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Xylan supplement improves 1,3-propanediol fermentation by *Clostridium butyricum*

Waraporn Apiwatanapiwat,[1,2] Pilanee Vaithanomsat,[2,3] Warunee Thanapase,[2] Khanok Ratanakhanokchai,[4] and Akihiko Kosugi[1,5,*]

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan,[1] Kasetsart Agricultural and Agro-Industrial Product Improvement Institute, Kasetsart University, 50 Chatuchak, Bangkok 10900, Thailand,[2] Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University (CASTNAR, NRU-KU), Kasetsart University, 50 Chatuchak, Bangkok 10900, Thailand,[3] School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkuntien, Bangkok 10150, Thailand,[4] and Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan[5]

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Lignocellulosic biomass as co-substrate enhances the 1,3-propanediol (1,3-PD) production of anaerobic fermenters by increasing their conversion yield from glycerol. To improve 1,3-propanediol (1,3-PD) production by this efficient approach, *Clostridium butyricum* I5-42 was supplemented with lignocellulosic biomasses (starch free fiber (CPF) from cassava pulp and xylan) as co-substrates. The 1,3-PD production and growth of *C. butyricum* were considerably higher in glycerol plus CPF and xylan than in glycerol alone, whereas another major polysaccharide (cellulose co-substrate) failed to improve the 1,3-PD production. *C. butyricum* I5-42 showed no degradation ability on cellulose powder, and only weak activity and slight growth on xylan. However CPF supplemented with xylan strongly enhanced the transcription levels of the major enzymes of 1,3-PD production (glycerol dehydratase, 1,3-propanediol dehydrogenase, and glycerol dehydrogenase). The intracellular redox reactions maintained equal balance in the supplemented media, suggesting that CPF plus xylan promotes 1,3-PD production in the reductive pathway. This promotion is probably mediated by NADH, which is effectively regenerated by small amounts of released oligosaccharides and subsequent activation of the glycerol oxidative pathway. Both supplements also improved the 1,3-PD production at high glycerol concentration. Therefore, supplementation with lignocellulolytic polysaccharides such as xylan can improve the production and productivity of 1,3-PD from glycerol in *C. butyricum*. Direct supplementation of CPF with xylan in 1,3-PD production has not been previously reported.

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[**Key words:** Xylan; *Clostridium butyricum*; 1,3-Propanediol; Cassava pulp; Hemicellulose; Glycerol]

Teraphthalic acid and the three-carbon diol 1,3-propanediol (1,3-PD) are important organic substrates for biopolymers such as polytrimethylene terephthalate (PTT). These biopolymers are used in apparel, upholstery, carpet, specialty resins and other materials requiring softness, comfort-stretch and dye ability (1). The global demand for PTT was approximately 400,000 tonnes in 2013, and that for 1,3-PD is expected to reach approximately 150 kt by 2019 (2). Thus, low-cost and high-yield production of 1,3-PD is important for competitiveness in the biopolymer market.

The main substrate of fermentative 1,3-PD production by microorganisms is glycerol, a by-product of biodiesel production (3,4). Facultative anaerobes such as *Klebsiella pneumoniae* and *K. oxytoca*, and strict anaerobes such as *Clostridium beijerinckii* (5), *C. butyricum* (6,7), and *C. diolis* (8,9), are the most widely investigated natural 1,3-PD bioproducers. Among these bacteria, non-pathogenic *Clostridium* sp. such as *C. butyricum* and *C. diolis* have been regarded as good producers of 1,3-PD. Whereas *Klebsiella* sp. are aerobic fermenters, *Clostridium* sp. convert glycerol to 1,3-PD anaerobically through the coenzyme B12-independent 1,3-PD biosynthetic pathway, enabling an economically feasible production process (10). However, the yield and productivity of 1,3-PD on glycerol are low because the growth and energy production are hampered by the low assimilation rate (7,11). Supplementing the glycerol medium with glucose is expected to enhance the growth and increase the 1,3-PD production, but represses the catabolites in *C. butyricum* (7,11). *C. butyricum* also produces solvents from polysaccharides such as starch (12). Recently, 1,3-PD production by *C. diolis* and *K. pneumoniae* has been reported in co-fermented glycerol and lignocellulosic hydrolysates such as xylose and arabinose (13,14). When added as co-substrate, the sugars glucose, sucrose, maltose, and xylose boost the conversion of 1,3-PD from glycerol. In our previous paper, we supplemented glycerol medium with cassava pulp (CP) in 1,3-PD production by *C. butyricum* (15). As a starchy-lignocellulosic biomass, CP is a promising substrate for biochemical production (16,17) because both of its major components, starch (50% dry basis) and lignocellulosic fiber (approximately 30% dry basis) (18), supplement the glycerol medium. The 1,3-PD productivity of *C. butyricum* in glycerol medium is undoubtedly improved by small amounts of CP, but excess CP supplementation

* Corresponding author at: Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan. Tel./fax: +81 29 838 6623.
E-mail address: akosugi@affrc.go.jp (A. Kosugi).

