ACETONE-BUTANOL-ETHANOL FERMENTATION FOR BIOENERGY USING VARIOUS SUBSTRATES IN DEFINED TYA MEDIA

Fermentasi Aseton-Butanol-Etanol untuk Bioenergi Menggunakan Berbagai Jenis Substrat dalam Media TYA Terdefinisi

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Abstrak

Serangkaian eksperimen telah dilakukan untuk meneliti kinerja dari Clostridium saccharoperbutylacetonicum strain N1-4 (ATCC 13564) dalam proses fermentasi aseton, butanol, dan etanol (ABE) menggunakan beberapa substrat yang belum pernah digunakan dalam penelitian-penelitian sebelumnya, seperti selobiose, dekstrin, berbagai macam tepung seperti jagung, kentang, tapioca, gandum dan sagu, serta karbohidrat kompleks lainnya seperti xylans, selulosa, dan carboxyl methyl cellulose (CMC). Penelitian ini khususnya untuk mempelajari pengaruh tipe, konsentrasi, dan rasio campuran dari berbagai substrat tersebut terhadap rasio dan produktivitas ABE yang dihasilkan. Hasil-hasil penelitian menunjukkan bahwa strain N1-4 ini dapat secara langsung memproduksi ABE dari berbagai macam substrat, dari jenis monosakarida sampai polisakarida berantai karbon enam (hexose) atau lima (pentose), kecuali CMC dan selulosa. Hasil penelitian juga membuktikan bahwa selobiosa, sebuah disakarida berantai karbon enam (hexose) dapat menjadi substrat yang lebih efisien untuk strain N1-4 daripada glukosa (sebuah monosakarida hexose) untuk memproduksi ABE di dalam medium TYA (Tryptone Yeast Agar). Terbukti pula bahwa rasio dari ABE yang diproduksi dalam proses situ sangat tergantung pada jenis dan konsentrasi dari substrate yang digunakan oleh strain N1-4.

Kata kunci: *aseton, butanol, etanol, clostridium saccharoperbutylacetonicum N1-4, fermentasi.*

Abstract

A series of experiments had been conducted to investigate the performance of *Clostridium saccharoperbutylacetonicum* strain N1-4 (ATCC 13564) in direct fermentation of acetone, butanol, and ethanol (ABE) using several substrates that have not been investigated previously using this strain, such as cellobiose, dextrin, starches of corn, potato, tapioca, wheat, and sago, as well as more complex carbohydrates such as xylans, cellulose, and carboxyl methyl cellulose (CMC), particularly to study the effect of such various substrate types and concentrations, as well as the substrates mixtures on the ABE ratio and productivity. The results showed that strain N1-4 could directly produce ABE from various substrates, from monosaccharide to polysaccharides of hexose or pentose sugars, except CMC and cellulose. The experiments also suggested that cellobiose (a hexose disaccharide) could be the more efficient substrate for strain N1-4 than glucose (a monosaccharide) to produce ABE in the TYA (Tryptone Yeast Agar) medium. It was proven also that the ABE ratio was very dependent on the type and concentration of substrates being used by strain N1-4.

Keywords**:** acetone, butanol, ethanol, clostridium saccharoperbutylacetonicum N1-4, fermentation

1. INTRODUCTION

The oil price in the world is now increasing, while on the other hand, the non-renewable supply of fossil fuels is decreasing over the time. Global crude oil production was predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 (Howard et al., 2003). Besides, the world is now also facing the environmental pollution problems due to the extensive use of fossil fuels in the industry or transportation. Therefore, it is urgent to find alternative renewable resources for the production of liquid fuels and chemicals. However, the use of edible agricultural crops for ABE production, such as corn in the USA or sugarcane in Brazil, can decrease the food stocks and raise the food prices. Fortunately, the huge amounts of residual biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals, and cheap energy sources from fermentation processes, etc. A wide variety of such biomass include the whole plants, the plant parts (e.g. seeds, stalks), the plant constituents (e.g. starch, lipids, protein, fibers), the processing bioproducts (e.g. distiller's grains, corn solubles), the materials of marine origin and animal byproducts, as well as the municipal and industrial wastes (Howard et al., 2003). Sadly, much of such residual biomass is often disposed by burning, which is not restricted to developing countries alone, but is considered as a global phenomenon (Levine, 1996).

Bioconversion of lignocellulosic materials to useful, higher value products normally require multi-step processes which include:

- pretreatment (mechanical, chemical, and/or biological) (Grethlein & Converse, 1991);
- hydrolysis of the polymers to produce readily metabolized molecules (e.g. hexose or pentose sugars);
- bio-utilization of these molecules to support microbial growth or to produce chemical products; and
- separation and purification of the products (Smith et al., 1987).

The cost of raw materials has been known to be the major limitation on the economic feasibility of 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 fermentation, for examples those who used simple sugars such as glucose, lactose, galactose and xylose (Bahl, et al., 1986; Keis et al., 2001; Shinto et al., 2008), or more complex substrates such as sago starch (Madihah et al. 2001), maltodextrin (Formanek et al., 1997), packing peanuts (Jesse et al., 2002), corn starch (McNeil & Kristiansen, 1986), maize and potato starch (Nicole et al., 1993), as well as xylans from different sources of hemicellulosic residues (Lemmel et al., 1985; Qureshi et al., 2006; Saha & Bothast, 1999), 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 *Clostridium acetobutylicum* or *C. beijerinckii*; consequently there is a lot of information regarding the ABE fermentation performed by such strains.

