Improvements of GC and HPLC Analyses in Solvent (Acetone-Butanol-Ethanol) Fermentation by *Clostridium saccharobutylicum* Using a Mixture of Starch and Glycerol as Carbon Source

Liew Shiau Tsuey, Arbakariya Bin Ariff*, Rosfarizan Mohamad, and Raha Abdul Rahim

Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor D.E., Malaysia

Abstract A study on the feasibility of using improved computer-controlled HPLC and GC systems was carried out to shorten the time needed for measuring levels of the substrates (glucose, maltose, and glycerol) and products (acetone, butanol ethanol, acetic acid, and butyric acid) produced by *Clostridium saccharobutylicum* DSM 13864 during direct fermentation of sago starch to solvent. The use of HPLC system with a single injection to analyse the composition of culture broth (substrates and products) during solvent fermentation was achieved by raising the column temperature to 80°C. Although good separation of the components in the mixture was achieved, a slight overlap was observed in the peaks for butyric acid and acetone. The shape of the peak obtained and the analysis time of 26.66 min were satisfactory at a fixed flow rate of 0.8 mL/min. An improved GC system was developed, that was able to measure the products of solvent fermentation (acetone, butanol, ethanol, acetic acid, and butyric acid) within 19.28 min. Excellent resolution for each peak was achieved by adjusting the oven temperature to 65°C.

Keywords: acetone-butanol-ethanol fermentation, solvent analysis, GC, HPLC, *Clostridium saccharobutylicum*

INTRODUCTION

Development of an anaerobic fermentation process for the production of such solvents as acetone, butanol, and ethanol continues to receive attention due to the potential application of such a process to biotechnology. The most important economic factor in solvent fermentation is the cost of raw material, which makes up about 60% of the overall cost [1]. Therefore, the use of sago starch, which is an inexpensive carbon source, would reduce the cost of raw material for solvent production in Malaysia. Direct fermentation of gelatinized sago starch to solvent has been developed successfully using batch cultures of *Clostridium saccharobutylicum* DSM 13864 [2]. During fermentation, gelatinized sago starch is first hydrolyzed to maltose and glucose by amylolytic enzymes (α-amylase and glucoamylase) secreted by the bacterium. The glucose is then converted to organic acids (acetic acid and butyric acid) as intermediate products prior to the formation of solvents (acetone, butanol, and ethanol) [3,4].

Methods used for measuring the substrates and products of this solvent fermentation are varied. Quantification of the volatile products using gas chromatography (GC) has been the most common procedure conducted, and various methods of GC analysis have been reported in the literature [5-7]. High performance liquid chromatography (HPLC) has also been used to analyse the solvent fermentation products [8-10]. However, poor resolution between butyric acid and acetone, and between acetone and ethanol using HPLC systems cannot adequately quantitate the concentrations of these materials. Improved acetone-butanol fermentation analysis using subambient HPLC column temperature has been reported by Buday *et al.* [8]. In our previous work, GC was only used to analyse solvents, whereas HPLC was used to analyse organic acids and reducing sugars. In this case, organic acids and reducing sugars were analysed separately using two different columns, an Aminex HPX-87H column and an NH_2 column. This method makes the analysis more complicated and time consuming. Furthermore, problems such as lower resolution, between acetone and ethanol occurred in GC analysis. At present, the potential use of glycerol for the improvement of direct fermentation of gelatinized sago starch to solvent is being studied in our laboratory. In solvent fermentation, NADH is required for the formation of butanol and ethanol, whereas abundant supply of ATP enhanced the production of acetone and butanol [11]. The major product of glycerol metabolism by *C. saccharobutylicum* is 1,3-propanediol [12]. Induction of 1,3-propanediol pathway in this bacte-

***Corresponding author**
Tel: +60-3-8946-7516 Fax: +60-3-8946-7510
e-mail: arbarif@biotech.upm.edu.my

rium is associated with higher accumulation of ATP and NADH. Thus, simultaneous monitoring of glycerol and other compounds in the culture broth is required to obtain complete data for better description of the fermentation process.

The present study was undertaken to study the feasibility of using of HPLC and GC as fast and efficient methods for the quantification of glucose, maltose, glycerol, acetone, butanol, ethanol, acetic acid, and butyric acid in culture broth withdrawn from solvent fermentation using a mixture of sago starch and glycerol as carbon source.

