

Full Length Research Paper

Improvement of gas chromatographic analysis for organic acids and solvents in acetone-butanol-ethanol fermentation from sweet sorghum juice

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Modern development for gas chromatographic peak recognition of organic acids and organic solvents in acetone-butanol-ethanol (ABE) fermentation was investigated under different temperature programmes. Either propanol or iso-butanol was used as an internal standard. The results showed that short retention time and fair recognition peak of the compounds were obtained under the following column temperature programme: 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C. The appearance of the chromatograms showed that iso-butanol allowed higher resolution and satisfactory peak shape for the tested compounds than propanol. Calibration curve between ratios of standard peak area per internal standard peak area and concentrations of the organic acids and organic solvents under these conditions had a good linear correlation with $R^2 \geq 0.998$. When ABE fermentation by *Clostridium beijerinckii* JCM1390 from sweet sorghum juice was carried out in a 2-L fermenter, the highest butanol concentration was 7.56 g/l with the butanol yield of 0.33 g/g sugar utilized.

Key words: Acetone-butanol-ethanol fermentation, column temperature programme, gas chromatography, sweet sorghum juice.

INTRODUCTION

Depletion of fossil fuels and fluctuating prices have rekindled and have been interesting in the development of renewable fuels/energy sources such as butanol (Qureshi and Blascheck, 2001). Butanol can be produced from a variety of renewable sources e.g. glucose, whey permeated, corn and cassava (Qureshi and Blascheck, 2001; Thang et al., 2010). This alcohol is widely used in the manufacturing of resin, cleaning fluids, plasticizers and in the action with acids to form esters (Wei et al., 2004). The production of a mixture of acetone, butanol

and ethanol fermentation by using the anaerobic bacterium *Clostridium acetobutylicum* continues to receive attention because of its potential commercial significance (Maddox et al., 1995).

Sweet sorghum [*Sorghum bicolor* (L.)] has been promised as a large scale energy crop because its stalks contain high fermentable sugar and it can be cultivated in nearly all temperatures and tropical climate areas (Sree et al., 1999; Laopaiboon et al., 2009). It is also one of the most drought resistant agricultural crops because of its capacity to remain dormant during the driest periods (Woods, 2000). The juice from sweet sorghum stalk can be efficiently converted into a biofuel, ethanol (Laopaiboon et al., 2009). However, there has been no report on using sweet sorghum juice as a substrate for butanol production.

A fermentation process producing acetone, butanol and ethanol has been known as acetone-butanol-ethanol (ABE) fermentation. ABE-producing clostridia possess two distinct characteristic phases in energy acquiring

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Abbreviations: ABE, Acetone-butanol-ethanol; GC, gas chromatography; FID, flame ionization detector; CMM, cooked meat medium; OFN, oxygen free nitrogen; TGY, tryptone-glucose-yeast extract; DNS, dinitrosalicylic acid.

pathway, that is, acidogenesis and solventogenesis (Jones and Woods, 1986). During acidogenesis, cell growth is exponential and products are acetic and butyric acids with ATP formation. In solventogenesis, cell growth enters stationary phase, the organic acids from the acidogenesis are reutilized and acetone, butanol and ethanol are produced (Tashiro et al., 2004). Therefore, it is necessary to identify and monitor concentrations of the organic acids and the organic solvents during fermentation process.

Most researchers have used gas chromatography or GC for acid and solvent analysis from ABE fermentation under various conditions and most of them used nitrogen as a carrier gas. Gapes et al. (1996) analyzed product concentrations with a glass column (inside diameter of 3.2 mm, length of 2.6 m) packed with Chromosorb 101 at a column temperature of 170°C and flame ionization detector (FID). Assobhei et al. (1998) used a 2-m long glass column packed with Porapak Q 100/120 mesh equipped with FID. The injector and detector temperatures were 220°C, the column temperature was programmed from 175 to 225°C. Montaya et al. (2000) found that the solvents were separated in a steel column packed with Chromosorb 102. The injector and detector temperatures were 200°C. The column temperature was programmed from 200 to 210°C, at a rate of 1°C/min. The injector and detector temperatures were 260°C. Qureshi et al. (2001) used GC equipped with FID and a capillary (0.53 mm, 30 m). The initial and final column temperatures were 60 and 160°C, respectively. Tashiro et al. (2004) determined ABE products of ABE fermentation by using GC equipped with FID and a 15 m capillary column. The column temperature was programmed from 50 to 170°C at the rate of 10°C/min. The injector and detector temperatures were 250°C and helium was used as a carrier gas. The two internal standards normally used for ABE analysis are propanol (Montaya et al., 2000; Qureshi et al., 2001) and iso-butanol (Gapes et al., 1996; Assobhei et al., 1998; Tashiro et al., 2004).

