Appl Biol Chem (2018) 61(4):469–472
https://doi.org/10.1007/s13765-018-0372-6

Online ISSN 2468-0842
Print ISSN 2468-0834

NOTE

Effects of nutritional enrichment on acid production from degenerated (non-solventogenic) *Clostridium acetobutylicum* strain M5

Ji Eun Woo[1] · Sang Yup Lee[2] · Yu-Sin Jang[1]

Received: 2 April 2018 / Accepted: 23 April 2018 / Published online: 4 May 2018

Abstract *Clostridium acetobutylicum* has been used as a microbial platform for the production of butanol, acetone, and butyrate from biomass. This study examined the effect of nutritional enrichment on the production of acetate and butyrate by *C. acetobutylicum* in culture, and tested whether this nutritional change could shift metabolic flux in these microbial cells. The degenerated (non-solventogenic) *C. acetobutylicum* M5 strain, which lacks the pSOL1 plasmid that contains genes responsible for solvent production, was cultured in the rich medium, *C. acetobutylicum* medium 1 (CAM1). As a control, M5 strain was also cultured in clostridial growth medium (CGM). Batch fermentation of M5 strain in CAM1 achieved a cell density of 23.7 (OD_{600}), which was 2.55 times that obtained when these cells were cultured in CGM. Fermentation in CAM1 yielded volumetric acetate and butyrate productivities of 0.42 g/L/h and 1.06 g/L/h, respectively, which were 2.33 and 1.33 times the values obtained in CGM. Nutritional enrichment also increased the acetate-to-butyrate ratio, which was 0.39 g/g for M5 cells grown in CAM1 and 0.25 g/g for those grown in CGM. These findings demonstrate that the tested nutritional enrichment triggers a metabolic shift in the acid production of a degenerated *C. acetobutylicum* in culture.

Keywords Acetate · Butanol · Butyrate · *C. acetobutylicum* medium · *Clostridium acetobutylicum*

✉ Sang Yup Lee
leesy@kaist.ac.kr

✉ Yu-Sin Jang
jangys@gnu.ac.kr

[1] Institute of Agriculture and Life Science (IALS), Department of Agricultural Chemistry and Food Science Technology, Division of Applied Life Science (BK21 Plus Program), Gyeongsang National University, Jinju 52828, Republic of Korea

[2] Department of Chemical and Biomolecular Engineering (BK21 Plus Program), BioProcess Engineering Research Center, Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea

Introduction

Clostridium acetobutylicum produces various metabolites (e.g., butanol, acetone, ethanol, butyrate, acetate, and hydrogen) from C5 and C6 sugars in nature [1]. *C. acetobutylicum* was used for the industrial production of butanol and acetone in the early to mid-1900s [2]. In recent decades, *C. acetobutylicum* has regained substantial attention as a producer of butanol, which is considered an alternative fuel to gasoline [3, 4]. *C. acetobutylicum* is also currently being evaluated in the context of producing butyrate and hydrogen for industrial applications [1].

Metabolically, *C. acetobutylicum* is characterized by a biphasic fermentation consisting of acidogenic and solventogenic phases. The major products of the acidogenic phase are acetate and butyrate, which are reassimilated during the solventogenic phase and used to produce solvents, such as acetone, butanol, and ethanol [2, 5, 6]. The acidogenic and solventogenic pathways have been experimentally targeted/disrupted with the goal of enhancing butanol production and butyrate production, respectively [3]. For example, butanol production was increased about 1.5-fold following the individual disruptions of the *pta* and *buk* genes, which encode two enzymes observed during the

acidogenic phase: phosphotransacetylase and butyrate kinase, respectively [7].

Researchers engineered a non-solventogenic strain simply by knocking out the aldehyde/alcohol dehydrogenase-encoding gene, *adhE1*, in *C. acetobutylicum* [8]. In a subsequent study, these *adhE1*-mutants were further engineered for butyrate production [9]. Prior to the development of an efficient gene-knockout tool, a non-solventogenic strain was constructed by random mutation, which was accompanied by curing (eliminating) the megaplasmid pSOL1 containing the *adhE1* gene, from *C. acetobutylicum* ATCC 824 [10]. A well-known degenerated (non-solventogenic) strain is *C. acetobutylicum* M5 [10, 11]; in this strain, complementation of the *adhE1* gene was found to recover the ability to produce butanol [12].

