MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 6, No 4, December 2012, p 139-147

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.6.4.1

Enhanced Acetone, Butanol, and Ethanol Fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) in a Chemically Defined Medium: Effect of Iron and Initial pH on ABE Ratio

HANIES AMBARSARI[1]*AND KENJI SONOMOTO[2,3]

[1]*Institute of Environmental Technology (BTL)-Indonesian, Agency for Assessment and Application of Technology (BPPT), Building 412 PUSPIPTEK Serpong, Banten 15314, Indonesia;*
[2]*Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan;*
[3]*Laboratory of Functional Food Design, Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

Batch studies were performed to investigate the performance of *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) in acetone-butanol-ethanol (ABE) fermentation as affected by iron and initial pH in defined TYA (Tryptone-Yeast extract-Acetate) media. Different concentrations of $FeSO_4.7H_2O$ in the TYA media were found to influence the ABE fermentation process resulting in different ABE ratios. From the experiment with different concentrations of $FeSO_4.7H_2O$, it was also found that lag phases at initial pH of 4.4 were longer than those at initial pH of 6.5, however they could still have higher ABE productivity values. Addition of 0.003 g L^{-1} $FeSO_4.7H_2O$ could give the highest ABE production with both initial pH of 6.5 and 4.4. With more than 0.01 g L^{-1} $FeSO_4.7H_2O$, ratios of acetone to butanol (0.50-0.53) were higher at initial pH of 4.4 than those (0.26-0.28) at initial pH of 6.5. Those differences were not obtained with low concentration of $FeSO_4.7H_2O$ among the same initial pH. It was also confirmed that initial pH affected ABE production significantly more than $FeSO_4.7H_2O$ by statistical analysis.

Key words: acetone-butanol-ethanol (ABE), *Clostridium saccharoperbutylacetonicum*, glucose, initial pH, iron, TYA medium

Suatu penelitian dilakukan untuk mengetahui kinerja dari mikroba *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) dalam proses fermentasi aseton-butanol-etanol (ABE) terutama pengaruh dari pH awal dan penambahan senyawa besi ke dalam media TYA (*Tryptone-Yeast extract-Acetate*) yang terdefinisi. Konsentrasi senyawa $FeSO_4.7H_2O$ yang berbeda di dalam media TYA ternyata dapat mempengaruhi proses fermentasi ABE dan menghasilkan rasio ABE berbeda. Dari penelitian dengan menggunakan konsentrasi senyawa $FeSO_4.7H_2O$ yang berbeda-beda, juga ditemukan bahwa fase lag pada perlakuan pH awal 4,4 lebih lama daripada perlakuan pH awal 6,5 tetapi dengan nilai produktivitas ABE yang lebih tinggi. Penambahan 0,003 g L^{-1} $FeSO_4.7H_2O$ dapat menghasilkan produksi ABE tertinggi pada kedua perlakuan pH awal 6,5 dan 4,4. Dengan penambahan lebih dari 0,01 g L^{-1} $FeSO_4.7H_2O$, rasio aseton terhadap butanol (0,50-0,53) lebih tinggi pada perlakuan pH awal 4,4 daripada perlakuan pH awal 6,5 (0,26-0,28). Perbedaan itu tidak ditemui pada penambahan senyawa $FeSO_4.7H_2O$ dengan konsentrasi yang lebih rendah pada pH awal yang sama. Dari analisa statistika terhadap data yang diperoleh dalam penelitian ini, dapat dibuktikan juga bahwa pengaruh pH awal secara signifikan lebih besar daripada pengaruh penambahan senyawa $FeSO_4.7H_2O$ terhadap proses fermentasi ABE di dalam media TYA yang terdefinisi.

Kata kunci: aseton-butanol-etanol (ABE), *Clostridium saccharoperbutylacetonicum*, glukosa, media TYA, pH awal

---

In the fermentation process, acetone, butanol, and ethanol are generally produced with butanol as the major product (Qureshi *et al*. 2006), hence it is generally called as butanol or ABE (acetone-butanol-ethanol) fermentation. ABE fermentation has been gaining a lot of interest worldwide due to the raising production cost of petroleum-based chemicals and fuels (Tashiro and Sonomoto 2010). In particular, butanol is the most promising solvent compared to acetone and ethanol, because butanol has a lower vapour pressure but a higher energy content, hence it is safer and more economical. Additionally, butanol-gasoline blends are less susceptible to separation because butanol has a higher tolerance to water contamination in the blends. This facilitates the use of butanol in existing gasoline supply and distribution channels. Butanol can also be blended with gasoline at higher concentrations than ethanol without requiring of

*Corresponding author; Phone: +62-21-7560919, Fax: +62-21-7563116, E-mail: hanies.ambarsari@gmail.com

vehicles modification (Hipolito *et al*. 2008; Qureshi and Blaschek 2001).