Although, GC conditions for acid and solvent quantification have been investigated by a number of researchers, product analysis by GC is also dependent on several parameters such as GC instrument, type of packing material in column, inside diameter and length of column, etc. Thus, the aim of this work is to develop a method to accurately detect the main products in the ABE fermentation broth, that is, acetic acid, butyric acid, acetone, butanol and ethanol using GC under different column temperature programmes. ABE fermentation from sweet sorghum juice by *C. beijerinckii* JCM1390 was also investigated.

MATERIALS AND METHODS

GC analysis

Standard solution for GC analysis consisted of acetic acid (Sigma-Aldrich, Germany), 20.0 g/l; acetone (Merck, Germany), 10.0 g/l; butyric acid (BDH, UK), 10.0 g/l; butanol (BDH, UK), 10.0 g/l;

ethanol (BDH, UK), 10.0 g/l of 20% (v/v) ortho-phosphoric acid (BDH, UK). The standard mixture was diluted at different concentrations by using the ortho-phosphoric acid as a diluent. Propanol (BDH, UK) at 8.0 g/l of 20% (v/v) ortho-phosphoric acid was used as an internal standard. The standard mixtures at various concentrations were mixed with the internal standard at the ratio of 1:1 (v/v). Then, 1 µl of the mixtures was injected at least three times into GC to ensure repeatability. The standard calibration curve between concentrations of the standard mixtures and the ratios of peak area (standard peak area per internal standard peak area) was established. Iso-butanol (BDH, UK) at concentration of 8.0 g/l (in 20% ortho-phosphoric acid) was also used as an internal standard. The standard mixtures at various concentrations were mixed with this internal standard as described for propanol.

GC analysis of organic acids and organic solvents was performed using a GC-14B (Shimadzu, Japan) gas chromatograph in a splitless mode. Separation took place in a 2-m long and 2-mm inner diameter stainless steel column packed with Parapak Q, 80/100 mesh (Resteck, USA). The injector was operated at 220°C and the FID was kept at 230°C. Nitrogen gas was used as a carrier gas at a pressure of 150 kPa. The column temperature was varied under five different column temperature programmes as stated in (A) - (E): (A) 145°C (isothermal programme); (B) 150°C (isothermal programme); (C) 10 min at 150°C, 10°C/min to 180°C, 20 min at 180°C; (D) 10 min at 150°C, 15°C/min to 180°C, 20 min at 180°C; (E) 10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C, 20 min at 200°C. Chromatographic data were acquired by C-R7A (Shimadzu, Japan).

ABE fermentation

Microorganism and inoculum preparation

C. beijerinckii JCM 1390 was maintained as spore suspension and stored at 4°C in sterile distilled H₂O. The spore suspension (0.5 ml) was heat shocked at 80°C for 10 min, cooled in iced-water for 1 min (Areesirisuk et al., 2006), and inoculated into 20 ml sealed serum bottle containing 10 ml cooked meat medium (CMM). The culture was incubated at 37°C until growth was observed (16 - 19 h). Before the inoculation, CMM in the bottle was rapidly sparged with filtered oxygen free nitrogen (OFN) gas to create a strictly anaerobic condition. Five ml of CMM cell suspension, which was highly motile cells, were transferred into tryptone-glucose-yeast extract (TGY) medium and incubated at 37°C for 8 h. This culture was used as an inoculum for butanol production.

Raw material and medium preparation

Sweet sorghum juice cv. K KU40 modified from cv. Keller was obtained from the Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand. After extraction, the juice was kept at -18°C until use.