On the other hand, the investigations employing strains of *C. saccharoperbutylacetonicum*, in particular strain N1-4 (ATCC 13564), were still limited. This strain was chosen in our lab for ABE fermentation 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 lower the ratio of acetone to butanol than with other industrial strains, i.e. 0.25:1 vs. 0.5:1 (Biebl, 1999). Several reports on direct ABE fermentation 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). Nevertheless, there was no sufficient information about how such substrates could affect the ABE ratio of acetone: butanol:ethanol directly produced by strain N1-4. Therefore, the objective of this experiment was to investigate the performance of strain N1-4 in direct fermentation of acetone, butanol, and ethanol using several substrates that have not been investigated previously using this strain, such as cellobiose, dextrin, starches of corn, potato, tapioca, wheat, and sago, as well as more complex carbohydrates such as xylans, cellulose, and carboxyl methyl cellulose (CMC), particularly to study the effect of such various substrate types and concentrations, as well as the substrates pretreatments and mixtures on the ABE ratio and productivity.

2. MATERIALS AND METHODS

2.1 Microorganisms

For a long-term stock culture, strain N1-4 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, 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 minutes and anaerobically incubated at 30°C for 28 h without agitation or pH control.

2.2 Pre-culture Media

The refresh culture was then transferred into TYA

(Tryptone-Yeast extract-Acetate) fresh medium to pre-culture the bacteria anaerobically at 30°C for 15 h without agitation and pH control. The TYA medium components per liter of distilled water were 6 g of bactotryptone (Difco, Detroit, MI, USA), 2 g of yeast extract (Difco), 3 g of CH_3COONH_4, 0.5 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 10 mg of $FeSO_4 \cdot 7H_2O$. 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 gl^{-1} glucose concentration before it was sterilized at 115°C for 15 min.

2.3 Main Culture Media

Main batch culture media for the fermentation experiment were prepared similarly to the pre-culture medium except the use of various substrates as the carbon sources, in addition to glucose as the control substrate. The main culture medium for each carbon substrate was prepared as described in the following sections with 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 48 h.

2.3.1 Effect of Substrate Types and Concentrations in Low or High Working Volumes

In the first experiment, there were thirteen various substrates including glucose, dextrin, soluble starch, and starches of corn, potato, sago, tapioca, and wheat, cellobiose, cellulose, carboxy-methylcellulose (CMC), xylose, and xylans (derived from birch wood source), each of which was added with the concentration of 10 and 20 gl^{-1} in a low working volume of 10 ml. During the subsequent experiment that was aimed to confirm the results obtained from the previous experiment in a higher working volume (250 ml), only dextrin, cellobiose, and xylans were used in addition to glucose as the control substrate. Each of such substrates was added as a concentrate and diluted with distilled water to a final total reducing sugar concentration of 10 or 20 gl^{-1}.

Another experiment was also performed to study the effect of increasing substrate concentrations by applying different concentrations of glucose and cellobiose (i.e. 10, 20, and 40 gl^{-1} of glucose; 10, 20, and 40 gl^{-1} of cellobiose) each in a working volume of 250 ml.

2.3.2 Effect of Substrate Mixtures

The effect of substrate mixtures was also investigated using the various mixture ratios of glucose and cellobiose or glucose and dextrin. The ratios being applied were 1:0, 1:1, 1:4, 2:3, 3:2, and 4:1 (g/g of glucose:cellobiose or glucose:dextrin) with the total substrate concentration of 20 gl^{-1} each and working volume of 250 ml.

2.3.3 Effect of Substrate Pre-treatments

The complex structures of cellulose and xylans have been known to hinder the availability of such carbohydrate for the direct ABE fermentation process, therefore several different techniques of physical and chemical pre-treatments were tried to be applied before such complex carbohydrates were directly used by strain N1-4. The pretreatment techniques were including the followings (each total substrate concentration was 10 gl^{-1}):

- Autoclaving for 90 min at 121°C and 15 atm pressure (Silverstein et al., 2007);
- Dilute acid hydrolysis using 2% H_2SO_4 (Silverstein et al., 2007);
- Alkaline hydrolysis using 2% NaOH (Silverstein et al., 2007);
- Solubilized by Altimizer system manufactured by Sugino Machine Ltd., JAPAN (see Picture 1).

Picture 1. The Altimizer system by Sugino Machine, Ltd., Japan

2.4 Sampling and Analysis

Sampling for the first experiment (using 10 ml working volume) was performed at 0-, 24-, and 48-h fermentation time, while for the subsequent experiments (using 250 ml working volume), it was performed periodically at every 4 or 6 hour. One set of the samples was centrifuged with 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).

2.5 Calculation

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

3. RESULTS AND DISCUSSION

3.1 Effect of Substrate Type and Concentration on Direct ABE Fermentation by Strain N1-4 in a Low Working Volume

Table 1 shows each concentration of acetone, butanol, ethanol, and ABE as well as the consumed substrate in gl^{-1} for all various substrates with two different concentrations (10 or 20 gl^{-1}) after 24 h of direct ABE fermentation period by strain N1-4 in a low working volume of 10 ml. It also indicates the ABE ratio with using butanol as the standard of ratio calculation. The butanol yield, ABE yield and productivity were calculated as mentioned above. It can be seen from these data that strain N1-4 could use many types of substrate to effectively produce the acetone, butanol, and ethanol, except in CMC and cellulose. In addition, it was indicated that the ABE ratio could be varied as affected by the substrate type and concen-tration, except in CMC and cellulose.