In a normal batch culture, solvent-producing *Clostridium* species produce hydrogen, carbon dioxide, acetate and butyrate during the initial growth phase (acidogenic phase), resulting in a decrease of the pH in the culture medium. The metabolism of the cells then switches to solvent production (solventogenic phase) as the culture enters the stationary growth phase, during which the reassimilation of acids occurs concurrently with the continued consumption of the carbohydrate and the medium pH normally increases (Jones and Woods 1986). The complex multibranched fermentation pathways of *Clostridium acetobutylicum* provide an excellent system for the study of metabolic regulation in clostridia. Of particular interest are the changes in carbon and electron flow during such fermentation and the regulation of these changes. It is very important to understand the factors involved in triggering the metabolic transition and the physiological state associated with the transition from the acidogenic to the solventogenic phase in which the production of solvents IS initiated and maintained. One of such factors is the role of nutrient limitation.

The effect of nutrient factors on the onset and maintenance of ABE production has been investigated by a number of workers, in both batch- and continuous-culture systems employing *Clostridium acetobutylicum*, *C. beijerinckii*, or *C. pasteurianum* strains (Heluane *et al*. 2011; Amador-Noguez *et al*. 2010; Awang *et al*. 1992; Bahl and Gottschalk 1984; Dabrock *et al*. 1992; Junelles, *et al*. 1988; Monot *et al*. 1982). The influence of pH has been recognized as a key factor in determining the outcome of ABE fermentation. Many of early reports noted that the initiation of ABE production occurred only after the pH had decreased to around 4.5 to 5.0, and recent studies also confirmed that cultures maintained at high pH produced mainly acids, whereas those maintained at a low pH predominantly produced ABE. Nevertheless, the pH range over which ABE formation may occur appears to vary quite widely depending on the particular strain and the culture conditions used. For examples, a number of *C. acetobutylicum* DSM strains could only produce ABE at below about pH 5.0 (Bahl *et al*. 1982; Nishio *et al*. 1983), while the *C. acetobutylicum* type culture strain ATCC 824 has been reported to produce good levels of ABE between pH 5.5 and 4.3 (Monot *et al*.1984).

However, to the best of our knowledge, only a limited number of studies have been done to investigate the nutritional and environmental factors affecting the ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564). In particular, the effects of nutrients contained in the TYA (Tryptone-Yeast extract-Acetate) medium, which has usually been used in the ABE fermentation process by strain N1-4, such as iron, phosphate, magnesium, ammonium, sulfate, and potassium sources have not been investigated in detail. There was a previous study by a research group in British Columbia, Canada investigating the influence of culture parameters on biological hydrogen production by *C. saccharoperbutylacetonicum* ATCC 27021, but they only focused on the hydrogen production and the parameters they studied were the carbon and nitrogen sources, iron, inoculum size, initial pH and agitation speed (Ferchichi *et al*. 2005). The objective of the present work is to study the effect of iron contained in the TYA media, as well as the initial pH, on the ABE fermentation by strain N1-4 using glucose as the substrate.

MATERIALS AND METHODS

Microorganism Preparation. 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, 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 minutes and anaerobically incubated at 30 °C for 28 h without agitation or pH control.

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 conventional 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 $MgSO_4.7H_2O$, 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 batch culture media for the fermentation experiment were prepared similarly to the pre-culture medium except the use of various concentrations of $FeSO_4.7H_2O$ contained in the TYA medium, as well as two different initial pH values, pH 6.5 and pH 4.4. The main culture medium for each

treatment was prepared in a 500 mL flask with the working volume of 250 mL, glucose concentration of 20 g L^{-1}, and the initial pH of 6.5 or 4.4 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 until the end of fermentation time.

Different concentrations of $FeSO_4.7H_2O$ were used in this experiment to investigate the effects of such different iron concentrations on the ABE fermentation by the strain N1-4 in the defined TYA media. The concentrations used were 0, 0.003, 0.01, and 0.025 g L^{-1} $FeSO_4.7H_2O$, each of which was applied at initial pH 6.5 or 4.4 with glucose concentration of 20 g L^{-1} as the substrate. The 0.01 g L^{-1} $FeSO_4.7H_2O$ with initial pH 6.5 was used as the control treatment since it was the condition normally defined for a TYA medium.

The followings are the treatment label codes applied during the experiment (G = glucose; F = $FeSO_4.7H_2O$; p = pH): TYA with no iron (0 g L^{-1} $FeSO_4.7H_2O$) (negative control), initial pH 6.5 (= GF0p6); TYA with no iron (0 g L^{-1} $FeSO_4.7H_2O$), initial pH 4.4 (= GF0p4), TYA containing 0.003 g L^{-1} $FeSO_4.7H_2O$ (iron-limited), initial pH 6.5 (= GF3p6); TYA containing 0.003 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (= GF3p4); TYA containing 0.01 g L^{-1} $FeSO_4.7H_2O$ (positive control), initial pH 6.5 (= GF10p6); TYA containing 0.01 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (= GF10p4); TYA containing 0.025 g L^{-1} $FeSO_4.7H_2O$ (iron-excess), initial pH 6.5 (= GF25p6); TYA containing 0.025 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (= GF25p4).

