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

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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.

[**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

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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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Analytical procedures The CPF (100 g) was mixed with 72% H_2SO_4 and maintained at room temperature for 1 h. The incubated samples were diluted to 4% H_2SO_4 with distilled water and autoclaved at 121°C for 1 h. The acid-soluble portion was assayed for its carbohydrate and organic acids compositions by high-performance liquid chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan) with a refractive index (Shimadzu RID-10A) detector on a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories) operated at 60°C with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Samples were centrifuged through a filter spin column at 13,000 rpm for 1 min. The 1,3-PD and glycerol concentrations were also measured by HPLC using a Shodex RSpak DE-613 column (Showa Denko K.K., Tokyo, Japan) at a constant temperature of 60°C. The mobile phase was MilliQ water with a flow rate of 0.6 ml/min. Samples for 1,3-PD assay were prepared by boiling and filtration through a 0.45-μm filter spin column (Millipore Corporation, Billerica, MA, USA).

RESULTS AND DISCUSSION

Effect of 1,3-PD production supplement with CPF by *C. butyricum* I5-42 CP consists of 50% (dry w/w) starch and 30% (dry w/w) fiber (18). We recently reported that supplementation with small quantities of CP not only improves the growth of *C. butyricum* I5-42 (an organism with high starch-degradation ability) (15) on M1 medium containing glycerol, but might also enhance its 1,3-PD production. On the contrary, excess CP-supplementation reduced the 1,3-PD production, because the high starch component was broken down by the organism's own amylase, releasing large amounts of glucose into the medium. The released glucose may have suppressed the inducible synthesis of glycerol dehydrogenase (GDH), dihydroxyacetone (DHA) kinase, glycerol dehydratase and 1,3-PD dehydrogenase by *C. butyricum*, especially during fermentation. However, once the starch is removed, the cellulose and hemicellulose in the remaining CP fiber (CPF) are abundant and promising bioresources (29). If CPF is supplemented with glycerol during 1,3-PD production, the starch might be separated from the CP and used to create valuable products such as bioethanol. To assess whether CPF-supplementation can improve the 1,3-PD production and cell growth of *C. butyricum*, we removed the starch from CP using commercial amylase and glucoamylase, and supplemented *C. butyricum* I5-42 with the resulting CPF. In M1 medium containing 20 g/l glycerol, the 1,3-PD concentration was 6.41 ± 0.37 g/l. When supplemented with 2 g/l CPF, the 1,3-PD concentration increased to 7.53 ± 0.35 g/l (Fig. 1A and Table 1).

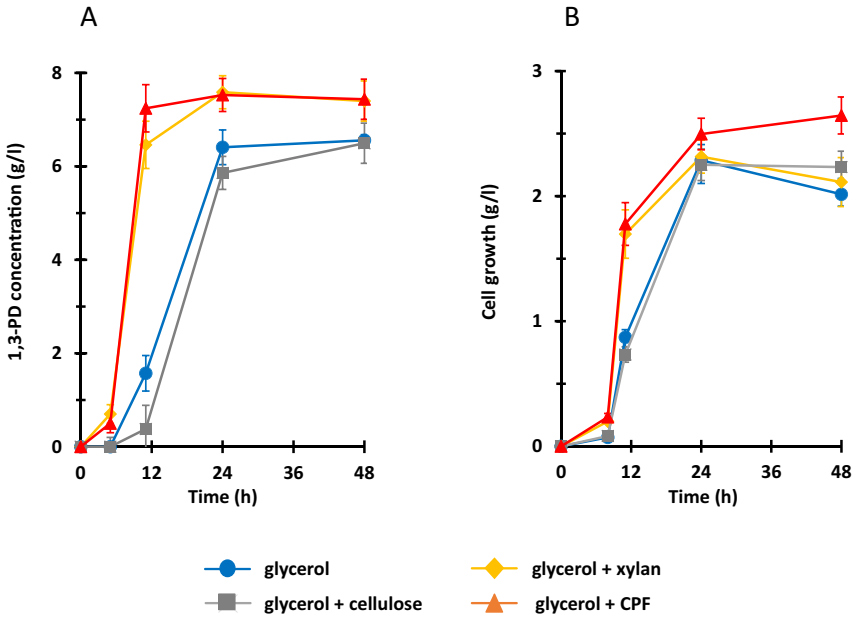

FIG. 1. Profiles of fermentative 1,3-PD production (A) and cell growth (B) by *C. butyricum* I5-42. Batch fermentation was carried out on M1 medium containing 20 g/l glycerol alone (blue), and glycerol supplemented with 2 g/l cellulose (gray), xylan (yellow) or CPF (red). Cell growth was assayed by the total cellular protein concentration. All data are the averages of three experiments. Error bars indicate the standard deviations of the three parallel replicates. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

TABLE 1. Effect of CPF, cellulose, and xylan supplements on 1,3-PD production by *C. butyricum* I5-42.

