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NOTE

Effects of Nutritional Enrichment on the Production of Acetone-Butanol-Ethanol (ABE) by *Clostridium acetobutylicum*

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***Clostridium acetobutylicum* is an industrially important organism that produces acetone-butanol-ethanol (ABE). The main objective of this study was to characterize the effects of increased cell density on the production of ABE during the phase transition from acidogenesis to solventogenesis in *C. acetobutylicum*. The increased ABE productivity of *C. acetobutylicum* was obtained by increasing the cell density using a newly designed medium (designated *C. acetobutylicum* medium 1; CAM1). The maximum OD_{600} value of *C. acetobutylicum* ATCC 824 strain obtained with CAM1 was 19.7, which is 1.8 times higher than that obtained with clostridial growth medium (CGM). The overall ABE productivity obtained in the CAM1-fermetation of the ATCC 824 strain was 0.83 g/L/h, which is 1.5 times higher than that (0.55 g/L/h) obtained with CGM. However, the increased productivity obtained with CAM1 did not result in an increase in the final ABE titer, because phase transition occurred at a high titer of acids.**

***Keywords*:** ABE, cell density, *Clostridium acetobutylicum*, phase transition, productivity

Biological production of acetone, butanol, and ethanol has attracted interest, due to recent climate change and on-going oil depletion. These compounds have various applications as solvents, as precursors for several valuable chemicals such as butyl acrylate, and as alternative fuels in case of butanol and ethanol (Jones and Woods, 1986; Durre, 2007; Ezeji *et al.*, 2007; Lee *et al.*, 2008; Jang *et al.*, 2012a). Several clostridial species including *Clostridium acetobutylicum* can produce acetone-butanol-ethanol (ABE) anaerobically (Jones and Woods, 1986; Durre, 2007; Lee *et al.*, 2008). The process of biological ABE production using clostridia was first developed in the 1910's and commercial production expanded worldwide. However, these biological processes lost their cost competitiveness after the 1950's due to advances in petrochemical synthesis.

The ABE fermentation of solventogenic clostridia is more complex than fermentations of other species in that they mainly produce acetic and butyric acids during the early growth phase (acidogenic phase), and then the metabolism shifts towards solvent production (solventogenic phase) as cells grow (Jones and Woods, 1986; Durre, 2007; Jang *et al.*, 2012b). This complex physiology is one of the major barriers in ABE production by solventogenic clostridia (Papoutsakis, 2008; Gu *et al.*, 2011). To revive the biological ABE processes economically, volumetric productivity as well as titer and yield of solvents need to be further improved. In general, the volumetric productivity of a metabolite is determined by cell density and specific productivity. It has been shown that increasing cell density by continuous fermentation with membrane cell-recycling greatly enhanced volumetric ABE productivity (Tashiro *et al.*, 2005; Malaviya *et al.*, 2012). However, the scaling-up of the membrane cell-recycling is not economically feasible due to the cost of the membrane itself, which must be changed and regenerated during the long-term fermentation because the accumulation of cells on the membrane surface can clog the membrane.

Cell density can be enhanced by supplying nutrients in excess. However, it is difficult to obtain high cell densities with the conventional medium used in ABE fermentation, in which nutrients including the nitrogen source and phosphate are not in excess (Monot *et al.*, 1982; Roos *et al.*, 1985; Bryant and Blaschek, 1988). In chemostat fermentations, the phosphate limitation is known to be beneficial for solvent production and the yield of butanol (Bahl *et al.*, 1982, 1986), but the effects of nutrient concentrations in the culture medium on solvent production by solventogenic clostridia are still controversial (Jones and Woods, 1986). In the present study, we enhanced the cell density of batch fermentations by cultivating *C. acetobutylicum* in a rich medium. Then, the effects of high cell density on the ABE production were investigated.