Sampling and Analysis. Sampling was performed periodically at every 6 h. One set of the samples was centrifuged with 20 400 x 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 and Statistical Analysis. As mentioned previously (Jesse *et al*. 2002), the ABE concentration (g L^{-1}) was defined as the difference between the ABE at the indicated fermentation time and that at the beginning of period. The ABE yield (ggT S^{-1}) 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 each product (acetone, butanol, or ethanol) and the butanol yield (ggT S^{-1}). The ABE productivity (g $L^{-1}h^{-1}$) 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.

Statistical analysis was carried out with Microsoft Office Excel 2003 ANOVA Two-Factor without Replication (Microsoft Inc., USA). It was defined that if P-value < 0.05 and F-value F-critical, it could be suggested that there was a significant effect of the treatment variations (nutrient concentrations or initial pH) on the ABE fermentation process (production of acetone, butanol, ethanol, or ABE) (Devore and Peck 1993).

RESULTS

Lag phases at initial pH of 4.4 were longer than those at initial pH of 6.5, however, the ABE production with initial pH 4.4 in each iron concentration applied was higher (Fig 1A and B, Fig 2A, Table 1). The ABE productivity was calculated at 48 h fermentation period, since the maximum ABE concentrations in almost all treatments, especially those with initial pH 4.4, could be obtained after this period. The highest ABE concentration (11 g L^{-1} as stated in Table 1) was achieved at 48 h by adding 0.003 g L^{-1} $FeSO_4.7H_2O$ into the TYA medium with initial pH 4.4. Comparing the treatments added by the same iron concentrations but different initial pH, it was revealed that all such treatments with initial pH 4.4 could obtain higher ABE

yield and productivity. Using 0.025 g L^{-1} $FeSO_4.7H_2O$ at initial pH 6.5, strain N1-4 could obtain 0.31 ggT S^{-1}, 0.41 ggT S^{-1}, and 0.18 g $L^{-1}h^{-1}$ for the butanol yield, ABE yield, and ABE productivity, respectively, but at initial 0.38 ggT S^{-1}, 0.51 ggT S^{-1}, and 0.22 g $L^{-1}h^{-1}$, respectively with initial pH 4.4, and 6.8 g L^{-1}, 0.32 ggT S^{-1}, 0.43 ggT S^{-1}, and 0.19 g $L^{-1}h^{-1}$, respectively with initial pH 6.5 (Table 1). Hence it could be proposed that the optimum