Our group recently designed a new rich medium, CAM1, and used it to examine the effect of nutritional enrichment on solvent production during the phase transition of *C. acetobutylicum* ATCC 824 [13]. However, no previous study has examined the effect of nutritional enrichment on the production of acetate and butyrate by *C. acetobutylicum*. In the present study, we cultured M5 strain in CAM1 and examined how the rich medium affected the culture of this acidogenic (degenerated) *C. acetobutylicum*.

Materials and methods

Strain and culture media

Degenerated *C. acetobutylicum* strain M5 was used in this study [10]. Clostridial growth media (CGM) was prepared as previously described [13], and CAM1 was prepared by supplementing CGM with 16 g/L tryptone and an additional 5 g/L yeast extract [13].

Fermentations

C. acetobutylicum M5 was cultured anaerobically in 500-mL flasks containing 200 mL CGM (or CAM1) medium [14]. Anaerobic batch fermentations were performed in a 5-L bioreactor (LiFlus-GX; Biotron, Kyunggi, Korea) containing 1.8 L of CGM (or CAM1) supplemented with 80 g/L glucose, at 37 °C with shaking at 200 rpm.

Analytical methods

Cell density was determined by measuring the optical density at 600 nm (OD_{600}) using an Ultrospec-3000 (Pharmacia Biotech, Uppsala, Sweden) [15]. The concentrations of acetate and butyrate were estimated using a gas chromatograph (GC-7890A; Agilent Technologies, Santa Clara, USA) equipped with an 80/120-Carbopack BAW (Supelco, Bellefonte, PA, USA) [16]. To determine the acetate titer, the amount of externally added sodium acetate was subtracted from the measured value. Glucose concentration was measured using a YSI-2300 (YSI Corporation, Yellow Springs, HI, USA) [17]. The ATP production was estimated from the consumption of glucose (2 ATPs per glucose), and the production of acetate and butyrate (1 ATP per each molecule).

Results and discussion

In a previous study, we examined the effect of nutritional enrichment on solvent production in *C. acetobutylicum* ATCC 824 [13]. We found that nutritional enrichment increased the specific growth rate and solvent productivity of ATCC 824, but decreased the solvent titer [13]. In this case, the acetate and butyrate formed during the acidogenic phase were consumed during the solventogenic phase; thus, we could not clearly determine the effect of nutritional enrichment on the production of acids by *C. acetobutylicum*.

To close this gap, we herein examined the effect of nutritional enrichment on the culture of a degenerated *C. acetobutylicum* strain. M5 cells were anaerobically cultured in a 5-L bioreactor containing CAM1 medium (Fig. 1). The pH was maintained above 5.5 using ammonia solution. The same batch culture was also performed in a bioreactor containing CGM medium, as a control (Fig. 2).

In the batch culture of M5 strain in CAM1, the OD_{600} reached 23.7 at 15.5 h after inoculation for a specific growth rate of 0.50 /h (Fig. 1, Table 1). This culture produced 7.3 g/L acetate and 18.6 g/L butyrate from 76.7 g/L glucose, yielding volumetric productivities of 0.42 and 1.06 g/L/h, respectively (Fig. 1, Table 1). In the batch culture of M5 strain in CGM, on the other hand, we