C. acetobutylicum strain ATCC 824 was provided by Terry Papoutsakis (University of Delaware, USA). For cultures, the *C. acetobutylicum* strain was grown in an anaerobic

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Table 1. Effect of tryptone and yeast extract on cell growth (OD_{600}) of *C. acetobutylicum* ATCC 824

	Control	Tryptone (g/L) with 10 g/L yeast extract				Yeast extract (g/L) with 16 g/L tryptone			
		8	16	24	32	5	10	15	20
Cell density (OD_{600})	7.1±0.29	10.9±0.39	12.5±0.53	12.0±0.88	11.8±2.23	12.1±1.41	13.0±1.03	10.5±0.32	10.7±0.35
Time (h)	24	41	35	47	35	38	35	41	38

chamber (Forma Scientific, USA) filled with 4% H_2 balanced with N_2 gas. The clostridial growth medium (CGM) containing 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.7 g $MgSO_4{\cdot}7H_2O$, 0.017 g $MnSO_4{\cdot}5H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 2 g $(NH_4)_2SO_4$, 1 g NaCl, and 2 g L-asparagine, 0.004 g *p*-aminobenzoic acid, 30 mmol $CH_3COONa{\cdot}3H_2O$, and 5 g yeast extract (all per L) was used for test tube, flask and bioreactor experiments throughout this study.

Batch fermentations were performed in a 5-L LiFlus GX fermentor (Biotron, Korea) containing 1.8 L of fresh medium supplemented with 80 g/L glucose. *C. acetobutylicum* cells were initially grown anaerobically at 37°C in flasks containing 200 ml of medium with the glucose concentration of 80 g/L in the anaerobic chamber. These cultures were inoculated for batch fermentations. The temperature and agitation were maintained at 37°C and 200 rpm, respectively. The pH was controlled using 28% (w/w) ammonia solution. Foam was controlled by the manual addition of Antifoam 204 (Sigma, USA). Anaerobicity of the bioreactor was maintained by oxygen-free, sterile nitrogen gas with a flow rate of 0.2 L/min.

The concentrations of acetate, butyrate, and ABE were determined by gas chromatography (GC 7890A, Agilent Technologies, USA) equipped with a flame ionization detector (FID; H_2 flow rate, 40 ml/min; air flow rate, 350 ml/min) and a glass-packed column (6.6% Carbowax 20M with 80/120 Carbopack B AW support; Supelco, USA). The residual glucose was measured by YSI 2700 biochemistry analyzer (YSI Corporation, USA).

In order to investigate the effects of tryptone and yeast extract supplementation on the density of the *C. acetobutylicum* ATCC 824 culture, we cultivated the strain under different nutrient conditions in test tubes containing 10 ml of medium. We prepared rich media having various compositions by adding 8, 16, 24, or 32 g/L of tryptone into CGM with 10 g/L of yeast extract and by adding 5, 10, 15, or 20 g/L of yeast extract with 16 g/L tryptone (all quantities shown as the final concentrations). Cell densities were monitored by optical density at 600 nm (OD_{600}) using the Ultrospec 3100 spectrophotometer (Amersham Biosciences, Sweden) at 3–4 h intervals during the 47 h fermentation. For each composition, the cultivation was performed twice, and the maximum OD_{600} values were determined and averaged. The cultures in CGM were used as a control. The highest cell density of 7.1 in control cultures was obtained at 24 h during culture (Table 1). With a fixed amount of yeast extract at 10 g/L, the highest cell density (OD_{600}) of 12.5 was observed at 35 h when 16 g/L tryptone was supplemented to the culture broth (Table 1). Using this information, we cultivated the strain in a fixed amount of tryptone at 16 g/L and varied the amount of yeast extract. With the 16 g/L tryptone supplement, the highest cell density of 13.0, which was 1.8 times higher than the control CGM cultivation, was observed at 35 h when 10 g/L yeast extract was supplemented (Table 1). We decided to call this composition, CAM1 (*C. acetobutylicum medium* 1). Providing more of either supplement beyond 16 g/L tryp-