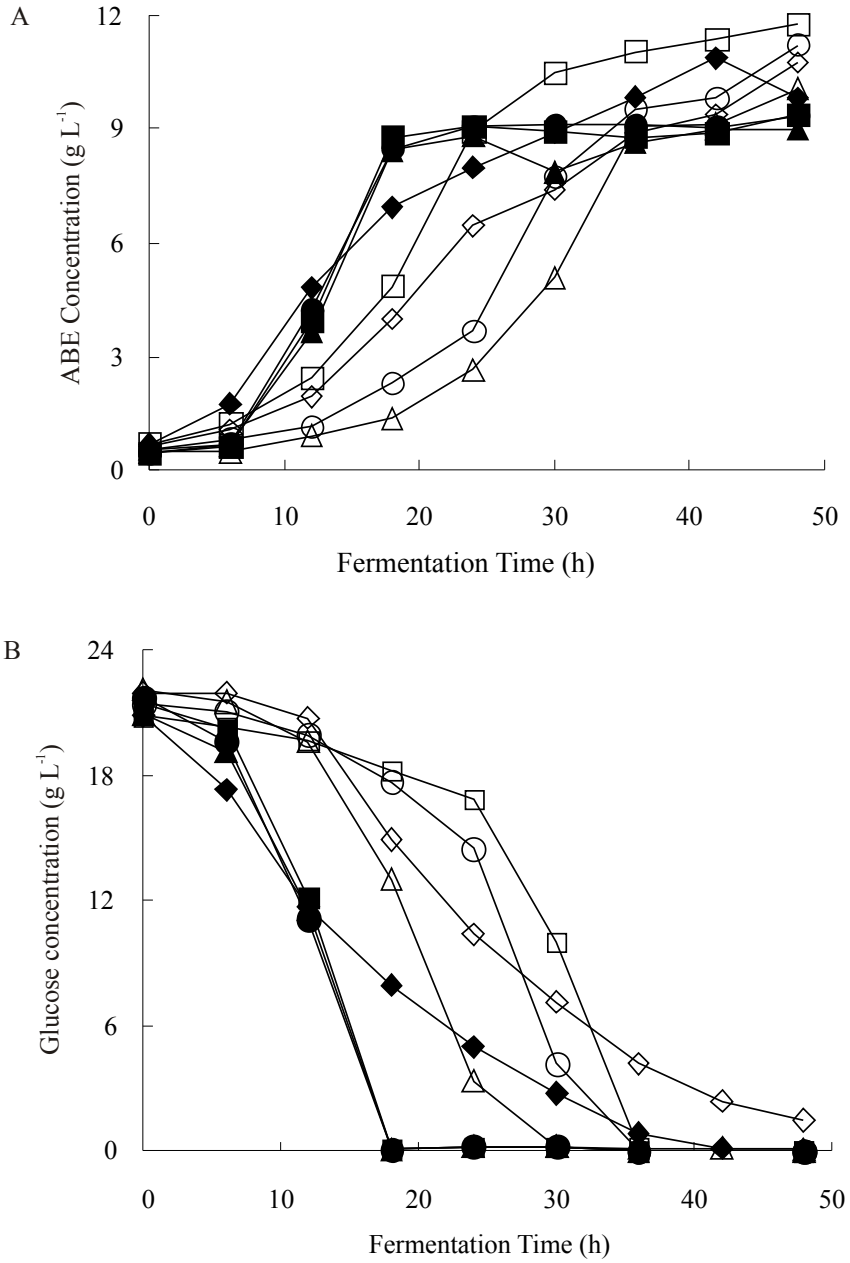

Fig 1 ABE concentration (A) and glucose consumption (B) during ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using glucose with different $FeSO_4.7H_2O$ concentrations in defined TYA media. Fermentation conditions: initial pH = 6.5 or 4.4; working volume = 250 ml; static batch culture without pH control. Symbols: (◆) GF0p6; (◇) GF0p4; (■) GF3p6; (□) GF3p4; (▲) GF10p6*; (△) GF10p4; (●) GF25p6; (○) GF25p4.

pH 4.4 it could obtain higher values of such parameters, which were 0.32 ggT S^{-1}, 0.50 ggT S^{-1}, and 0.22 g $L^{-1}h^{-1}$, respectively (Table 1).

Addition of 0.003 g L^{-1} $FeSO_4.7H_2O$ gave the highest ABE production with either initial pH 4.4 or 6.5, with the values of butanol concentration, butanol yield, ABE yield, and ABE productivity were 7.9 g L^{-1}, condition for strain N1-4 to produce high ABE in a TYA medium with glucose as the substrate is by using 0.003 g L^{-1} $FeSO_4 \cdot 7H_2O$. Interestingly, the treatment GF0p4 (using no iron with initial pH 4.4) could be suggested as the more efficient condition than treatment GF10p6* (using 0.01 g L^{-1} $FeSO_4.7H_2O$ with initial pH 6.5 as the positive control treatment), since strain N1-4 in GF0p4

Table 1 Effect of different $FeSO_4.7H_2O$ concentrations and initial pH on ABE fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) using glucose in defined TYA media at 48 h fermentation time

C-Sources	Acetone ($g\ L^{-1}$)	Butanol ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	ABE ($g\ L^{-1}$)	Consumed substrate ($g\ L^{-1}$)	ABE ratio	Butanol yield ($ggT\ S^{-1}$)	ABE yield ($ggT\ S^{-1}$)	ABE productivity ($g\ L^{-1}h^{-1}$)
GF0p6	3.0	5.7	0.34	9.1	21	0.53:1:0.06	0.28	0.44	0.19
GF0p4	3.4	6.3	0.47	10	20	0.53:1:0.07	0.31	0.49	0.21
GF3p6	2.1	6.8	0.20	9.1	21	0.31:1:0.03	0.32	0.43	0.19
GF3p4	2.5	7.9	0.26	11	21	0.32:1:0.03	0.38	0.51	0.22
GF10p6*	1.7	6.1	0.37	8.2	21	0.28:1:0.06	0.29	0.39	0.17
GF10p4	3.0	6.0	0.51	9.6	22	0.50:1:0.08	0.27	0.43	0.20
GF25p6	1.7	6.8	0.35	8.9	22	0.26:1:0.05	0.31	0.41	0.18
GF25p4	3.5	6.8	0.33	11	21	0.51:1:0.05	0.32	0.50	0.22

The followings are the treatment label codes applied during the experiment (G = glucose; F = $FeSO_4.7H_2O$; p = pH): TYA with no iron (0 g L^{-1} $FeSO_4.7H_2O$) (negative control), initial pH 6.5 (= GF0p6); TYA with no iron (0 g L^{-1} $FeSO_4.7H_2O$), initial pH 4.4 (= GF0p4); TYA containing 0.003 g L^{-1} $FeSO_4.7H_2O$ (iron-limited), initial pH 6.5 (= GF3p6); TYA containing 0.003 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (= GF3p4); TYA containing 0.01 g L^{-1} $FeSO_4.7H_2O$ (positive control), initial pH 6.5 (= GF10p6*); TYA containing 0.01 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (=GF10p4); TYA containing 0.025 g L^{-1} $FeSO_4.7H_2O$ (iron-excess), initial pH 6.5 (= GF25p6); TYA containing 0.025 g L^{-1} $FeSO_4.7H_2O$, initial pH 4.4 (= GF25p4).

could obtain higher yields of butanol and ABE as well as higher ABE productivity than in GF10p6*. In addition, strain N1-4 in treatment GF0p6 (the negative control treatment) could also produce higher ABE yield and productivity than in GF10p6*.