Carbon source	GC (g/l)	SC (g/l)	PD (g/l)	Conversion (mol/mol)	Productivity (g/l/h)	Specific PD productivity (g PD/g cells)	Product concentration (g/l)		
							Lactate	Acetate	Butyrate
Glycerol	20	–	6.41 ± 0.37	0.57 ± 0.02	0.26 ± 0.02	3.19	1.32 ± 0.02	0.39 ± 0.11	2.65 ± 0.17
Glycerol + CPF	20	2	7.53 ± 0.35	0.66 ± 0.02	0.31 ± 0.01	2.85	1.61 ± 0.55	0.17 ± 0.01	1.72 ± 0.10
Glycerol + cellulose	20	2	6.49 ± 1.00	0.58 ± 0.09	0.27 ± 0.08	2.91	0.77 ± 0.20	0.29 ± 0.04	3.43 ± 0.33
Glycerol + xylan	20	2	7.40 ± 0.59	0.61 ± 0.04	0.31 ± 0.02	3.51	0.81 ± 0.02	0.20 ± 0.04	1.91 ± 0.02
Cellulose	–	10	N.D.	N.D.	N.D.	N.D.	<0.01	<0.01	<0.02
Xylan	–	10	N.D.	N.D.	N.D.	N.D.	<0.1	<0.05	<0.1
Glycerol	60	–	7.51 ± 0.32	0.66 ± 0.04	0.31 ± 0.01	4.66	1.54 ± 0.02	0.45 ± 0.12	3.10 ± 0.12
Glycerol + CPF	60	2	10.2 ± 0.58	0.89 ± 0.07	0.41 ± 0.03	4.62	2.17 ± 0.02	0.22 ± 0.02	1.30 ± 0.02
Glycerol + cellulose	60	2	7.32 ± 0.31	0.65 ± 0.04	0.30 ± 0.01	4.58	0.86 ± 0.03	0.28 ± 0.14	3.84 ± 0.30
Glycerol + xylan	60	2	9.82 ± 0.19	0.81 ± 0.02	0.42 ± 0.02	4.63	1.10 ± 0.02	0.29 ± 0.16	1.03 ± 0.30

Values are the averages and standard deviations of three independent experiments. GC, initial glycerol concentration; SC, concentration of supplemented polysaccharides; PD, 1,3-PD concentration; Specific PD productivity, 1,3-PD concentration (g/L) divided by the cellular concentration (g/l); -, not included. N.D., not detected. Organic acids in the supernatants of 48-h old cultures were analyzed by HPLC.

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The 1,3-PD yield and productivity under CFP supplementation were 0.662 ± 0.02 mol 1,3-PD/mol glycerol and 0.31 ± 0.01 g/l/h respectively, higher than in glycerol alone (0.570 ± 0.02 mol 1,3-PD/mol glycerol and 0.26 ± 0.02 g/l/h, respectively; Table 1). The growth of *C. butyricum* I5-42 also slightly improved, from 2.01 ± 0.12 g cell protein/l to 2.64 ± 0.15 g cell protein/l (Fig. 1B and Table 1). These results indicate the feasibility of boosting 1,3-PD biosynthesis by CPF. The effects of glucose and starch supplements, and lignocellulosic hydrolysates such as xylose and arabinose, on glycerol metabolism have been reported in *Clostridium* and *Klebsiella* (7,13–15). When co-fermented with glycerol, these sugars feasibly improve the 1,3-PD conversion in these microbes. However, the monosaccharide supplements must be extracted by pretreating the biomass with expensive chemicals, which increases the cost of 1,3-PD production. If the CPF supplementation requires no pretreatment (i.e., if the polysaccharides can be directly applied as supplements), the fermentation efficiency of 1,3-PD by these microbes, and the economic viability of the fermentation, can be greatly improved.

