like in *C. acetobutylicum* (Allcock and Woods 1981; Lee *et al.* 1985) or cellobiose phosphorylase like in *C. thermocellum* as described above, and where the enzyme activity takes place, whether extracellularly, intracellularly, or in the cell membrane.

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