Data in Table 1 also indicated the different performance of strain N1-4 in direct ABE fermentation using more complex carbohydrates such as starches, CMC or cellulose. The results

Table 1. Data of Direct ABE Fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) at 24 h Fermentation Time

C-Sources	Acetone (gl^{-1})	Butanol (gl^{-1})	Ethanol (gl^{-1})	ABE (gl^{-1})	Consumed substrate (gl^{-1})	ABE ratio	Butanol yield ($ggTS^{-1}$)	ABE yield ($ggTS^{-1}$)	ABE productivity ($gl^{-1}h^{-1}$)
Glucose10	0.73	3.1	0.16	4	10	0.24:1:0.05	0.3	0.39	0.17
Glucose20	0.97	5.9	0.5	7.4	19	0.16:1:0.08	0.31	0.39	0.31
Dextrin10	0.32	2.1	0.22	2.6	5	0.15:1:0.10	0.42	0.53	0.11
Dextrin20	0.76	4.8	0.3	5.8	12	0.16:1:0.06	0.4	0.48	0.24
Soluble Starch10	0.36	2.6	0	2.9	5.8	0.14:1:0	0.44	0.5	0.12
Soluble Starch20	0.72	4.2	0.3	5.2	8.8	0.17:1:0.07	0.48	0.59	0.22
Sago10	0.44	3	0	3.5	6.5	0.15:1:0	0.47	0.54	0.14
Sago20	0.68	3.7	0.24	4.6	11	0.19:1:0.07	0.34	0.42	0.19
Tapioca10	0.57	3.1	0	3.7	6.6	0.18:1:0	0.47	0.56	0.15
Tapioca20	0.83	5	0.29	6.1	17	0.17:1:0.06	0.29	0.36	0.26
Corn10	0.6	2.8	0.06	3.5	7.6	0.21:1:0.02	0.37	0.46	0.14
Corn20	0.67	4.1	0.29	5.1	11	0.16:1:0.07	0.4	0.49	0.21
Wheat10	0.57	2.9	0	3.5	6.1	0.19:1:0	0.48	0.57	0.15
Wheat20	0.69	4.1	0.26	5	15	0.17:1:0.06	0.27	0.33	0.21
Potato10	0.56	2.7	0	3.2	6.9	0.21:1:0	0.39	0.47	0.13
Potato20	0.8	4.5	0.28	5.6	12	0.18:1:0.06	0.37	0.46	0.23
Cellobiose10	0.74	3.2	0.27	4.3	10	0.23:1:0.08	0.31	0.41	0.18
Cellobiose20	1.4	6.4	0.59	8.3	21	0.21:1:0.09	0.31	0.4	0.35
CMC10	0.06	0.06	0	0.12	0.74	1:01:00	0.08	0.16	0.01
CMC20	0	0	0	0	0	0	0	0	0
Cellulose10	0.03	0.01	0	0.04	1.7	3:01:00	0	0.02	0
Cellulose20	0	0	0	0	2.5	0	0	0	0
Xylose10	0.5	2.8	0.27	3.6	10	0.18:1:0.10	0.28	0.36	0.15
Xylose20	0.5	2.7	0.32	3.5	12	0.19:1:0.12	0.22	0.29	0.15
Xylan10	0.18	0.43	0	0.6	6.4	0.42:1:0	0.07	0.09	0.02
Xylan20	0.21	0.74	0.22	1.2	6.5	0.28:1:0.30	0.11	0.18	0.05

Note:
Substrate concentration 10 or 20 gl^{-1}; working volume 10 ml
Number following the substrate indicates the concentration of the substrate in gl^{-1}

obtained in the fermentation of starches showed that different sources of starches could affect the ABE production as well as the ABE ratio. Between starches of sago and corn, the ABE ratio in 10 gl^{-1} of sago fermentation was 0.15:1:0, while in 10 gl^{-1} of corn fermentation it was 0.21:1:0.02. Such ratio could further be changed by applying different concentration of the starches, by using 20 gl^{-1} of sago the ABE ratio was 0.19:1:0.07, while by using 20 gl^{-1} of corn the ABE ratio was 0.16:1:0.07. On the other hand, even though there was some amount of total sugar being consumed, but the ABE concentration was very low or even zero, which indicated that strain N1-4 could not utilize CMC and cellulose to directly produce the ABE.

Moreover, the results in Table 1 also proved that strain N1-4 could utilize the pentose sugar such as xylose and xylans to produce ABE directly. In xylans fermentation, there was a significant different in the ABE ratio between the fermentation of 10 gl^{-1} of xylans (the ratio of 0.42:1:0) and the fermentation of 20 gl^{-1} of xylans (the ratio of 0.28:1:0.30), producing ABE concentration of 0.60 gl^{-1} and 1.2 gl^{-1} after 24 h (Table 2.1) or 1.6 gl^{-1} and 3.0 gl^{-1} after 48 h (data not shown), respectively.

It could be suggested from the results of this section that cellobiose could be more efficient substrate for strain N1-4 to produce higher butanol and ABE than glucose. It could also be concluded that strain N1-4 could utilize different sources of starches and xylans, but could not use CMC and cellulose to produce ABE directly. The ABE ratio was found to be varied by using various types and concentrations of the substrates.