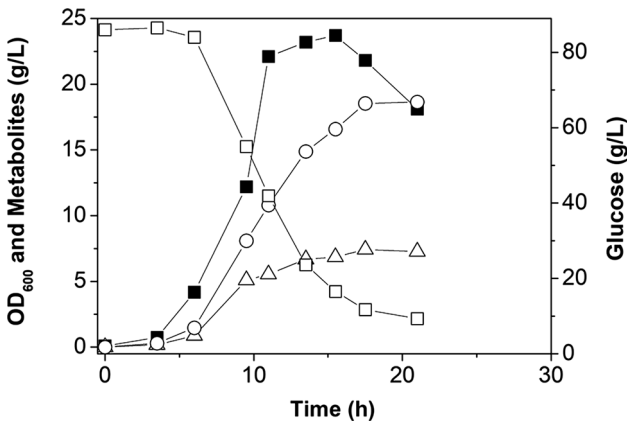

Fig. 1 Batch-fermentation profiles of *C. acetobutylicum* M5 in CAM1. Symbols are: OD_{600} (black squares), glucose (open squares), acetate (upward triangles), butyrate (open circles)

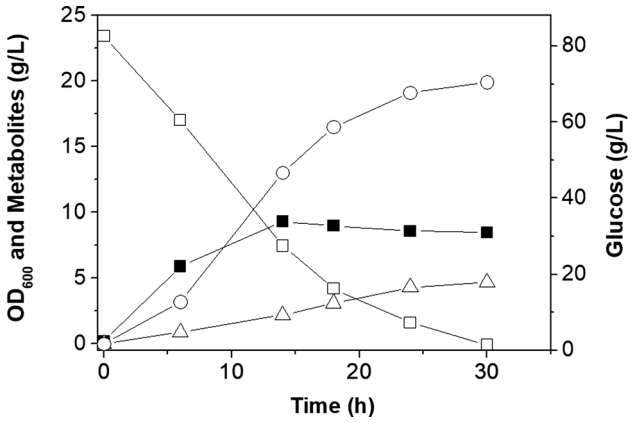

Fig. 2 Batch-fermentation profiles of *C. acetobutylicum* M5 in CGM. Symbols are: OD_{600} (black squares), glucose (open squares), acetate (upward triangles), butyrate (open circles)

obtained an OD_{600} of 9.3 at 14.0 h and a specific growth rate of 0.27 /h (Fig. 2, Table 1). This culture produced 5.0 g/L acetate and 19.9 g/L butyrate from 81.0 g/L glucose, yielding volumetric productivities of 0.18 and 0.80 g/L/h, respectively (Fig. 2, Table 1). Under nutritional enrichment, the acetate and butyrate titer was not improved, relatively, compared with the increase in cell density. This means that the concentration of both acids in the fermentation broth might have exerted stress to the cells [13].

The results obtained from fermentations performed using *C. acetobutylicum* M5 in CAM1 and CGM are listed in Table 1. The cell mass and specific growth rate obtained from the CAM1 culture were 2.55 and 1.85 times higher, respectively, than those obtained from the CGM culture. With respect to organic acid production, nutritional enrichment increased the acetate titer by 1.46 times but slightly decreased the butyrate titer by 0.94 times. The acetate-to-butyrate (AA/BA) ratios were 0.25 and 0.39 g/g for the CGM and CAM1 cultures, respectively. Similarly, the volumetric acetate and butyrate productivities were enhanced by 2.33 and 1.33 times, respectively, under nutritional enrichment.

The increase in the AA/BA ratio in CAM1-grown M5 cultures could reflect that ATP production was not sufficient for intracellular processes under high cell density. Indeed, our metabolic pathway analysis calculated that the volumetric ATP productions were 1203 mM for CGM-grown cultures and 1184 mM for CAM1-grown cultures (Fig. 3), indicating that the specific ATP productions were 129 mM/OD and 50 mM/OD, respectively. Thus, it seems

Table 1 Comparison of major parameters obtained from the fermentations using *C. acetobutylicum* M5 in CGM and CAM1

Culture medium	Cell density (OD_{600})	Specific growth rate (/h)	Glucose consumption (g/L)	Acetate (g/L)	Butyrate (g/L)	AA/BA ratio[a] (g/g)	Volumetric acetate productivity (g/L/h)	Volumetric butyrate productivity (g/L/h)
CGM	9.3	0.27	81.0	5.0	19.9	0.25	0.18	0.80
CAM1	23.7	0.50	76.7	7.3	18.6	0.39	0.42	1.06
CAM1/CGM	2.55	1.85	0.95	1.46	0.94	–	2.33	1.33