The ABE ratio in treatments using different iron concentrations but the same initial pH could be changed, as well as the ABE ratio in treatments using the same iron concentration but different initial pH values (Table 1). The ABE ratio with initial pH 6.5 using different iron concentrations in treatment GF0p6 was 0.53:1:0.06, but in treatment GF3p6 it changed to 0.31:1:0.03, then to 0.28:1:0.06 in treatment GF10p6 (the positive control), and to 0.26:1:0.05 in treatment GF25p6. Similarly, the ABE ratio with initial pH 4.4 in treatment GF0p4 was 0.53:1:0.07, but then it could change to 0.32:1:0.03 in treatment GF3p4, or to 0.50:1:0.08 in treatment GF10p4, or to 0.51:1:0.05 in treatment GF25p4. The ABE ratio changes in treatments by using the same iron concentration but different initial pH values was clearly demonstrated in treatments which used 0.010 g L^{-1} or higher concentrations. In treatment GF0p6 the ABE ratio was 0.53:1:0.06, which was almost similar to the ratio in treatment GF0p4 (0.53:1:0.07). Also in treatment GF3p6 the ratio was 0.31:1:0.03, almost similar to that in treatment GF3p4 (0.32:1:0.03). However, in treatment GF10p6 the ratio was 0.28:1:0.06, that very much different from the ratio in treatment GF10p4 (0.50:1:0.08); also the ratio in treatment GF25p6 was 0.26:1:0.05, very much different from the ratio in treatment GF25p4 (0.51:1:0.05). Since the ethanol concentration was very low compared to those of acetone and butanol, the ABE ratio could be expressed as the ratio of acetone to butanol only. It was found that with more than 0.01 g L^{-1} $FeSO_4.7H_2O$, ABE ratios of acetone to butanol (0.50-0.53) were higher at initial pH of 4.4 than that (0.26-0.28) at initial pH of 6.5, while those differences were not obtained with low concentration of $FeSO_4.7H_2O$ among the same initial pH (Table 1).

The pH on all media with initial pH 6.5 demonstrates a similar trend, which was decreasing up to 6 h, then increasing as the ABE production initiated, except in the medium GF0p6 that slightly decreasing again after 18 h up to the end of sampling period (Fig 2B). The trend of pH change in media with initial pH 4.4 was different from those with initial pH 6.5. The pH on all media with initial pH 4.4 were all increasing from the beginning until the end of fermentation time, except in the treatment GF0p4 in which the pH started to decrease after 30 h.

Initial pH affected total ABE significantly more than $FeSO_4.7H_2O$ by statistical analysis (Table 2). Data in Table 2 indicate that only the initial pH effect on ABE has the P-value < 0.05 (P-value = 0.003) and F-value > F-critical, meaning that the initial pH had a significant effect on the ABE production by strain N1-4, and that the effect of initial pH was much greater than

the effect of $FeSO_4.7H_2O$ different concentrations on the ABE production in the TYA media using glucose as the substrate.

Finally, from the experiment using different concentration of $FeSO_4.7H_2O$, it can be concluded that $FeSO_4.7H_2O$ by statistical analysis.