There have been a number of studies about the effect of nutrient limitation on the onset and maintenance of ABE production, in both batch- and continuous-culture systems (Jones & Woods, 1986). 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 & Engasser, 1983; Monot et al., 1982). In particular, when glucose was present below the threshold level of about 7-10 gl^{-1}, then no shift to ABE 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 of *C. acetobutylicum* strains, 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 ABE production (Jones & Woods, 1986). Surprisingly, however, our results showed that strain N1-4 could directly ferment all of the substrates being utilized in the first experiment, except CMC and cellulose, each with a threshold concentration of 10 gl^{-1}, producing acetone and butanol. Even, strain N1-4 could also produce ABE by using 5 gl^{-1} of cellobiose (data not shown). It seemed that strain N1-4 could have a lower threshold level of substrate concentration to produce ABE than the strains of *C. acetobutylicum*.

It was found in our first experiment, which was then confirmed in our subsequent experiment, that the ABE ratio directly produced by strain N1-4 was very dependent on the type and concentration of substrates being used. These results were in accordance with previous studies employing the strains of *C. acetobutylicum*, which suggested that depending on the nature of the carbohydrate and the culture conditions, the ratio of conversion to ABE can vary (Bahl, et al., 1986; Matta-El-Ammouri et al., 1987; Monot et al., 1982). A previous study employing *C. thermocellum* also showed the similar phenomenon (Brener & Johnson, 1984). Our results were also in agreement with those of Awang et al. (1992) employing *C. acetobutylicum* P262 (now *C. saccharobutylicum*). 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 strain N1-4 could produce higher butanol in a substrate-dependent pathway (Shinto et al., 2008; Shinto et al., 2007).

Our results revealed that strain N1-4 could directly ferment the starches to produce relatively high ABE concentrations. As shown in Table 1, the yields of butanol and ABE were relatively higher in starch fermentation than those of glucose fermentation. Our results were in agreement with the results by Shaheen et al. (2000) who observed that strain N1-4 could perform well in the maize starch medium producing high proportion of butanol. It could be suggested, therefore, that the strain N1-4 could have a relatively high amylase enzyme activity for direct fermentation of starches. The ability of *Clostridium* sp. to produce amylolytic enzymes such as amylases, pullulanase and glucoamylase for direct fermentation of starches to ABE has been demonstrated previously (Jones & Keis, 1995). Additionally, there have been several studies on the utilization of various types of starchy materials for ABE production by *C. acetobutylicum* (Jones & Woods, 1986; Madihah et al., 2001). On the other hand, however, there was only a very few previous studies on direct ABE fermentation using starchy materials by strain N1-4. Therefore, our experiment at this time could be considered as a novel study on direct ABE fermentation on various starches by strain N1-4, especially to investigate the effect of starch type and concentration on the ABE ratio, since it was known previously that starches from different sources show different types of physico-chemical properties depending on the relative amylase and amylopectin contents (Sim et al., 1991).

In contrast, the ABE production in xylans fermentation by strain N1-4 was much lower than those in glucose, cellobiose, and dextrin fermentations. On the other hand, the butyrate production was much higher even though needed

a longer period in xylans fermentation than that in the other substrates fermentation. Our results in xylans fermentation were also similar with the results obtained 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). Similar with our results, 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. Moreover, the results on CMC or cellulose fermentation indicated that strain N1-4 could not directly use such complex carbohydrates to produce significant amount of ABE (Table 1). The very complex structure of cellulose, especially its crystallinity which limits the hydrolysis of cellulose, was most probably the main cause of very low ABE concentrations directly produced by strain N1-4 (Hendriks & Zeeman, 2009). Poor growth, butanol production and sporulation in cultures containing CMC were also found in previous studies employing *C. acetobutylicum* (Lee et al.,1985a). It could also be predicted that such strains could not have a significant cellulase enzyme activity for direct utilization of the CMC or cellulose. It can be suggested that some pre-treatment methods could be applied to hydrolyze the CMC or cellulose into simpler sugars such as cellobiose or dextrin before being used by strain N1-4 or *C. acetobutylicum* to produce a significant concentration of ABE.

It was confirmed in our subsequent experiment using a higher working volume that the concentrations of acetone, butanol, and ethanol as well as the butanol yield, ABE yield and productivity in cellobiose fermentation by strain N1-4 were indeed higher than those in glucose fermentation. The higher production in cellobiose fermentation than in glucose fermentation by strain N1-4 was similar to the results obtained in a previous study (Awang et al., 1992) employing *C acetobutylicum* P262 (now *C. saccharobutylicum*). 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.

Based on the results of our experiment, it could be suggested that the different performances on direct ABE fermentation by strain N1-4 using various types and concentrations of the substrates, in particular between 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* 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). 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 (Muir et al., 1985). 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). Therefore, it is suggested that a further experiment needs to be performed to investigate what type of enzyme involved in the direct utilization of cellobiose by strain N1-4, whether it is cellobiase like in *C. acetobutylicum* (Allcock & Woods, 1981, Lee et al., 1984) 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.

3.2. Effect of Substrate Type and Concentration on Direct ABE Fermentation by Strain N1-4 in a High Working Volume

A subsequent experiment, which was then conducted by using glucose, dextrin, cellobiose, and xylans as the substrates of choice each with two different concentrations (10 or 20 gl^{-1}) and a higher working volume (250 ml), confirmed that the substrate types and concentrations could indeed vary the ABE ratio directly produced by strain N1-4. The data in Table 2 clearly show that the ABE ratio obtained in the direct fermentation using different substrate types but the same concentrations, as well as by using the same substrate type but different concentrations, generally could be varied significantly. Particularly between glucose and cellobiose, the ABE ratio of 0.22:1:0 using 10 gl^{-1} glucose could be changed into 0.29:1:0.05 ratio using 20 gl^{-1} glucose, further which could be changed into 0.30:1:0.06 ratio by using 10 gl^{-1} cellobiose or into 0.41:1:0.07 ratio by using 20 gl^{-1} cellobiose. The difference of ABE ratio between glucose and dextrin fermentation was not relatively significant, that was 0.22:1:0 with 10 gl^{-1} of either glucose or dextrin and 0.29:1:0.05 with 20 gl^{-1} glucose compared to 0.24:1:0.04 with 20 gl^{-1} 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 gl^{-1} or 20 gl^{-1} of xylans, respectively, compared to those of glucose fermentation.