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decreases the 1,3-PD productivity because the glucose released from starch degradation becomes sufficient to repress the glycerol metabolism and 1,3-PD biosynthesis (15). Thus, although co-substrate is an important influencer of 1,3-PD production yields, some co-substrates contain carbon sources that initiate catabolite repression in 1,3-PD production by *C. butyricum*. Such co-substrates must be avoided.

Thus far, cost-effective depolymerization techniques for lignocellulosic biomass have received wide attention (19,20). For example, dilute sulfuric acid pretreatment is a popular and efficient approach for inducing enzymatic saccharification of corn straw. However, the pretreatment of lignocellulose by acid hydrolysis, steam explosion, and high-temperature steaming (20,21) generate several groups of fermentation inhibitors such as furfural, phenolic compounds, and acetic acid (22,23). Therefore, lignocellulosic hydrolysate co-substrates are of limited use in 1,3-PD production. If untreated lignocellulosic biomass could be directly utilized as the co-substrate in 1,3-PD production, we could improve the effectiveness and economy of 1,3-PD production. Although co-fermentation of glycerol and lignocellulosic hydrolysates has been studied in 1,3-PD production by *Clostridium* spp. (13), 1,3-PD production under direct supplementation with lignocellulosic biomass has not been reported.

The present study investigates whether direct application of lignocellulosic biomass as co-substrate can improve 1,3-PD production by *C. butyricum* I5-42. To examine the direct effects of lignocellulosic biomass, we first prepared starch-free cassava pulp fiber (CPF) as the co-substrate for 1,3-PD production. We then selected the major components of CPF (cellulose and xylan) as co-substrates, and investigated their effects on cell growth and the 1,3-PD biosynthesis process. The xylan co-substrate increased the cell mass and growth rate, thereby benefitting the 1,3-PD synthesis. The study confirmed that direct supplementation with xylan boosts the 1,3-PD production of *C. butyricum* from glycerol. For the first time, we show that lignocellulosic biomass and hemicellulolytic components can enhance 1,3-PD production without requiring pretreatment.

MATERIALS AND METHODS

Bacterial strain and growth conditions *C. butyricum* I5-42 was originally isolated from soil (15). The prepared medium (M1) contained the following chemicals (per liter): K_2HPO_4 (3.4 g), KH_2PO_4 (1.3 g), $(NH_4)_2SO_4$ (2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), $CaCO_3$ (2 g), $FeSO_4 \cdot 7H_2O$ (5 mg), yeast extract (Difco Laboratories, Detroit, MI, USA) (1 g), and trace-element solution (2 ml), pH 7.0 (6). All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All *C. butyricum* media were degassed in boiling water and bubbled with high purity nitrogen gas. The pre-cultures were grown overnight to early stationary phase in 5 ml screw-capped bottles with butyl rubber stoppers. Two milliliters of cell suspension were incubated into 100 mL fermentation medium and incubated at 37°C for 48 h at 150 rpm.

Preparation starch free fiber (CPF) from CP CP was obtained from Sanguan Wongse Industrial Co., Ltd. (Nakhon Ratchasima, Thailand) (15). The CP was dried for 3 days at 70°C and ground with a 0.5-mm mesh screen (ZM-100; Retsch, Haan, Germany). Dried CP (100 g) was added to a 500 ml autoclavable bottle containing 300 ml of deionized water. The bottle was autoclaved for 20 min at 121°C. To liquefy the starch in the CP, the autoclaved suspension was incubated with α-amylase (Sigma–Aldrich, St. Louis, MO, USA) and glucoamylase (Sigma–Aldrich) (at final concentrations of 100 U and 30 U, respectively) for 2 days at 50°C with shaking. The fiber fraction was separated by centrifugation (10,000×*g*, 15 min) and washed twice with deionized water. The fraction was oven-dried at 70°C for 3 days to measure the moisture content and its composition was analyzed.

Supplemented glycerol fermentation The fermentation medium contained 100 ml of M1 medium, 20 or 60 g/l of glycerol, and 2 g/l of supplement (CPF, cellulose, xylan, glucose, or xylose). All carbon sources were purchased from Wako Pure Chemical Industries and Sigma–Aldrich. The fermentation medium was inoculated with 2% (v/v) of the pre-cultured *C. butyricum*. The culture was incubated for 48 h at 37°C with stirring at 150 rpm.

Measurement of *C. butyricum* growth properties The growth of *C. butyricum* was determined by measuring the total protein concentration (24). Cell growth in the co-fermentation medium with glycerol and supplement (CPF, cellulose, xylan, glucose or xylose) was assayed by the increasing protein concentration in the pellet. Briefly, cells were lysed in a NaOH/SDS solution containing 0.2 M NaOH (Wako Pure Chemicals) and 0.2% (w/v) SDS (Wako Pure Chemicals). Cell debris and residual solids were pelleted from the NaOH/SDS solution by centrifugation (9700×*g* for 5 min), and the protein concentration in the supernatant was estimated using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard.