MATERIALS AND METHODS

High Performance Liquid Chromatography (HPLC)

The experiments were conducted using HPLC (Waters 2690 Alliance Separation Module) equipped with column heater module and a refractive index (RI) detector (Waters 2410). Separation of compounds was achieved by using a Shodex KC-811 packed column (8 × 300 mm). The column was packed with a sulfonated rigid styrene divinylbenzene copolymer providing high efficiency separation of low molecular weight organic acids and water soluble organics like alcohol. The column provided both ion exclusion and reversed-phase modes of chromatography. Separation employing the KC-811 column could be controlled by varying mobile phase concentration, column temperature, and flow rate. The mobile phase was 3 mM H_2SO_4 which was filtered using Whatman cellulose nitrate membrane filter paper (0.45 μm pore size; 47 mm diameter). The flow rate was set at 0.8 mL/min, the column temperature was 80°C, and the injection volume was 10 μL. Data acquisition software (Millennium 32 v) was used to integrate the data.

Gas Chromatography (GC)

The GC used in this study was an Agilent 6890 with a flame ionization detector. Separation took place in a 2 m capillary column packed with polar BP 20 (SGE), using nitrogen as the carrier gas. This type of column is normally used to analyze alcohols, solvents, free acids, fatty acid methyl esters, aromatics, and essential oils. The GC oven temperature was initially controlled at 65°C for 8 min, then it was increased at a rate of 20°C per min to a final temperature of 140°C, which was held for 10 min. The temperature of the injector was set at 220°C and the detector was set at 270°C. The injection volume was 1 μL. Data acquisition software (Chemstation, Agilent Technologies, Hewlett Packard, Wilmington, Delaware, USA) was used to integrate the data.

Preparation of Standards

Chromatographic standards were obtained commercially. Acetone (99.99%) and butanol (99.94%) were from Fisher Scientific Co., while ethanol (99.8%) and acetic acid (99.8%) came from Merck. Agros Organics was the source of butyric acid (99.0%). Fluka provided the maltose (98.0%), glucose (98.0%) was purchased from HmbG Chemicals, and glycerol came from Sigma. Before injection into GC and HPLC, standards were filtered through Whatman nylon filter paper (0.45 μm pore size; 13 mm diameter) to remove insoluble materials that could block the column. All samples were stored frozen in sealed vials to maintain the stability of volatile components. During thawing, the vials were shaken vigorously by hand for 2 min.

Microorganism and Fermentation Condition

The bacterium *C. saccharobutylicum* DSM 13864 was maintained as spore suspension in sterile Reinforced Clostridium Medium (RCM) and stored under anaerobic conditions at 4°C. RCM was also used for the preparation of inoculum.

The solvent fermentation protocol as suggested by Madihah *et al.* [2] was used in this study. Stock culture was initially heat shocked at 80°C for 90 s, cooled in ice-cold water for 1 min and then transferred into modified Duran Schott bottles. Each Schott bottle was then incubated in an incubator at 35°C. When growth appeared, 10% (v/v) of cell culture was inoculated to 1.5 L of a medium consisting of the following: 50.0 g/L sago starch, 0.75 g/L KH_2PO_4, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 5.0 g/L yeast extract, 2.0 g/L NH_4NO_3, 0.5 g/L NaCl, 0.001 g/L resazurin, 0.5 g/L cysteine, 0.001 g/L *p*-amino benzoic acid, 0.0008 g/L biotin, and 6 g/L glycerol. The accumulation of ATP and NADH in the culture for enhancement of solvent production can be triggered by the addition of glycerol in medium formulation [10,14]. Gelatinized starch was prepared by heating the slurry to slightly above 80°C. The medium was autoclaved at 121°C for 20 min. Sterile medium in the 2-liter stirred tank fermentor was kept under anaerobic conditions by sparging an oxygen free nitrogen gas before and after inoculation. In all experiments, the pH of the media was adjusted to 6 using 3 M HCl, temperature was controlled at 35°C, and agitation speed was fixed at 100 rpm.

RESULTS AND DISCUSSION

Determination of Substrates, Solvents, and Organic Acids Using HPLC

In the preliminary studies, standard mixtures were tested. These consisted of glucose, maltose, glycerol, solvents (acetone, butanol, ethanol), and organic acids (acetic acid, butyric acid) with a concentration of 10 g/L for each compound. Results of our analysis showed that differences in concentrations of mobile phase (H_2SO_4) ranging from 3 to 12 mM had very little influence on the retention times of the compounds tested. Lower concentrations of mobile phase caused a slight increase in the retention time of the compounds (data not shown). Therefore, 3 mM H_2SO_4 was used as a mobile phase for

Table 1. Retention times achieved in HPLC analysis using different column temperatures at a fixed flow rate of 0.8 mL/min.