Effect of polysaccharide components of CPF on 1,3-PD production According to the hydrolysis analysis, CPF mainly consists of cellulose (48.3 ± 2.3% (w/w)) and xylan (10.2 ± 1.1% (w/w)). To understand which components improve the 1,3-PD production in *C. butyricum* I5-42, M1 medium was supplemented not with CPF, but with each polysaccharide in the CPF. Specifically, M1 medium containing 20 g/l glycerol was supplemented with 2 g/l cellulose or xylan. The resulting 1,3-PD concentrations were 6.49 ± 1.00 g/l and 7.40 ± 0.59 g/l, respectively (Fig. 1A and Table 1). The corresponding productivities were 0.27 ± 0.08 g/l/h and 0.31 ± 0.02 g/l/h, respectively (Table 1). Similar results were obtained in CPF-supplemented medium (with a 1,3-PD concentration and productivity of 7.53 ± 0.35 g/l and 0.31 ± 0.01 g/l/h, respectively). These results indicate that xylan can remarkably improve the 1,3-PD production and productivity in *C. butyricum* I5-42. In medium supplemented with xylan, the cell growth during the 11-h fermentation time was much higher than in glycerol alone (Fig. 1B). However, the maximum growth almost equaled that in glycerol alone and in glycerol supplemented with xylan (2.01 ± 0.12 g cell protein/l and 2.11 ± 0.21 g cell protein/l, respectively). In addition, the specific 1,3-PD productivities were not different in glycerol alone and glycerol supplemented with xylan. These results suggest that the 1,3-PD production may be enhanced by the accelerated growth rate and fermentation speed of *C. butyricum* I5-42 in glycerol co-fermented with CPF or xylan, rather than an increase of 1,3-PD production efficiency per cell. A high productivity rate usually confers a large economic advantage, as shortening the fermentation time will decrease the number of required fermentation vessels and the operation cost (30).

C. butyricum exhibits weak or no enzymatic activity against polysaccharides such as cellulose and xylan (31). To confirm whether *C. butyricum* I5-42 can utilize these polysaccharides for cell growth, 1,3-PD production, and degradation, we prepared *C. butyricum* fermentations in M1 medium containing glycerol and these polysaccharides as sole carbon source. 1,3-PD production failed on all three polysaccharides, and no growth was observed on cellulose. Weak xylanase activities were observed in the extracellular fraction of *C. butyricum* 15–42 grown on M1 medium containing xylan, and the organism grew slightly on M1 medium containing xylan as the sole carbon source. These results suggest that *C. butyricum* I5-42 can utilize xylan to a limited extent through endo-β-1,4-xylanase activity (Table 2). To confirm whether hydrolysates of the polysaccharides improve 1,3-PD production by *C. butyricum* I5-42, we supplemented glycerol-containing medium with 2 g/l of the corresponding monosaccharides of cellulose and xylan (glucose and xylose, respectively). Supplementation with all three sugars negatively affected the 1,3-PD production and growth (Table S2). The inhibition strengthened with increasing monosaccharide concentration. As an example, we consider glucose. Glucose suppresses the inducible synthesis of glycerol dehydrogenase (GDH), dihydroxyacetone kinase (DHAK), glycerol dehydratase (GlyD) and 1,3-PD dehydrogenase (1,3-PDDH), especially during fermentation (7,11). Therefore, it appears that 1,3-PD biosynthesis by *C. butyricum* is repressed when monosaccharides such as glucose and xylose exist in the glycerol medium. No β-xylosidase activity was detected in the extracellular fractions (Table 2), indicating that *C. butyricum* 15–42 may utilize xylobiose and xylooligosaccharides from xylan through the phosphotransferase (PTS) system (32). These oligosaccharides can be simultaneously phosphorylated and translocated across the cell membrane (33). Many solventogenic and cellulolytic *Clostridia* accumulate carbohydrates through the PTS system (34,35). When the glycerol medium was supplemented with high concentrations of polysaccharides such as cellulose and xylan, the 1,3-PD production and productivity of *C. butyricum* I5-42 were robust to rising xylan and cellulose concentrations in the glycerol medium. Interestingly, xylan appears to improve the 1,3-PD yields and productivity without inhibition at high concentrations. Similarly improved 1,3-PD production has been reported in *C. diolis* DSM15140 (13) and *K. pneumoniae* (14). In these studies, the co-substrate comprised mixed mono-sugars from lignocellulosic hydrolysates. However, the effects of the polysaccharides described in this paper were not observed in the earlier studies.