The sweet sorghum juice was supplemented with 1 g/l of yeast extract (Oxoid, UK) (Qureshi and Blaschek, 1999) and pH of the juice was adjusted to 5.5 with 1.0 N NaOH (BDH, UK). The juice was then transferred into a 2-L fermenter (B. Braun Biotech International, Biostat B, Germany) with a final working volume of 1.5 L and autoclaved at 110°C for 25 min. Three stock solutions (A, B and C) were prepared (Qureshi and Blaschek, 1999). The stock solution A was composed of K₂HPO₄ (BDH, UK), 50 g/l; KH₂PO₄ (BDH, UK), 50 g/l and ammonium acetate (BDH, UK), 220 g/l. The stock solution B was composed of para-amino-benzoic acid (BDH, UK), 0.1 g/l; thiamine (BDH, UK), 0.1 g/l and biotin (Fluka, Switzerland), 0.001 g/l. The stock solution C was composed of MgSO₄·7H₂O (Riedel-DeHaen, Germany), 20 g/l; MnSO₄·H₂O (BDH, UK), 1 g/l; FeSO₄·7H₂O (Riedel-DeHaen, Germany), 1 g/l

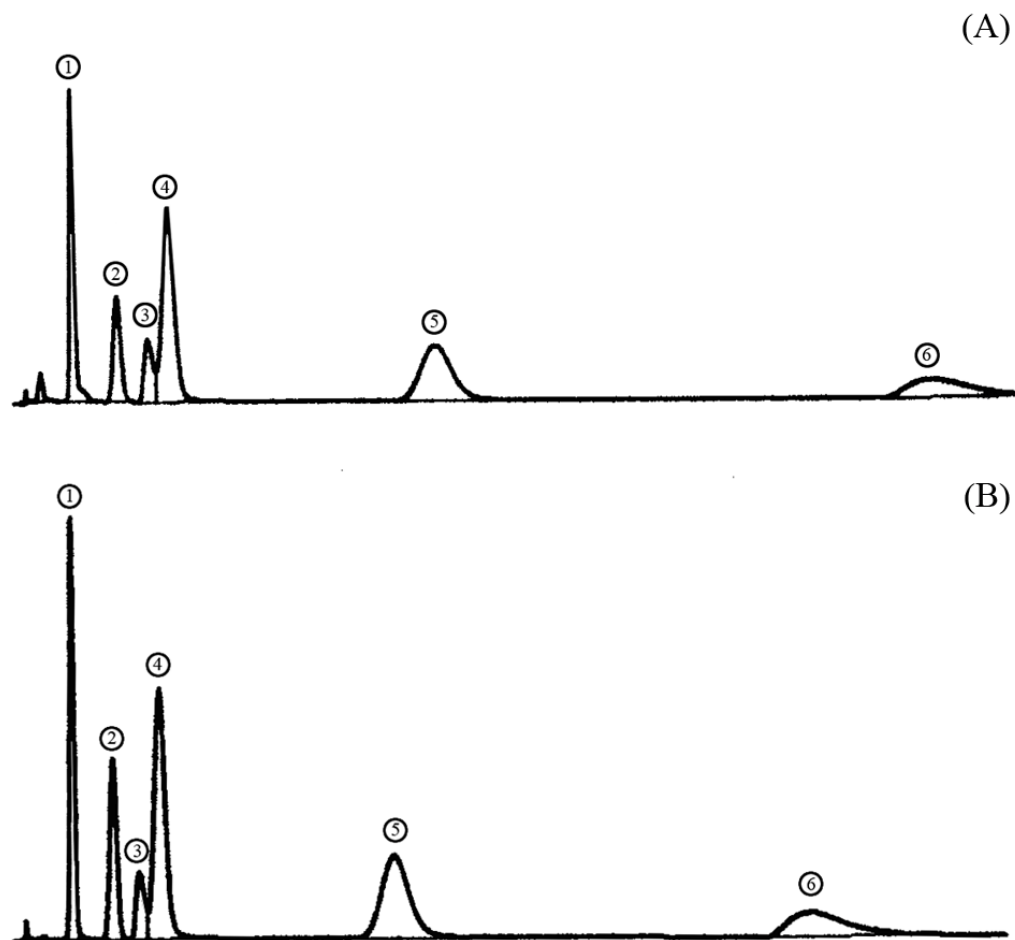


Figure 1. GC chromatogram of organic acid and organic solvent standards under isothermal temperature programme. A, Condition A (isothermal programme at 145°C); B, condition B (isothermal programme at 150°C); ①, ethanol; ②, acetone; ③, acetic acid; ④, propanol; ⑤, butanol; ⑥, butyric acid.

and NaCl (BDH, UK), 1 g/l. The three stock solutions were sterilized by filtration through a 0.2 µm pore-size cellulose acetate membrane filter before adding (15 ml each) into the sterile juice.