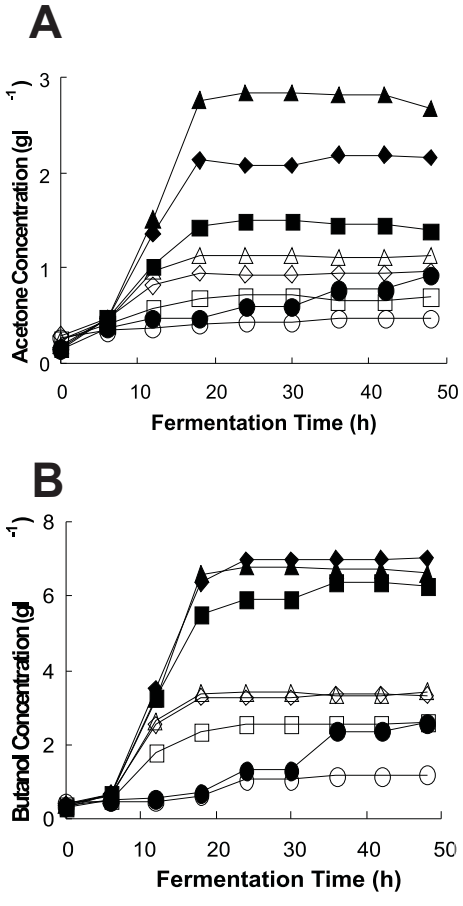
As shown in Figure 1 and Table 2, there was also a difference in the performances of strain N1-4 between glucose and cellobiose fermentation. The ABE concentration directly produced using both

Table 2. Effects of Substrate Type And Initial Concentration on ABE Fermentation by *C. Saccharoperbutylacetonicum* N1-4 (ATCC 13564) at 24 h Fermentation Time

C-Sources	Acetone (gl^{-1})	Butanol (gl^{-1})	Ethanol (gl^{-1})	ABE (gl^{-1})	Consumed substrate (gl^{-1})	ABE ratio	Butanol yield ($ggTS^{-1}$)	ABE yield ($ggTS^{-1}$)	ABE productivity ($gl^{-1}h^{-1}$)
Glucose10	0.64	2.9	0	3.5	10	0.22:1:0	0.28	0.34	0.15
Glucose20	1.9	6.6	0.35	8.9	22	0.29:1:0.05	0.31	0.41	0.37
Cellobiose10	0.91	3	0.19	4.1	10	0.30:1:0.06	0.29	0.4	0.17
Cellobiose20	2.7	6.4	0.43	9.5	20	0.41:1:0.07	0.32	0.47	0.4
Dextrin10	0.47	2.2	0	2.6	7.6	0.22:1:0	0.29	0.35	0.11
Dextrin20	1.4	5.6	0.22	7.2	22	0.24:1:0.04	0.26	0.33	0.3
Xylans10	0.16	0.61	0	0.77	4.8	0.26:1:0	0.13	0.16	0.03
Xylans20	0.44	0.99	0	1.4	7.8	0.44:1:0	0.13	0.19	0.06

Note: Substrate concentration: 10 or 20 gl^{-1}; working volume 250 ml
Number following the substrate indicates the concentration of the substrate in gl^{-1}

concentrations of cellobiose (10 gl^{-1} or 20 gl^{-1}) was higher (4.1 gl^{-1} or 9.5 gl^{-1}) than those using the same concentrations of glucose (3.5 gl^{-1} or 8.9 gl^{-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 gl^{-1} glucose (6.6 gl^{-1}) which was slightly higher than that in the fermentation of 20 gl^{-1}cellobiose (6.4 gl^{-1}). In addition, the yields of butanol and ABE as well as the ABE productivity at 24 h were all higher in cellobiose fermentation than those in glucose fermentation. On the other hand, the xylans fermentation was producing lower concentrations of acetone, butanol, and ethanol than the glucose fermentation at 24 h.



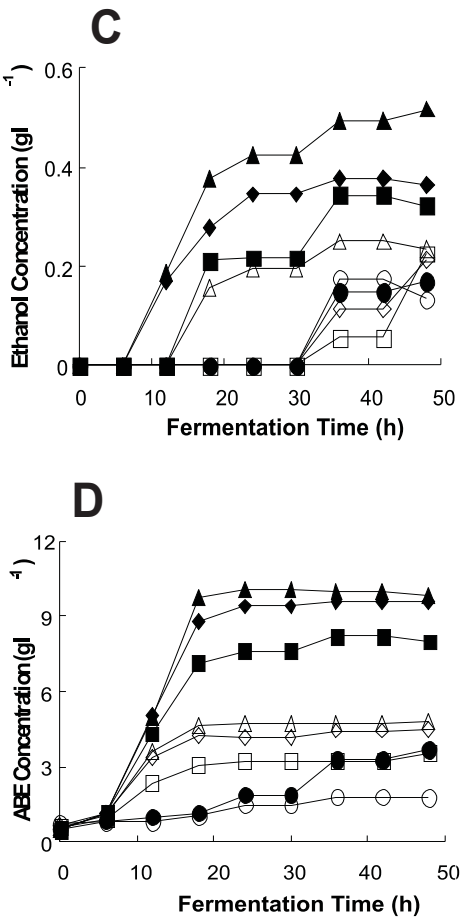


Figure 1. Concentrations of acetone (A), butanol (B), ethanol (C), and ABE (D) directly produced by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 ml; static batch culture without pH control. Symbols: (◇) glucose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