DISCUSSION

During the course of glucose fermentation by

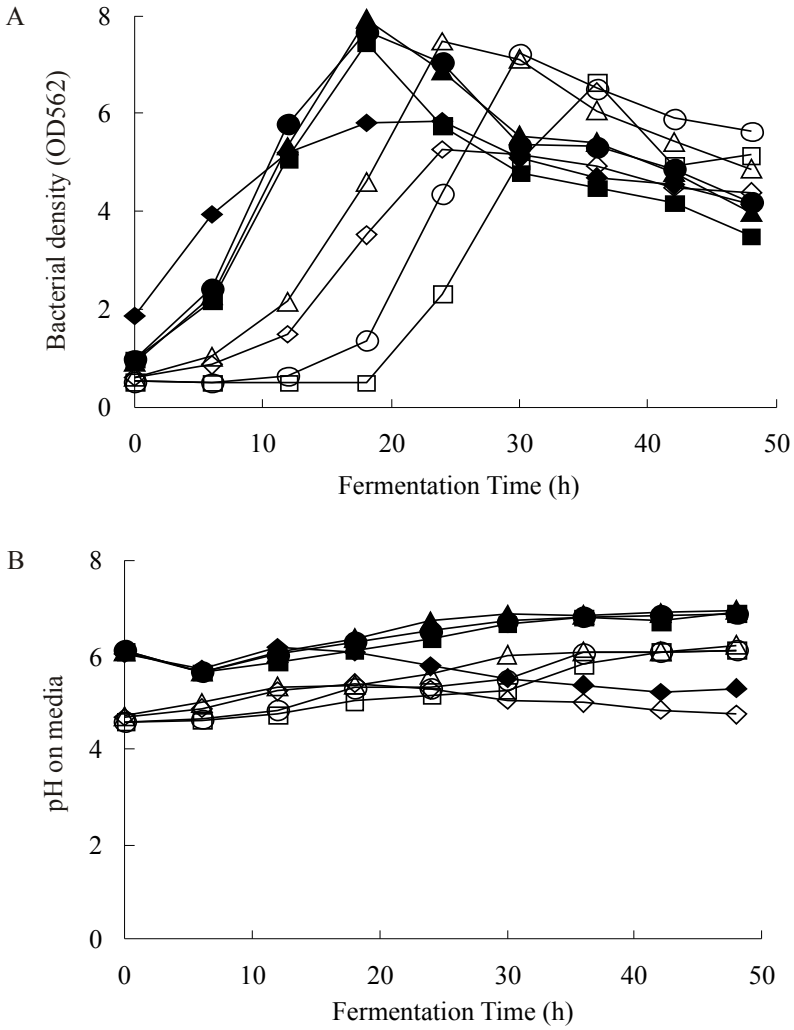

Fig 2 Bacterial density (A) and pH on media (B) during ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using glucose with different $FeSO_4.7H_2O$ concentrations in defined TYA media. Fermentation conditions: initial pH = 6.5 or 4.4; working volume = 250 mL; static batch culture without pH control. Symbols: (◆) GF0p6; (◇) GF0p4; (■) GF3p6; (□) GF3p4; (▲) GF10p6*; (△) GF10p4; (●) GF25p6; (○) GF25p4.

even though the lag phases at initial pH of 4.4 were longer than those at initial pH of 6.5, however they could still have higher ABE productivity values. It was demonstrated that the addition of 0.003 g L^{-1} $FeSO_4.7H_2O$ gave the highest ABE production with both initial pH of 6.5 and 4.4. With more than 0.01 g L^{-1} $FeSO_4.7H_2O$, ABE ratios of acetone to butanol (0.50-0.53) were higher at initial pH of 4.4 than that (0.26-0.28) at initial pH of 6.5, while those differences were not obtained with low concentration of $FeSO_4.7H_2O$ among the same initial pH. It was also confirmed that initial pH affected total ABE significantly more than

ABE-producing Clostridia bacteria, the electron flow is first directed to hydrogen production and the carbon flow to acid biosynthesis (acetic and butyric acids). The breakdown of glucose to acetyl coenzyme A provides the cell with reducing equivalents. To recycle NAD^+, NADH is oxidized by NADH ferredoxin reductase, while hydrogenase oxidizes reduced ferredoxins to produce molecular hydrogen (Adams *et al*. 1980). On the other hand, the solventogenic phase is initiated with accumulation of the fatty acids; both electron and carbon flows are directed to ABE production via ferredoxins $NAD(P)^+$ reductase also

Table 2 Results of statistical analysis using ANOVA Two-Factor without replication for the effect of $FeSO_4.7H_2O$ concentrations and initial pH on ABE fermentation by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) using glucose as the substrate in defined TYA media

Source of Variation	F	P-value	F-critical
$FeSO_4.7H_2O$ concentrations effect on acetone	1.4	0.40	9.3
Initial pH effect on acetone	7.5	0.072	10
$FeSO_4.7H_2O$ concentrations effect on butanol	5.9	0.090	9.3
Initial pH effect on butanol	2.1	0.24	10
$FeSO_4.7H_2O$ concentrations effect on ethanol	6.2	0.084	9.3
Initial pH effect on ethanol	4.4	0.13	10
$FeSO_4.7H_2O$ concentrations effect on ABE	8.4	0.057	9.3
Initial pH effect on ABE	78	0.003	10

aldehyde and alcohol dehydrogenases. ABE-producing Clostridia bacteria were known to posses the metalloenzymes, hydrogenase and NADH-ferredoxin reductase, which mediate hydrogen formation containing iron clusters and a unique type of Fe-S centre termed the H_2 cluster (Adams 1990; Santangelo *et al*. 1995). The iron clusters serve to transfer electrons between the hydrogen cluster and the external electron carrier (Adams 1990). Hence, it was expected that iron supplementation would facilitate hydrogenase biosynthesis and hydrogen evolution, whereas iron limitation could severely affect the enzyme's biosynthesis and function which would then shift the metabolic pathway to ABE production (Bahl *et al*. 1986; Ferchichi *et al*. 2005; Peguin and Soucaille 1995).

Our results demonstrated that under $FeSO_4.7H_2O$-limited conditions, strain N1-4 could produce higher ABE concentrations than under positive control conditions which were usually applied in our ABE group using high iron concentration. These were in agreement with previous studies employing *C. acetobutylicum* (Bahl *et al*. 1986; Junelles *at al*. 1988). The data obtained by Junelles *et al.* (1988) clearly showed that $FeSO_4.7H_2O$ limitation causes a decrease of hydrogenase specific activity and under $FeSO_4.7H_2O$ limitation, acid production is limited but butanol production specifically enhanced by alteration of the carbon and electron pathways present in *C. acetobutylicum*. Their results were in agreement with those of Kim *et al.* (1984), Meyer *et al.* (1986), Dabrock *et al*. (1992), and of Datta and Zeikus (1985), who used carbon monoxide to inhibit hydrogenase either in *C. acetobutylicum* or *C. pasteurianum*.