Fermentation conditions and analyses

OFN gas was quickly flushed into the sterile medium in the fermenter to create a strictly anaerobic condition before the *C. beijerinckii* JCM 1390 in TGY medium (5%, v/v) was inoculated. The fermentation was operated at the agitation rate of 50 rpm, 37°C and OFN gas was flushed continuously across the surface of the medium at the rate of 0.2 ml/min until gases in the fermentation broth were generated by visual observation. The samples were withdrawn at time intervals for analysis.

Acid and solvent concentrations of the supernatant were analyzed by GC under the optimum conditions obtained. Total sugar and reducing sugar concentrations of the supernatant were determined by phenol-sulphuric acid and dinitrosalicylic acid (DNS) method, respectively (Scherz and Bonn, 1998). The butanol yield ($Y_{p/s}$) and volumetric butanol productivity (Q_p) were calculated by the following equations:

$$Y_{p/s} = \frac{P}{TS}$$

$$Q_p = \frac{P}{t}$$

Where, P is the butanol concentration (g/l), TS is the total sugar utilized (g/l) and t is the fermentation time (h).

RESULTS AND DISCUSSION

Gas chromatographic peak identification

Isothermal programme

Figure 1 shows the chromatogram of organic acid and organic solvent analysis under isothermal temperature programme and propanol as an internal standard. Under condition A (145°C), all peaks of the standards could be

Table 1. Retention time of organic acids and organic solvents under different GC conditions.

GC condition*	Retention time (min)						
	Ethanol	Acetone	Acetic acid	Propanol	Iso-butanol	Butanol	Butyric acid
A with propanol**	3.890	6.774	8.769	10.113	-	27.359	59.491
B**	3.513	5.983	7.549	8.801	-	22.983	48.071
C**	3.477	5.929	7.474	8.723	-	16.201	23.612
D**	3.516	5.999	7.583	8.830	-	15.961	23.421
E**	3.480	5.940	7.500	8.743	-	15.875	21.028
D with iso-butanol***	3.782	6.174	8.760	-	15.029	16.712	24.844

*Condition A: Isothermal programme at 145°C; condition B: isothermal programme at 150°C; condition C: 10 min at 150°C, 10°C/min to 180°C and 20 min at 180°C; condition D: 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C and condition E: 10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C and 20 min at 200°C. ** ***propanol or iso-butanol as the internal standard, respectively.

separated, but butanol and butyric acid peaks had band broadening and the retention time of the last peak was too long (Figure 1A). To make the last two peaks sharper and reduce analysis time, column temperature was increased from 145 to 150°C (condition B). The results indicated that retention time of the standard mixtures was decreased with increasing column temperature (Figure 1B and Table 1). Nonetheless, butanol and butyric acid peaks still had band spreading. Thus, column temperature programme conditions were applied.

Temperature programme

In order to reduce retention time and alleviate band broadening, column temperature programme under condition C (10 min at 150°C, 10°C/min to 180°C and 20 min at 180°C) was tested. The results indicated that retention time of the standard mixtures was significantly decreased (Figure 2A). In particular, the retention time of butanol and butyric acid peaks were reduced to approximately 10 and 51%, respectively, when compared to those under condition B. This suggested that temperature programme could improve chromatogram for the standard mixture separation. However, a tailing peak of butyric acid was observed. To develop the butyric acid peak, rate of increasing column temperature was adapted to condition D (10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C). The chromatogram and retention time of ethanol, acetone, acetic acid, propanol, butanol and butyric acid under condition D (Figure 2B) were similar to those under condition C (Figure 2A). This implied that chromatographic separation could not always be improved by increasing accelerating rate of column temperature only.