Figure 2 further demonstrated the different performance of strain N1-4 in the utilization or

production of acids and total sugar using different substrates. Figure 2A shows that the acetate reutilization in xylans fermentation was much lower than those of the other substrates. 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. 2B), 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.

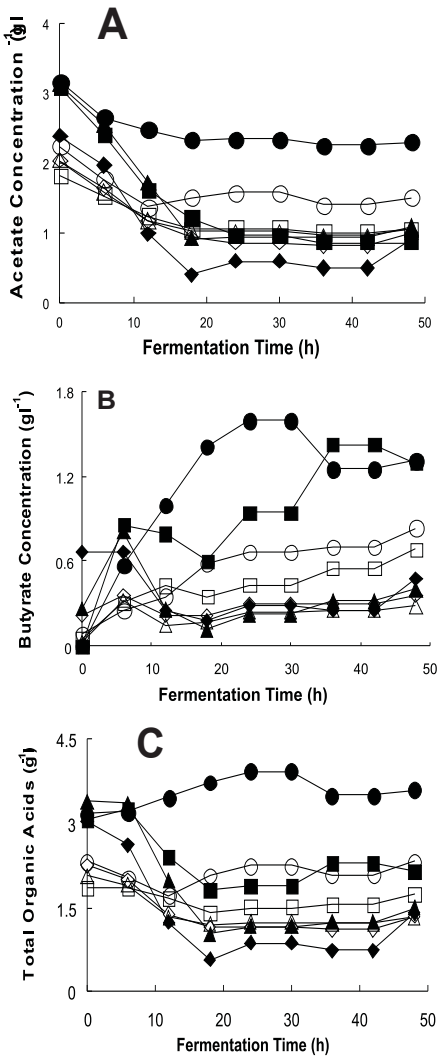


Figure 2. Acetate concentration (A), butyrate concentration (B), and consumption of total sugar (C) during direct ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 ml; static batch culture without pH control. Symbols: (◇) glucose 10 gl^{-1}; (◆)glucose 20 gl^{-1}; (△)cellobiose 10 gl^{-1}; (▲)cellobiose 20 gl^{-1}; (□)dextrin 10 gl^{-1}; (■) dextrin 20 gl^{-1}; (○)xylans 10 gl^{-1}; (●)xylans 20 gl^{-1}

The pH change in the xylans medium was also different from those in the other substrates media (Fig. 3B). The pH in xylans medium was relatively constant up to 12 h period and start decreasing until 24 h before increasing again, while the pH in the other substrates media showed the similar decrease up to 6 h, which was then followed by the similar increase until the end of fermentation time. The trend of pH change in the xylans fermentation was relatively correlated with that of the ABE production (Fig. 1D) as well as that of butyrate production and utilization (Fig. 2B). Additionally, Figure 2B also indicated that strain N1-4 could produce butyrate up to 6 h in glucose, cellobiose, and dextrin fermentation, whereas in xylans fermentation the butyrate was produced up to 24 h with higher concentration than the other substrates, which in turn caused the pH in the media decreasing during such different fermentation time (Fig 3B).

Figure 3A illustrates the different pattern of the bacterial density (OD_{562}) using different substrates by strain N1-4. There was a long lag period up to 12 h in xylans fermentation before strain N1-4 started to grow exponentially until 24 h and then entered the stationary phase. In contrast, by using the other substrates strain N1-4 could directly grow exponentially until 18 h before the bacterial density declined.

Table 3. Effects of Glucose or Cellobiose Increasing Concentrations on ABE Fermentation by *C. Saccharoperbutylacetonicum* N1-4 (ATCC 13564) at 24 h Fermentation Time

C-Sources	Acetone (gl^{-1})	Butanol (gl^{-1})	Ethanol (gl^{-1})	ABE (gl^{-1})	Consumed substrate (gl^{-1})	ABE ratio	Butanol yield ($ggTS^{-1}$)	ABE yield ($ggTS^{-1}$)	ABE productivity ($gl^{-1} h^{-1}$)
glucose10	0.64	2.9	0	3.5	10	0.22:1:0	0.28	0.34	0.15
glucose20	1.7	6.1	0.32	8.1	20	0.28:1:0.05	0.3	0.4	0.34
glucose40	2.8	9	0.6	12	31	0.31:1:0.07	0.29	0.4	0.52
cellobiose10	0.91	3	0.19	4.1	10	0.30:1:0.06	0.29	0.4	0.17
cellobiose20	1.9	6	0.36	8.3	20	0.31:1:0.06	0.3	0.41	0.34
cellobiose40	3	8.8	0.53	12	36	0.35:1:0.06	0.24	0.34	0.51

Note: Substrate concentration: 10, 20, or 40 gl^{-1}; working volume 250 ml
Number following the substrate indicates the concentration of the substrate in gl^{-1}