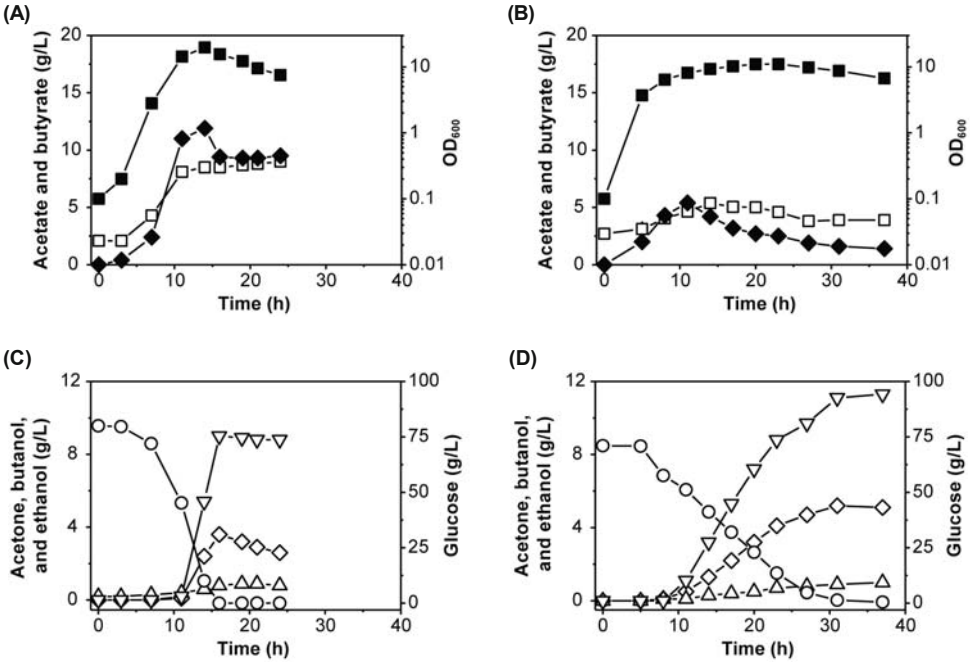

Fig. 1. Time-course profiles of 2 L-scale pH-controlled batch fermentations of *C. acetobutylicum* ATCC 824 with (A) CAM1 and (B) CGM. Symbols are: (■) OD_{600}; (□) acetate; (◆) butyrate; (○) glucose; (▽) butanol; (△) ethanol; and (◇) acetone.

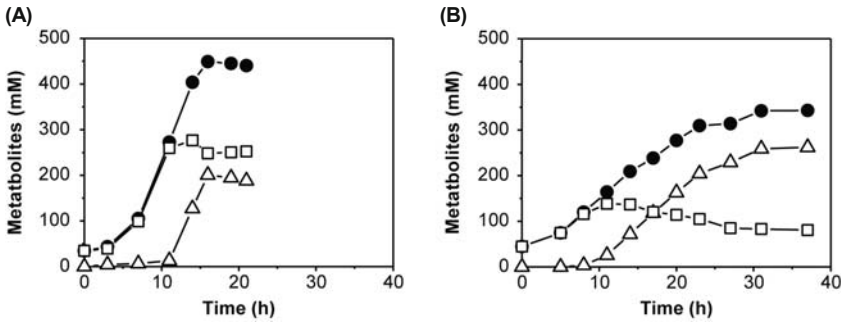

Fig. 2. Time-course profiles of total acids (aceate and butyrate) and ABE during pH-controlled batch fermentations of *C. acetobutylicum* ATCC 824 with (A) CAM1 and (B) CGM. Symbols are: (□) total acids; (△) total ABE; and (●) sum of total acids and total ABE.

tone or 10 g/L yeast extract did not improve the cell density.

To examine whether CAM1 would improve the ABE productivity of *C. acetobutylicum* ATCC 824, batch-fermentations were carried out with either CGM or CAM1 as medium. Higher overall ABE productivity, specific growth rate, glucose consumption rate, and maximum volumetric productivity of ABE were observed for *C. acetobutylicum* ATCC 824 grown in CAM1 as compared to that in CGM. The highest cell density of 19.7 (OD_{600}) was obtained at 14 h (Fig. 1A) in the CAM1 fermentation, as compared to just 11.0 at 20 h in the CGM fermentation (Fig. 1B). The maximum specific growth rate obtained in the CAM1 fermentation was 0.38 h^{-1}, which is 1.58 times higher compared to the value obtained in the CGM fermentation (0.24 h^{-1}). The glucose consumption rate in the CAM1 fermentation was also higher than that in the CGM fermentation (Fig. 1). In the CAM1 fermentation, glucose was completely consumed at 16 h after inoculation (Fig. 1A), whereas 37 h was required for the complete consumption of glucose in the CGM fermentation (Fig. 1B). As a result, batch fermentation of ATCC 824 with CAM1 showed an increased overall ABE productivity of 0.83 g/L/h, which is 1.51 times higher than that obtained with CGM (0.55 g/L/h; Table 2). The CAM1/CGM ratio of overall ABE productivity (1.51) showed a slight difference compared to that of the highest cell mass (1.79). One possible explanation is acid toxicity (Kell *et al.*, 1981; Nicolaou *et al.*, 2010), because phase transition with a high concentration of acids had a negative impact on solvent production, which will be discussed later. In a study using a non-solventogenic *C. acetobutylicum*, 276 mM of total acids, which is similar to our result (Fig. 2A), were produced (Sillers *et al.*, 2008). This suggests that this amount of total carboxylic acids might be the threshold for *C. acetobutylicum* metabolism. Furthermore, increased acid production possibly led to accelerated sporulation, which also causes cell death. Although the mechanism of clostridial sporulation initiation has not been elucidated yet, previous studies suggested that intracellular butyryl phosphate can act as an initiator of sporulation in *C. acetobutylicum* (Paredes *et al.*, 2005; Zhao *et al.*, 2005). Increased acid production in the CAM1 medium possibly resulted in an increase of the intracellular butyryl phosphate level, leading to the drastic decrease of metabolic activity of *C. acetobutylicum*.