To alleviate the tailing peak of butyric acid, the column temperature programme of condition E (10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C and 20 min at 200°C) was operated (Figure 2C). Under condition E, the retention time of butyric acid peak was decreased by 56% when compared with that under

isothermal condition B, whereas those of ethanol, acetone, acetic acid, propanol and butanol were still similar to those under condition C and D (Table 1). However, the baseline of chromatogram under condition E was not good enough. Therefore, it was not selected for further studies.

Under condition D, the peaks of acetic acid and propanol were overlapped and the acetic acid peak was closed to the acetone peak (Figure 2B). Therefore, another internal standard normally used, iso-butanol (Gapes et al., 1996; Assobhei et al., 1998; Tashiro et al., 2004), was tested to improve the chromatogram. The appearance of the chromatogram demonstrated that iso-butanol allowed high resolution and satisfactory peak shape for the tested compounds (Figure 3). Analysis of the chromatogram using propanol compared with that using iso-butanol revealed that the organic acids and solvents under using the latter internal standard were separated better than those using the former one (Figures 2B and 3). Therefore, the column temperature programme under condition D and iso-butanol as an internal standard were chosen to evaluate calibration curves of standard mixtures for ABE production.

The standard mixtures at various concentrations were injected into the GC under condition D. The calibration curves between ratios of standard peak area per internal standard (iso-butanol) peak area and concentrations of the organic acids and organic solvents were established (Figure 4). The results showed that the ratios of standard peak area per internal standard peak area and concentration of the standards had a good linear correlation with $R^2 \geq 0.998$. Under condition D, the sensitivity or detection limit of ethanol, acetone, acetic acid, butanol and butyric acid were 0.31, 0.31, 1.25, 0.63 and 0.63 g/l, respectively.

ABE fermentation from sweet sorghum juice

Batch ABE fermentation of *C. beijerinckii* JCM 1390 was performed to evaluate the GC condition developed and to study the possibility of the use of the sweet sorghum juice

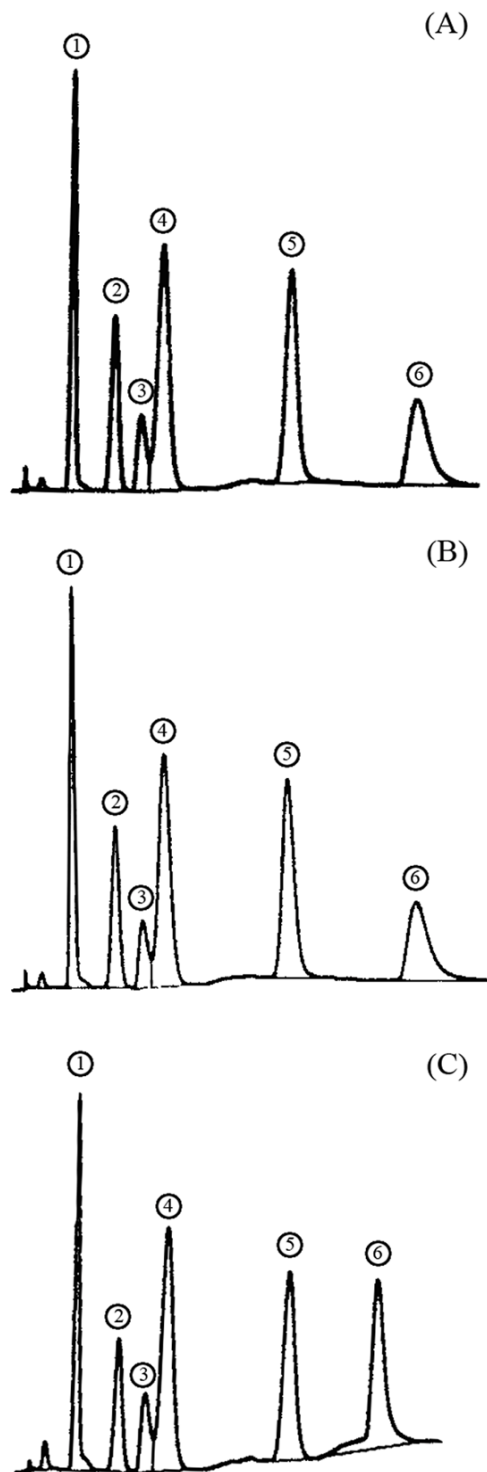


Figure 2. GC chromatogram of organic acid and organic solvent standards under column temperature programme. A, Condition C (10 min at 150°C, 10°C/min to 180°C and 20 min at 180°C); B, condition D (10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C); C, condition E (10 min at 150°C, 15°C/min to 180°C, 5 min at 180 °C, 10°C/min to 200°C and 20 min at 200°C); ①, ethanol; ②, acetone; ③, acetic acid; ④, propanol; ⑤, butanol; ⑥, butyric acid.