Column temperature	Retention time (min)							
	Maltose	Glucose	Glycerol	Acetic acid	Butyric acid	Acetone	Ethanol	Butanol
30°C	7.64	8.49	11.20	12.74	17.51	17.87	16.54	28.80
60°C	7.62	8.53	11.23	12.37	16.44	16.92	17.08	28.39
80°C	7.61	8.58	11.22	12.05	15.65	16.10	17.08	26.66

subsequent experiments. In addition, improvement for the separation of butyric acid and acetone was also not observed with variation in gradient mode.

The retention times of the compounds analysed showed varied reaction to changes in column temperature (Table 1). For instance, when the column temperature was set at 30°C, an elution order of ethanol > butyric acid > acetone > butanol was observed. On the other hand, when column temperature was increased to 60~80°C, the following elution order applied: butyric acid > acetone > ethanol > butanol. This result suggests that the diffusivity of butyric acid and acetone may be increased at higher temperature, thereby decreasing the resistance to mobile phase mass transfer.

Obviously, column temperature was an important parameter in optimizing the analyses of butyric acid, acetone, butanol, and ethanol using KC-811. However, the elution order of maltose, glucose, glycerol, and acetic acid was unaffected by variations in column temperature. Retention times of maltose, glucose, and glycerol, which were all eluted within 12 min, were only slightly affected by differences in temperature. This observation indicates that these components did not react significantly to changes in column temperature.

In all cases, baseline separation was achieved for all compounds analyzed except for butyric acid, acetone, and ethanol. When the temperature of the column was set at 30°C, butyric acid and acetone were eluted as a single peak at 17.93 min (Fig. 1A). When temperature was set at 60°C, unexpected results were observed where butyric acid, acetone, and ethanol were eluted together as one peak at 16.92 min (Fig. 1B). Resolution of these compounds was not improved, although the flow rate of the mobile phase was decreased from 1.0 to 0.2 mL/min.

Very little improvement in resolution was observed when the temperature of the column was increased up to 80°C. In this case, butyric acid and acetone were detected as an overlapping peak (Fig. 1C). Although baseline separation was achieved for ethanol, unsatisfactory resolution of the overlapping peaks for butyric acid and acetone still resulted in a poor quantification of all compounds analysed in the mixture. The resolution, R, can be defined as:

$$R = [2(T_{R(b)} - T_{R(a)})/(W_{B(b)} + W_{B(a)})] \quad (1)$$

where, $T_{R(a)}$ is the retention time of peak a (in min), $T_{R(b)}$ is the retention time of peak b (in min), $W_{B(a)}$ is the base width of peak a, and $W_{B(b)}$ is the base width of peak b.

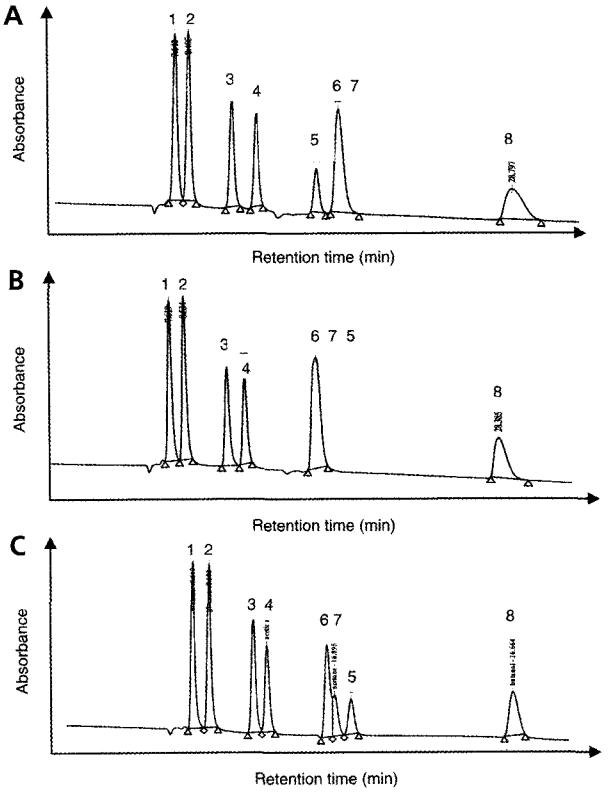

Fig. 1. HPLC chromatograms of a mixture of standards at flow rate 0.8 mL/min and mobile phase 3 mM H_2SO_4 at different temperatures (A) 30°C; (B) 60°C; and (C) 80°C.