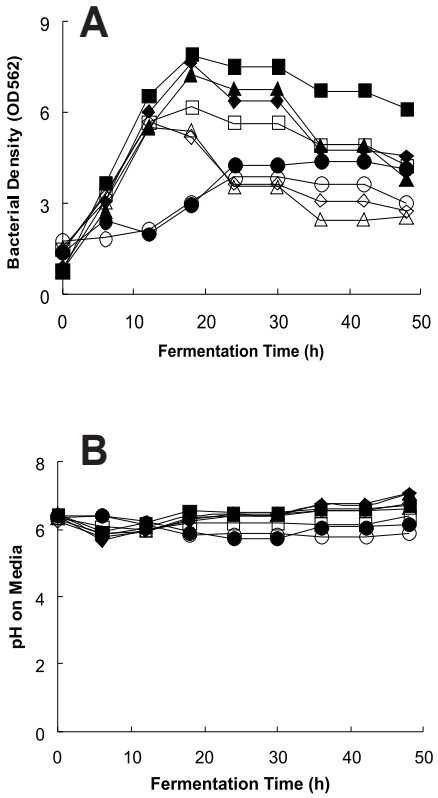


Figure 3. Bacterial density (A) and pH (B) during direct ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using different substrates. Fermentation conditions: initial pH = 6.5; working volume = 250 ml; static batch culture without pH control. Symbols: (◇) glucose 10 gl^{-1}; (◆)glucose 20 gl^{-1}; (△)cellobiose 10 gl^{-1}; (▲)cellobiose 20 gl^{-1}; (□)dextrin 10 gl^{-1}; (■) dextrin 20 gl^{-1}; (○)xylans 10 gl^{-1}; (●)xylans 20 gl^{-1}

From the results of this experiment, it could be confirmed that the substrate types and concentrations could indeed vary the ABE ratio directly produced by strain N1-4. It could also be suggested that the ABE ratio generally could be varied using different substrate types but the same concentrations, as well as by using the same substrate type but different concentrations. In addition, strain N1-4 was found to be able to utilize the xylans derived from birch wood to directly produce ABE in defined TYA media.

Our results showed that the higher the substrate concentration, the higher ABE concentration could be produced, but not necessarily in the same increasing ratio. However, the yields of butanol and ABE were not affected by the increasing substrate concentrations, especially in cellobiose fermentation. Even by using the excessive cellobiose (80 gl^{-1}), the yields of butanol and ABE were the lowest (data not shown). On the other hand, by using 5 gl^{-1} of cellobiose the yields of butanol and ABE were the highest (data not shown). Cellobiose was chosen as the carbon source in this 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 & Ezeji, 2008).

3.3 Effect of Substrate Increasing Concentrations on Direct ABE Fermentation by Strain N1-4 in a High Working Volume

Table 3 indicated the effects of increasing substrate concentrations on direct ABE fermentation by strain N1-4. The results show that the increasing substrate concentrations could increase the ABE concentration, but not necessarily in the same increasing ratio. In glucose fermentation, the ABE concentration could increase from 3.5 gl^{-1} to 8.1 and 12 gl^{-1} (130% and 250% increase) by increasing the initial substrate from 10 gl^{-1} to 20 and 40 gl^{-1}, respectively. Similarly, in cellobiose fermentation using the increasing initial substrate concentrations from 10 gl^{-1} to 20 and 40 gl^{-1}, the ABE concentration could be enhanced from 4.1 gl^{-1} to 8.3 and 12 gl^{-1}, respectively.

The ABE ratio in glucose and cellobiose fermentation was slightly affected by the increasing initial substrate concentration (Table 3). 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 strain N1-4. By using 40 gl^{-1} of substrate, the total sugar utilization by strain N1-4 seemed to be incomplete, as there was some residual substrate left in the glucose or cellobiose media.

Table 4. Effect of Different Substrate Mixture Ratios Between Glucose and Cellobiose on ABE Fermentation by *C. Saccharoperbutylacetonicum* N1-4 (ATCC 13564) At 24 h Fermentation Time

C-Sources	Acetone (gl^{-1})	Butanol (gl^{-1})	Ethanol (gl^{-1})	ABE (gl^{-1})	Consumed substrate (gl^{-1})	ABE ratio	Butanol yield ($ggTS^{-1}$)	ABE yield ($ggTS^{-1}$)	ABE productivity ($gl^{-1}h^{-1}$)
glucose	2	6.4	0.51	8.9	18	0.31:1:0.08	0.36	0.5	0.37
cellobiose	2.6	6.4	0.43	9.5	20	0.41:1:0.07	0.32	0.48	0.4
glu1:cello1	2.3	6.2	0.42	9	17	0.38:1:0.07	0.36	0.52	0.38
glu1:cello4	2.2	5.8	0.24	8.2	19	0.38:1:0.04	0.3	0.43	0.34
glu4:cello1	2.3	5.5	0.28	8.1	23	0.42:1:0.05	0.24	0.35	0.34
glu2:cello3	2	5.9	0.26	8.2	29	0.33:1:0.04	0.21	0.29	0.34
glu3:cello2	2.2	5.1	0.32	7.6	20	0.42:1:0.06	0.25	0.38	0.32

Table 5. Effect of Different Substrate Mixture Ratios between Glucose and Dextrin on ABE Fermentation by *C. Saccharoperbutylacetonicum* N1-4 (ATCC 13564) at 24 h Fermentation Time