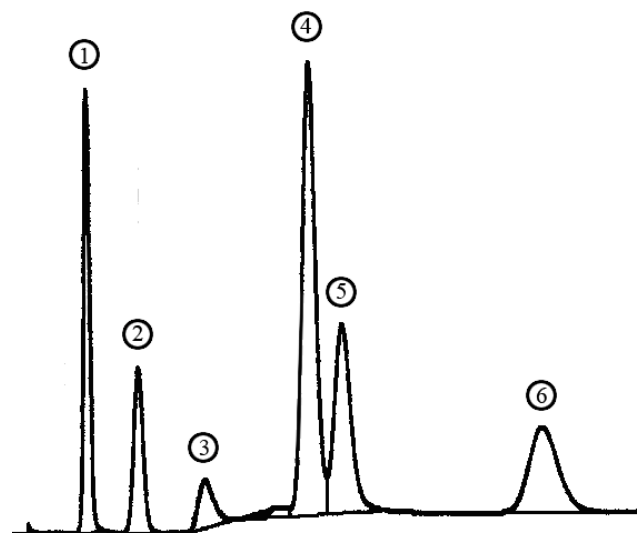


Figure 3. GC chromatogram of organic acid and organic solvent standards under condition D (10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C) using iso-butanol as an internal standard. ①, Ethanol; ②, acetone; ③, acetic acid; ④, iso-butanol; ⑤, butanol; ⑥, butyric acid.

as a substrate for butanol production. The profiles of total sugar, reducing sugar, pH, organic acids and organic solvents during fermentation are shown in Figure 5. Total sugar and reducing sugar were decreased in the first 75 h. After that, their concentrations rarely changed. The total sugar and reducing sugar utilized were similar with the values of 24.91 and 20.19 g/l, respectively, indicating that almost all sugar utilized were glucose and fructose because they were the main reducing sugar in the sweet sorghum juice (Laopaiboon et al., 2009). When the products from the fermentation were monitored (Figure 5), the results showed that two distinct characteristic phases, acidogenesis and solventogenesis, occurred.

Acidogenesis phase of *C. beijerinckii* JCM 1390 had occurred since the beginning of the fermentation. Acetic acid concentration was increased to 2.89 g/l at 38 h and slightly decreased after that, while butyric acid concentration was increased to 2.45 g/l at 38 h and was highest (3.05 g/l) at 95 h. The accumulation of the acetic and butyric acids resulted in a decrease in pH of the broth from 5.51 at the beginning to 4.98 at 38 h. pH of the broth was slightly increased after that and remained constant at 5.13. Similar results were observed by Wang et al. (2005) who studied ABE production by *C. beijerinckii* NCIMB 8052 grown on TGY medium containing 0.5% (w/v) glucose at 37°C. They reported that pH of the broth was decreased with an increase in organic acids.

Solventogenesis phase was observed after approximately 20 h of the fermentation (Figure 5). Butanol concentration was rapidly increased after 38 h and the highest value was obtained at 95 h. Ethanol and acetone were produced at very low levels with the maximum