Resolution is always measured to describe how well compounds have been separated. The higher the resolution result to reduce overlap between two peaks. In order to improve resolution, the flow rate was decreased from 0.8 to 0.4 mL/min. In this case, further improvement in resolution was not achieved, but the analysis time was increased from 27 to 54 min. Retention times obtained for each component in individual injections at different column temperatures showed that closer retention times were obtained for butyric acid, acetone, and ethanol. This resulted in overlapping peaks.

When the flow rate was constant at 0.8 mL/min. and the column temperature was controlled at 30°C, HPLC analysis took 28.80 min. At 60°C, analysis took 28.39 min. However, the analysis time was shortened to 26.66 min when a higher column temperature of 80°C was applied. According to Pecina and Bonn [13], the selection

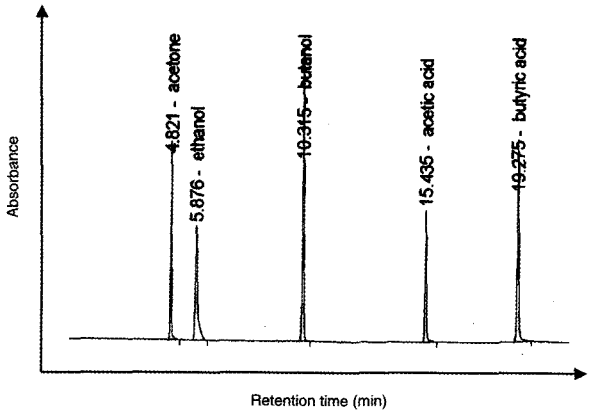

Fig. 2. GC chromatogram for the analysis of solvent fermentation products.

Table 2. Retention times in minutes obtained for products analysed by gas chromatography

Initial oven temperature	Retention time (min)				
	Acetone	Ethanol	Butanol	Acetic acid	Butyric acid
65°C	4.82	5.88	10.32	15.44	19.28
115°C	3.74	3.90	4.69	8.39	10.91

of a particular column temperature may frequently enhance the resolution for overlapping substances and may substantially reduce the analysis time in some instances. Despite the overlapping peaks for butyric acid and acetone, the shape of other peaks and the analysis time of about 26.66 min are satisfactory.

Determination of Solvent Fermentation Products Using GC Analysis

In the preliminary experiment, a standard mixture was analysed using GC. This mixture consisted of solvents and organic acids, with a concentration of 10 g/L for each compound. The oven temperature was initially controlled at 115°C for 8 min, and then it was programmed to increase at 20°C/min increments to a final temperature of 170°C. This final temperature was maintained for 10 min hold. Using this strategy, the use of BP 20 was found to be excellent for separation of butanol, acetic acid, and butyric acid (Table 2). However, the separation of acetone and ethanol peaks was very poor. Overlapping peaks occurred for these two compounds and resulted in lower resolution. An elution order of acetone > ethanol > butanol > acetic acid > butyric acid was attained in the chromatogram. According to Fowlis [14], if the compounds are not completely resolved, then any quantification will be an approximation and the answer will vary depending upon how the data system handles the signal. Therefore, conditions must be changed in order to provide baseline separation for accurate quantitative analysis.

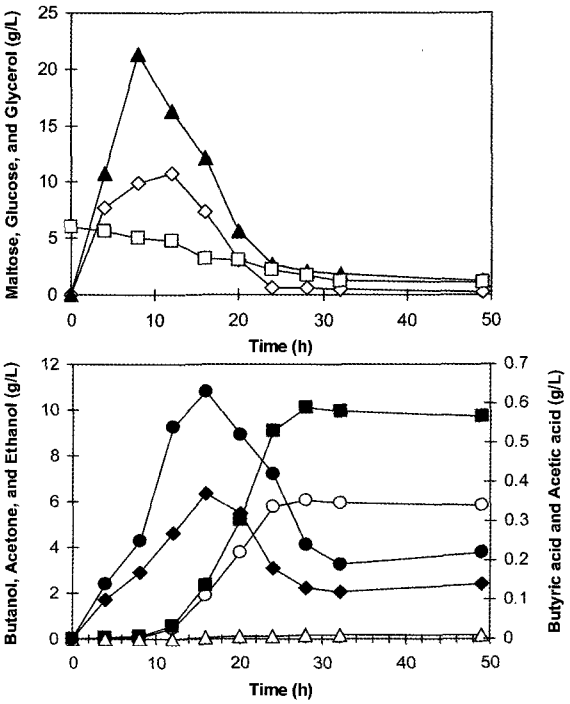

Fig. 3. Profiles of substrates and products during solvent fermentation by *C. saccharobutylicum* DSM 13864. Substrates were determined using HPLC and products were measured with GC. Symbols: (▲) glucose; (◊) maltose; (□) glycerol; (o) acetone; (■) butanol; (Δ) ethanol; (♦) butyric acid, and (•) acetic acid.