C-Sources	Acetone (gl^{-1})	Butanol (gl^{-1})	Ethanol (gl^{-1})	ABE (gl^{-1})	Consumed substrate (gl^{-1})	ABE ratio	Butanol yield ($ggTS^{-1}$)	ABE yield ($ggTS^{-1}$)	ABE productivity ($gl^{-1}h^{-1}$)
glucose	1.8	6.1	0.29	8.2	23	0.29:1:0.05	0.27	0.36	0.34
dextrin	1.3	5.6	0.22	7.1	22	0.24:1:0.04	0.26	0.33	0.3
glu1:dext1	1.9	5.9	0.3	8.1	25	0.33:1:0.05	0.23	0.32	0.34
glu2:dext3	1.9	5.9	0.34	8.1	14	0.32:1:0.06	0.42	0.58	0.34
glu3:dext2	2.2	5.5	0.31	8	19	0.39:1:0.06	0.29	0.42	0.33
glu1:dext4	1.5	6.1	0.28	8	18	0.25:1:0.05	0.33	0.43	0.33
glu4:dext1	1.8	6.6	0.32	8.7	21	0.28:1:0.05	0.31	0.41	0.36

Note: Substrate total concentration: 20 gl^{-1}; working volume 250 ml
Number following the substrate indicates the ratio of the substrate in gg^{-1}

Interestingly, the yields of butanol and ABE (0.29 $ggTS^{-1}$ and 0.40 $ggTS^{-1}$, respectively) obtained by using 10 gl^{-1} cellobiose were higher than those obtained by using 40 gl^{-1} cellobiose (i.e. 0.24 $ggTS^{-1}$ and 0.34 $ggTS^{-1}$, respectively). On the other hand, such a phenomenon could not be found in glucose fermentation. It was the ABE productivity that was directly affected by the increasing substrate concentrations, in both glucose and cellobiose fermentations; the higher the initial substrate concentration, then the higher the ABE productivity.

3.4 Effect of Substrate Mixture Ratios on Direct ABE Fermentation by Strain N1-4 in a High Working Volume

The studies with glucose and cellobiose mixtures were done because of the relatively high butanol production from cellobiose fermentation, the likelihood that cellobiose and glucose mixtures would be found in substrates derived from cellulose, the projected industrial importance of cellulosic substrates (Gibbs, 1983), and the possibility of an effect of glucose on cellobiose fermentation (Awang et.al., 1992). In our experiment, the fermentation of glucose and cellobiose mixture only with a ratio of 1:1 (glucose:cellobiose) could produce higher yields of butanol and ABE, as well as ABE productivity, while the other ratios only produce lower ABE productivity and yields, than by using glucose alone (Table 4). Interestingly, there were lower ABE concentrations in all mixtures of glucose and cellobiose than in the TYA medium using cellobiose only (Table 4). It could be suggested that glucose might have a deleterious effect on ABE production in culture containing cellobiose as stated previously (Awang et al., 1992).

In addition, even though the butanol yield was higher, but the butanol concentration produced by using such a ratio 1:1 in the glucose and cellobiose mixture was slightly lower than that by using the glucose or the cellobiose alone (Table 4). Our results were similar with those by Awang et al. (1992) who employed *C. acetobutylicum* P262 (now *C. saccharobutylicum*), by which the butanol concentration produced in cultures containing a 1:1 ratio of the two sugars was also low. It was suggested that instability of cellobiase or cellobiose phosphorylase in the solventogenic phase of the culture may have accounted for this phenomenon (Allcock and Woods, 1981; Awang et al., 1992).

On the other hand, there were higher ABE concentrations in all mixtures of glucose and dextrin than in the TYA medium using dextrin only (Table 5). It seemed that the addition of glucose in dextrin fermentation using TYA media could increase the ABE concentration directly produced by strain N1-4.

Table 4 indicated the results obtained in the ABE fermentation by strain N1-4 using the mixtures of glucose and cellobiose with different ratios. It can be seen that by using the ratio of 1:1 (g:g of glucose:cellobiose), strain N1-4 could produce slightly higher ABE yield and productivity than by using glucose alone. On the other hand, other ratios of the mixture glucose:cellobiose did not cause higher yields of butanol and ABE in the fermentation processes by strain N1-4. Interestingly, however, there were lower ABE concentrations in all mixtures of glucose and cellobiose than in the TYA medium with cellobiose only.

Table 5 revealed the results obtained in the ABE fermentation by strain N1-4 using the mixtures of glucose and dextrin with different ratios. It can be seen that by using the ratio of 2:3, 3:2, 1:4, and 4:1 (g:g of glucose:dextrin), strain N1-4 could all produce higher yields of butanol and ABE than by using glucose alone. On the other hand, the ratio of 1:1 of glucose:dextrin did not cause higher yields of butanol and ABE in the fermentation processes by strain N1-4. Interestingly, there were higher concentrations of ABE in all mixtures of glucose and dextrin than in the TYA medium with dextrin only. It could be suggested that the addition of glucose in cellobiose fermentation by strain N1-4 could decrease the ABE concentration produced in the TYA medium, whereas on the other hand, the addition of glucose in dextrin fermentation could increase it.

4. CONCLUSION

Finally it can be concluded that strain N1-4 could directly produce ABE from various substrates, from monosaccharide to polysaccharides of hexose or pentose sugars, except CMC and cellulose. Cellobiose (a hexose disaccharide) could be the more efficient substrate for strain N1-4 than glucose (a monosaccharide) to produce ABE in the TYA medium. The ABE ratio was very dependent on the type and concentration of substrates being used by strain N1-4.

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