A previous study by Bahl *et al*. (1986) also revealed that under $FeSO_4.7H_2O$ limitation, lactate instead of butyrate and acetate became the predominant product at pH >5, whereas at lower pH the lactic acid was replaced by butanol production; the highest butanol production was found at pH 4.4 . Our results similarly display the higher lactate production under $FeSO_4.7H_2O$-limited TYA medium with initial pH 6.5 than that with initial pH 4.4 (data not shown). On the other hand, under $FeSO_4.7H_2O$-limited TYA medium with initial pH 4.4 there was a higher butanol production than that with initial pH 6.5. This could be suggested to be caused by a partial inactivation of the hydrogenase enzyme at low pH as found by Andersch *et al*. (1983); inhibition of hydrogenase enzyme resulted in an increase in the butanol production.

In addition, ABE-producing Clostridia bacteria need iron ions for the growth and ABE production (Gottschalk 1986), therefore Fe^{2+} is supplemented in the medium at 10^{-5} - 10^{-4} M. It was found in a study by Ogata *et al*. (1986) also employing strain N1-4, that Fe^{2+} was accumulated from the medium by the cells in proportion to their growth, and more of it was selectively accumulated into the cell walls than any of the other metal ions. They also found that the Fe ions were removed from the medium throughout the log phase and the removal rate was very high at the middle to late log phase (6-12 h). It was then confirmed that there is a close relationship between Fe^{2+} uptake and cell growth with butanol production. Nevertheless, it was not clear that Fe^{2+} had a high affinity with the peptidoglycan moiety of cell wall by *in vivo* and *in vitro*

accumulation tests; the protoplasmic membrane also contained Fe at a high level. The selective assimilation of Fe in cell wall peptidoglycan was thought to be the first step in its transport into the protoplasmic membrane and protoplasm. There, many ferredoxins and other Fe-containing compounds must act in cell growth and butanol production. Interestingly, the data of our study show that strain N1-4 could still grow and produce ABE yields, although in lower states than the other treatments, in the negative control treatments with no addition of $FeSO_4.7H_2O$ into the TYA media at both initial pH values (pH 4.4 or pH 6.5). Therefore, it could be suggested that the $FeSO_4.7H_2O$ is not the limiting factor for the growth and ABE production of strain N1-4 using glucose as the substrate in the chemically defined TYA medium.

Our statistical analysis resulted that the initial pH had a significant effect on the ABE production by strain N1-4, and that the effect of initial pH was much greater than the effect of $FeSO_4.7H_2O$ concentrations on ABE production in the TYA media using glucose as the substrate. These were in accordance with previous studies employing a number of *C. acetobutylicum* DSM strains, which reported the ABE production only below about pH 5.0 using such strains (Andersch *et al*. 1983; Bahl *et al*. 1984; Nishio *et al.* 1983). Additionally, it was known than at pH 6.0 only 6% of the total amount of butyrate is in the undissociated form, whereas at pH 4.5, 66% occurs in the undissociated form (Jones and Woods 1986); therefore it could be the reason of higher ABE production with initial pH 4.4 than with initial pH 6.5 as obtained in our experiment. To the best of our knowledge, our study can be considered as a novel study about the effect of nutrients and initial pH on the ABE fermentation by strain N1-4 in the defined TYA media.

REFERENCES

Adams MW. 1990. The structure and mechanism of iron-hydrogenases. Biochim Biophys Acta. 1020(2):115-145. doi:10.1016/0005-2728(90)90044-5.

Adams MW, Mortenson LE, Chen JS. 1980. Hydrogenase. Biochim Biophys Acta. 594(2-3):105-176.

Amador-Noguez D, Feng XJ, Fan J, Roquet N, Rabitz H, Rabinowitz JD. 2010. Systems-level metabolic flux profiling elucidates a complete bifurcated tricarboxylic acid cycle in *Clostridium acetobutylicum*. J Bacteriol. 192(17): 4452-4461. doi: 10.1128/JB.00490-10.

Andersch W, Bahl H, Gottschalk G. 1983. Level of enzymes involved in acetate, butyrate, acetone, and butanol formation by *Clostridium acetobutylicum*. Eur J Appl Microbiol Biotechnol. 18(6):327-332. doi:10.1007/BF00504740.

Awang GM, Ingledew WM, Jones GA. 1992. The effect of fermentable carbohydrate on sporulation and butanol production by *Clostridium acetobutylicum* P262. Appl Microbiol Biotechnol. 38(1): 12-16.doi:10.1007/BF00169411.

Bahl H, Andersch W, Braun K, Gottschalk G. 1982. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. Eur J Appl Microbiol Biotechnol. 14(1):17-20. doi:10.1007/BF00507998.

Bahl H, Gottschalk G. 1984. Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. Biotechnol Bioeng Symp. 14(1):215-223.

Bahl H, Gottwald M, Kuhn A, Rale V, Andersch W, Gottschalk G. 1986. Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. Appl Environ Microbiol. 52(1):169-172.