Quantitation of *dhaB1*, *dhaT* and *dhaD* mRNAs In the mid-exponential phase, *C. butyricum* I5-42 cells were collected from 20 g/l glycerol and mixed with xylan, cellulose or CPF (each 2 g/l). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Frederick, MD, USA), then treated with DNase I (Takara Bio, Shiga, Japan). The cDNA was synthesized using an iScript Advanced cDNA Synthesis Kit for quantitative real-time PCR (qPCR) (Bio-Rad Laboratories, Hercules, CA, USA) with random primers and an RNA template. The designed primer sequences are shown in Table S1. The qPCR assays were performed in the CFX96 Real-time system (Bio-Rad Laboratories) according to the manufacturer's instructions.

The qPCR analysis was then carried out using an SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad Laboratories) under the following conditions: initial denaturation at 95°C for 30 s, followed by 5 s at 95°C and 10 s at 57°C (40 cycles). After each run, the specificity of the PCR product was checked by constructing a melting curve. All samples were analyzed in at least two independent experiments with three replicates in each run. The transcription levels of the target genes were normalized against the transcript levels of the 16S rRNA gene.

Preparation of crude cell-free extracts and extracellular fractions The grown *C. butyricum* I5-42 cells were harvested by centrifugation (9000×*g*) for 5 min at 4°C and washed with 100 mM potassium bicarbonate buffer (pH 9.0) containing 2 mM dithiothreitol (DTT). The pelleted cells were suspended in the same buffer and disrupted by sonication (four 2-min sonications with a 30-s rest interval at output level 4) (TAITEC Corporation, Japan). The crude cell-free extract was obtained by centrifugation at 13,000×*g* for 10 min at 4°C. For measuring the extracellular cellulose and xylan degradation abilities of the cells, ammonium sulfate (Wako Pure Chemical) was gently added to the cell-free supernatant (maintained at 4°C) up to 80% saturation, and stirred for 15 h. After centrifugation (30 min at 15,000×*g*, 4°C), the pellet was resuspended in 50 mM sodium phosphate buffer, pH 7.0, and loaded onto an Econo-Pac 10DG column (Bio-Rad) equilibrated with 50 mM phosphate buffer, pH 7.0. The total proteins were eluted from the column with distilled water and concentrated through Amicon Ultra centrifugal filters (Merck Millipore Corp., Darmstadt, Germany). The proteins were used as the extracellular fraction of *C. butyricum* I5-42.

Enzyme assay 1,3-PD dehydrogenase activity was determined spectrophotometrically by the procedure of Boenigk et al. (25). The assay mixture (final volume 1 ml) contained 30 mM $(NH_4)_2SO_4$, 100 mM potassium carbonate (pH 9.0), 2 mM DTT, 2 mM NAD^+, and 100 mM 1,3-PD. The glycerol dehydratase activity was assayed by a method derived from the procedure by Toraya et al. (26), which determines the NADH consumption when the aldehydes formed by dehydratase are reduced to their corresponding alcohols by an excess of yeast alcohol dehydrogenase. The assay mixture (final volume 1 ml) contained 0.03 M $(NH_4)_2SO_4$, 0.1 M 1,2-propanediol, 0.1 M potassium carbonate buffer pH 7.0, 2 mM DTT, and 10 mM NADH. Coenzyme B12 (10 μM) or *S*-adenosylmethionine (4 mM) was added or omitted from this reaction mixture. The NADH consumption was followed continuously at 340 nm. All enzyme assays were performed under anaerobic conditions at 37°C. One unit of enzyme activity defines the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min at 37°C. The xylanase and cellulase activities were measured by determining the amount of reducing sugar released from beechwood xylan and cellulose powder (Sigma–Aldrich), respectively (27). The reaction mixture contained 0.9 ml of 0.5% (w/v) xylan or cellulose substrates in 0.1 M sodium acetate buffer at pH 6.0, and 50 μg extracellular protein prepared from 0.1 ml of the culture supernatant. After 10 min incubation, the reaction was stopped by boiling, and the mixture was separated by centrifugation at 12,100×*g* for 10 min (28). The released reducing sugars were quantified by the Somogyi–Nelson method with xylose as the standard. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar in 1 min under the above conditions (27,28). β-Xylosidase activity was determined by measuring the p-nitrophenol released from *p*-nitrophenyl β-D-xyloside (Sigma–Aldrich) (28). All assays were duplicated on two different cell extracts, and the reported values are the averages and standard deviations of four assays. The protein contents of the extracts were determined by the BCA (Thermo Fisher Scientific) method with BSA as the standard.

Determination of the NAD/NADH ratio The intracellular concentrations of NADH and NAD^+ were determined using an Amplite Fluorimetric NAD/NADH Ratio Assay Kit from AAT Bioquest, Inc. (Sunnyvale, CA, USA) according to the manufacturer's instructions. Equal amounts of NAD^+ and NADH (25 μl) were treated with or without the NADH and NAD^+ extraction solution for 15 min, and then neutralized with the extraction solutions at room temperature. After adding 75 μl of the NADH reaction mixture, the signal was acquired at Ex/Em = 540/590 nm (cutoff at 570 nm) for 30 min. In wells showing NADH reactions, the blank signal was subtracted from the signal values. All reactions were performed in a labeled 96-well plate.

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