[a]Acetate/butyrate ratio

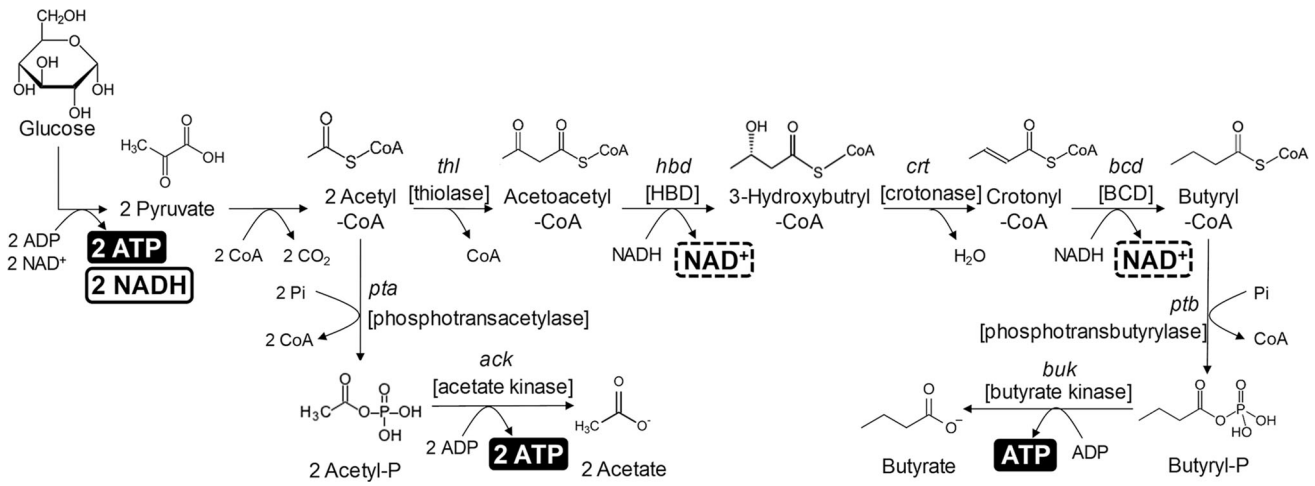

Fig. 3 Metabolic pathway of a degenerated *C. acetobutylicum* M5. HBD, 3-hydroxybutyryl-CoA dehydrogenase; and BCD, butyryl-CoA dehydrogenase

that the metabolic flux was controlled such that ATP production was maximized through the acetate pathway, with NAD^+ regeneration occurring through the butyrate pathway (Fig. 3). In *C. acetobutylicum*, the acetate pathway is favored over the butyrate pathway for ATP production: the former produces one ATP from one molecule of acetyl-CoA, while the latter produces one ATP from two molecules of acetyl-CoA (Fig. 3). However, the actual ATP requirement might be not dramatic in CAM1-grown cultures, because the ATP cost for growth might be affected by addition of tryptone and yeast extract, which contain amino acids, nucleotides and vitamins.

In sum, we herein report that a cell density (OD_{600}) of 23.7 was achieved in batch fermentation of *C. acetobutylicum* M5 with the rich medium, CAM1. This is the highest value reported to date for this strain. Nutritional enrichment increased the AA/BA ratio in M5 cultures, apparently reflecting the increased need for ATP under high cell density. As the cell mass increased, the volumetric productivities of acetate and butyrate were also enhanced in CAM1-grown M5 cultures compared to control (CGM-grown) cultures.

Acknowledgments We would like to thank E.T. Papoutsakis and G. Bennett for providing strain M5. This work was supported by grants from the Ministry of Science and ICT (MSIT) through the NRF of Korea (NRF-2016R1D1A3B04933184, NRF-2012M1A2A2026556, and NRF-2012M1A2A2026557).

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