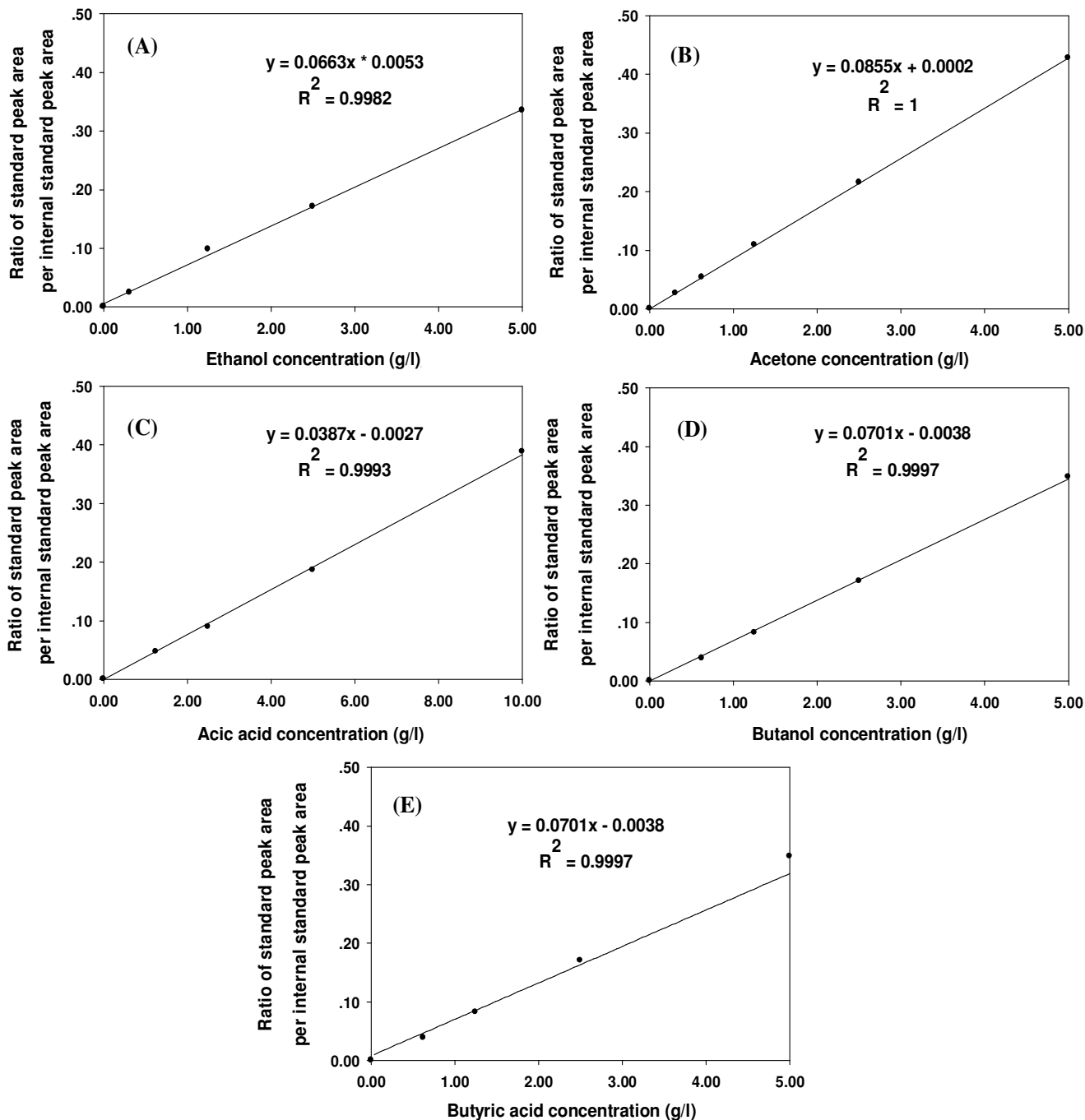


Figure 4. Calibration curves of ethanol (A), acetone (B), acetic acid (C), butanol (D) and butyric acid (E) using iso-butanol as an internal standard.

values of 0.72 and 0.66 g/l, respectively. *C. beijerinckii* JCM 1390 produced butanol much higher than ethanol and acetone. The highest butanol concentration, $Y_{p/s}$ and Q_p were 7.56 g/l, 0.30 g/g and 0.08 g/l/h, respectively. The results obtained from this study were satisfactory in terms of very low ethanol and acetone produced when compared to those of Tsuey et al. (2006)

in which the acetone concentration was about 60% of butanol concentration.