Table 2. Batch fermentation profiles of *C. acetobutylicum* ATCC 824 with either CGM or CAM1

Fermentation properties	Media		CAM1/CGM
	CGM	CAM1	
Highest cell mass (OD_{600})	11.0	19.7	1.79
Specific growth rate (/h)	0.24	0.38	1.58
Final ABE titer (g/L)	17.2	13.4	0.78
Overall ABE productivity (g/L/h)	0.55	0.83	1.51
Consumed glucose (g/L)	70.5	79.9	-

Fermentation with CAM1 resulted in positive effects on cell growth and ABE productivity of *C. acetobutylicum* ATCC 824, but the final ABE titer unexpectedly decreased (13.4 g/L vs. 17.2 g/L with CGM; Table 2). To examine whether this result was due to altered phase transition from acidogenic to solventogenic phase, the production behavior of both organic acids and ABE were further studied. As seen in Fig. 2, solvent production began at 11 h in both the CAM1 and CGM fermentations of *C. acetobutylicum* ATCC 824. The production profile of total acids (acetate and butyrate) in the CAM1 fermentation was quite different from that obtained in the CGM fermentation (Fig. 2). The CGM fermentation showed increasing production (138.5 mM at peak) of total acids until 11 h, and a subsequent gradual decreasing to 80.8 mM until 37 h during the solventogenic phase (Fig. 2B). However, a maximum 259.7 mM of total acids accumulated in the CAM1 fermentation within 11 h (Fig. 2A), which is 1.9 times higher compared to the value obtained from the CGM fermentation. Peak production of acids in the CGM fermentation was 138.5 mM during the acidogenic phase, whereas it was 276.6 mM during the solventogenic phase in the CAM1 fermentation (Fig. 2). After peak production of acids in the CAM1 fermentation during the solventogenic phase, just 24.5 mM of acids were reassimilated, and the final acid residue was 252.1 mM. Phase transition with a high concentration of acids (259.7 mM) resulted in 449.0 mM production of total acids and solvents together when the production of ABE peaked (Fig. 2A). However, phase transition with a low concentration of acids (138.5 mM) resulted in 342.8 mM final production of total acids and solvents for the CGM fermentation (Fig. 2B). The higher concentration of acids at transition phase (at 11 h) in the CAM1 fermentation might have exerted stress in the cells during the solventogenic phase, thereby causing an earlier cell death after reaching maximum OD_{600}. This reduced cell viability during stationary phase possibly contributed to the lower final ABE titer in the CAM1 fermentation compared to that obtained in the CGM fermentation. In the case of the CGM fermentation, however, organic acids, which had been produced at relatively low levels, were almost all converted to

ABE (Fig. 2B). The CAM1 composition dramatically reduced the time required to reach the maximum cell density. However, the higher maximum cell density obtained in CAM1 was unexpectedly followed by a rapid decline and accompanied by increased production of organic acids including acetic and butyric acids. Similar behaviors were observed with CAM1 medium in the non-solventogenic M5 strain and solventogenic M5(pIMP1E1AB) strain (Lee *et al.*, 2009), which was previously engineered for butanol production using the M5 strain (data not shown).

In this work, supplementation of CGM with tryptone and yeast extract successfully enhanced cell density for *C. acetobutylicum* and ABE productivity in the batch fermentation. Although the supplemented components have complex compositions, this is the first report on achieving high productivity of ABE via increasing the cell density in batch fermentations through medium optimization. We also demonstrated that excess production of organic acids in the rich medium resulted in a negative effect on the ABE titer. Further physiological studies should be conducted to investigate the effects of medium supplementation on the *C. acetobutylicum* physiology and sporulation, with a view to increasing the ABE titer as well as the cell density in the rich medium.

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