Dabrock B, Bahl H, Gottschalk G. 1992. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl Environ Microbiol. 58(4):1233-1239.

Datta R, Zeikus JG. 1985. Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. Appl Environ Microbiol. 49(3):522-529.

Devore J, Peck R. 1993. Statistics: The exploration and analysis of data. 2nd Ed. Belmont, California 94002: Wadsworth Publishing Co.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem. 28(3): 350-356. doi:10.1021/ac60111a017.

Ferchichi M, Crabbe E, Hintz W, Gil G-H, Almadidy A. 2005. Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021. World J Microbiol Biotechnol. 21(6-7):855-862. doi: 10.1007/s11274-004-5972-0.

Formanek J, Mackie R, Blaschek HP. 1997. Enhanced butanol production by *Clostridium beijerinckii* BA101 grown on semi-defined P2 medium containing 6% maltodextrin or glucose. Appl Environ Microbiol. 63(6):2306 -2310.

Gottschalk G. 1986. Bacterial metabolism. 2nd Ed. NY, Tokyo: Springer-Verlag.

Heluane H, Evans MR, Dagher SF, Bruno-Barcena JM. 2011. Meta-analysis and functional validation of nutritional requirements of solventogenic Clostridia growing under butanol stress conditions and coutilization of D-glucose and D-xylose. Appl Env Microbiol. 77(13): 4473-4485. doi: 10.1128/AEM.00116-11.

Hipolito CN, Crabbe E, Badillo CM, Zarrabal OC, Mora MAM, Flores GP, Cortazar MAH, Ishizaki A. 2008. Bioconversion of industrial wastewater from palm oil processing to butanol by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). J Clean Prod. 16(5):632 -638. doi: 10.1016/j.jclepro.2007.02.005.

Jesse TW, Ezeji TC, Qureshi N, Blaschek HP. 2002. Production of butanol from starch-based waste packing peanuts and agricultural waste. J Ind Microbiol Biotechnol. 29(3):117-123. doi: 10.1038/sj.jim.7000285.

Jones DT, Woods DR, 1986. Acetone-butanol fermentation revisited. Microbiol Rev. 50(4): 484 -524.

Junelles AM, Janati-Idrissi R, Petitdemange H, Gay R. 1988. Iron effect on acetone-butanol fermentation. Curr Microbiol.17(5):299-303. doi: 10.1007/BF01571332.

Kim BH, Bellows P, Datta R, Zeikus JG, 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl Environ Microbiol. 48(4): 764-770.

Meyer CL, Roos JW, Papoutsakis ET. 1986. Carbon monoxide gasing leads to alcohol production and butyrate uptake without acetone formation in continuous culture of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol. 24(2):159-167. doi: 10.1007/BF01982561.

Monot F, Engasser JM, Petitdemange H, 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol. 19(6):422-426. doi: 10.1007/BF00454381.

Monot F, Martin JR, Petitdemange H, Gay R. 1982. Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. Appl Environ Microbiol. 44(6):1318-1324.

Nishio N, Biebl H, Meiners M. 1983. Effect of pH on the production of acetone and butanol by *C. acetobutylicum* in a minimum medium. J Ferment Technol. 61(1):101-104.

Ogata S, Fujino K, Yoshino S, Hayashida S, Yuita K. 1986. Selective accumulation of iron ion into the cell wall of *Clostridium saccaharoperbutylacetonicum* ATCC 13564. J Gen Appl Microbiol. 32(2): 101-109.

Peguin S, Soucaille P. 1995. Modulation of carbon and electron flow in *Clostridium acetobutylicum* by iron limitation and methyl viologen addition. Appl Environ Microbiol. 61(1): 403-405.

Qureshi N, Blaschek HP. 2001. ABE production from corn: A recent economic evaluation. J Ind Microbiol Biotechnol. 27(5):292-297. doi: 10.1038/sj.jim.7000123.

Qureshi N, Li XL, Hughes S, Saha BC, Cotta MA. 2006. Butanol production from corn fiber xylan using *Clostridium acetobutylicum*. Biotechnol Prog. 22: 673-680. doi: 10.1021/bp050360w.

Santangelo JD, Durre P, Woods DR. 1995. Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262. Microbiology. 141(1): 171-180. doi: 10.1099/00221287-141-1-171.

Tashiro Y, Sonomoto K. 2010. Advances in butanol production by clostridia. In: Mendez-Vilas, A. (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (Microbiology Book Series, number #2). FORMATEX, Spain:1383-1394.

Tashiro Y, Shinto H, Hayashi M, Baba S, Kobayashi G, Sonomoto K. 2007. Novel high-efficient butanol production from butyrate by non-growing *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) with methyl viologen. J Biosc Bioeng. 104(3): 238-240. doi: 10.1263/jbb.104.238.

Tashiro Y, Takeda K, Kobayashi G, Sonomoto K, Ishizaki A, Yoshino S, 2004. High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed-batch culture with pH-stat continuous butyric acid and glucose feeding method. J Biosci Bioeng. 98(4): 263-268. doi: 10.1263/jbb.98.263.