Conclusions

GC conditions have been developed to improve the gas chromatographic peak and retention time of the main

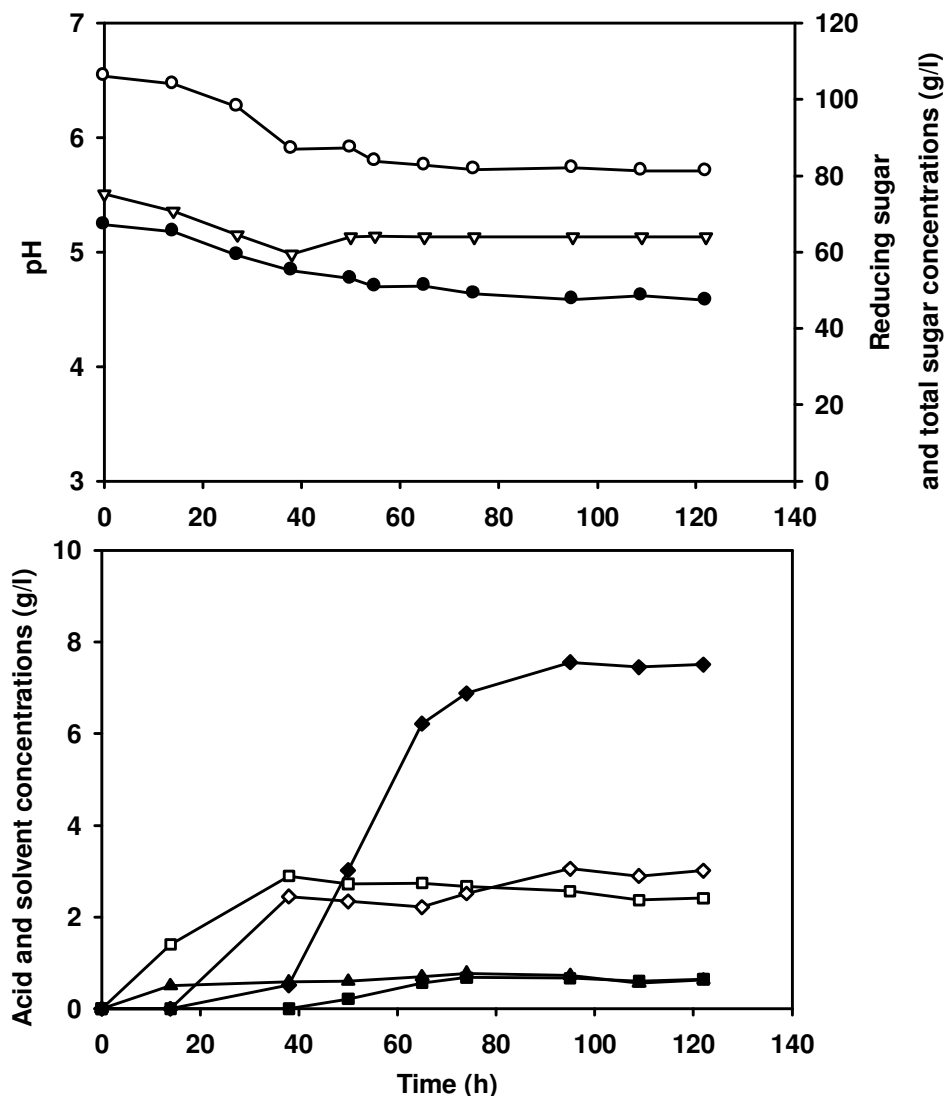


Figure 5. Batch culture profiles and product formation of *C. beijerinckii* JCM 1390 from the sweet sorghum juice. ▽, pH; ●, reducing sugar; ○, total sugar; ■, acetone; ◆, butanol; ▲, ethanol; □, acetic acid; ◇, butyric acid.

organic acids and organic solvents produced in ABE fermentation under different temperature programmes. The optimum conditions for identification and monitoring of ABE concentration during fermentation process of the acids and the solvents using GC were 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C and iso-butanol was used as an internal standard. The ratio of standard peak area per internal standard peak area and concentrations of the standards had a good linear correlation with $R^2 \geq 0.998$. The ABE fermentation of *C. beijerinckii* JCM 1390 demonstrated that sweet sorghum juice could be used as a substrate for butanol production. However, low butanol production rate or in our study needs to be improved. The effects of initial pH and total sugar to improve the rate of butanol production from sweet sorghum juice are under investigated.

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