Excellent baseline separation was achieved for acetone and ethanol when the oven temperature was decreased (Fig. 2). All compounds analyzed were separated completely when the initial oven temperature was set at 65°C for 8 min, and then increased at increments of 20°C per min to a final temperature of 140°C. This temperature was held until the last peak eluted. The calculated resolution between acetone and ethanol was increased from 3.39 to 13.20.

Results from this study indicate that oven temperature was the most important parameter for enhancing the separation of solvent fermentation products when GC was used for the analysis. In all cases, the injector temperature was set at 220°C and the detector temperature was 270°C. However, the time required for analysis was increased from 10.91 to 19.28 min due to a decrease in oven temperature. It is important to note that the sharp peak observed in the chromatogram indicated greater column efficiency.

Analysis of Fermentation Samples

The measurement system was evaluated by analyzing samples of a batch fermentation of *C. saccharobutylicum* DSM 13864. Samples were taken at time intervals and processed immediately for analysis. Glucose, maltose, and glycerol were analyzed using HPLC whereas solvents and organic acids were analyzed using GC. The profiles

Table 3. Typical example of concentration of substrates and products during fermentation of 50 g/L sago starch to solvent by *C. saccharoacetobutylicum* with addition of 6 g/L glycerol. Values were determined using improved HPLC and GC methods for samples taken at 28 h. The initial culture pH was 6 and not controlled, temperature was controlled at 35°C, and agitation speed was fixed at 100 rpm throughout the fermentation.

Substrates/Products	HPLC (g/L)	GC (g/L)
Maltose	2.14	
Glucose	0.56	
Glycerol	1.66	
Acetic acid	0.18	0.13
Butyric acid		0.24
Acetone		6.03
Butanol	9.96	10.11
Ethanol	0.13	0.16

of substrate and product concentration during fermentation are shown in Fig. 3. During the early stages of growth, sago starch was hydrolyzed into maltose and glucose by the amylolytic enzymes secreted by the bacterium. Rapid consumption of glucose was observed after 10 h of fermentation while glycerol was gradually consumed throughout the fermentation. Solvent fermentation by *C. saccharobutylicum* DSM 13864 using a mixture of sago starch and glycerol as carbon source can be divided into two phases, the acidogenic phase and the solventogenic phase. An acidogenic phase was observed during the first 15 h of fermentation, as indicated by rapid production of organic acids (acetic and butyric acids). The fermentation entered the solventogenic phase after about 15 h, where the metabolism of cell undergoes a shift to produce solvent (acetone, butanol, and ethanol) by reassimilation of organic acid.

Table 3 shows the comparison of GC and HPLC results for samples taken at 28 h during solvent fermentation by *C. saccharobutylicum*. Acetic acid, butanol, and ethanol were clearly detected by both GC and HPLC methods where the results were in good agreement. Since the separation was not very clear, butyric acid and acetone could not be detected by HPLC. However, they were detected at very good resolution using GC. On the other hand, maltose, glucose, and glycerol were detected with good high degree of resolution using the HPLC method.

CONCLUSIONS

Mixtures of sugars, glycerol, solvents, and organic acids were tested using HPLC column KC-811. The column temperature was found to be an important parameter in improving separation. By increasing the column temperature to 80°C, with a flow rate fixed at 0.8 mL/min, all components were separated completely. The separation of butyric acid and acetone was also improved. However, the similar retention times obtained between butyric acid and acetone resulted in low resolution. On the other hand, in the study involving GC analysis, oven temperature was found to be important in enhancing the performance of the BP20 capillary column. The resolution between acetone and ethanol was greatly increased when the oven temperature was decreased to 65°C. When the new method was applied, baseline separation was achieved for solvents and organic acids.

Attempts to analyze all the substrates, solvents, and organic acids using HPLC as a single analysis step was not successful. However, the analysis method was greatly improved in terms of resolution and speed. The separation of solvents and organic acids could be conducted within 19.28 min using GC whereas glucose, maltose, and glycerol can be determined in 11.22 min using HPLC.

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