TABLE 2. Xylan degradation abilities in the supernatants of *C. butyricum* I5-42 cultures.

Extracellular fractions	Enzymatic activity (units/mg proteins)	
	β-1,4-Xylanase	β-Xylosidase
Glucose	0.030 ± 0.01	0.004 ± 0.001
Xylose	0.091 ± 0.01	0.002 ± 0.001
Cellulose	0.042 ± 0.05	0.005 ± 0.001
Xylan	0.062 ± 0.01	0.004 ± 0.001
Glycerol + xylan	0.063 ± 0.03	0.004 ± 0.001

Extracellular fractions were prepared from culture supernatants of *C. butyricum* I5-42 grown in M1 medium supplemented with each carbon source. Concentrations of the carbon and glycerol sources in M1 were 2 g/l and 20 g/l, respectively. Values are the averages and standard deviations of three independent experiments.

Effect of CPF and xylan on *dhaB1*, *dhaT* and *dhaD* mRNA levels and the enzymatic activities of their encoded proteins When supplemented with CPF and xylan, *C. butyricum* I5-42 significantly increased its 1,3-PD yield and productivity from those of glycerol and cellulose-supplemented glycerol. Therefore, CPF and xylan must influence the glycerol metabolism pathway in *C. butyricum*. The primary enzymes in *C. butyricum*'s 1,3-PD production pathway are GlyD, 1,3-PDDH, and GDH, encoded by the *dhaB1*, *dhaT* and *dhaD* genes, respectively (11,36). To understand the effects of cellulose and xylan on the glycerol metabolism pathway in this organism, the transcriptional regulation of these genes was evaluated in *C. butyricum*. The mRNA levels were similar in the cellulose supplemented medium and in glycerol alone. In M1 medium containing 20 g/l glycerol and CPF or xylan as supplement, the transcription levels of *dhaB1* and *dhaT* were 2.7–4.9 times and 4.2–6.2 times higher, respectively, than in glycerol alone and cellulose-supplemented glycerol (Fig. 2). These results suggest that 1,3-PD production is enhanced by stimulation of the *dhaB1* and *dhaT* transcription levels in the glycerol metabolism of *C. butyricum*. In contrast, the *dhaD* mRNA levels in CPF- and xylan-supplemented glycerol were approximately 2–4 times lower than in glycerol alone and cellulose-supplemented glycerol (Fig. 2). Therefore, CPF and xylan might repress GDH expression and prevent *C. butyricum* from entering the oxidative pathway in glycerol degradation. To

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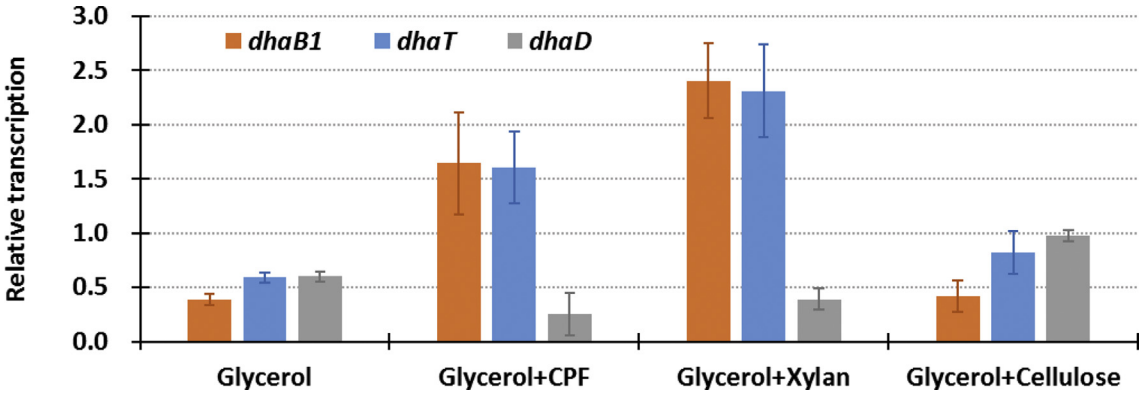

FIG. 2. Profiles of transcriptional levels of the major genes involved in glycerol metabolism by *C. butyricum* I5-42. Cells were cultured in M1 medium containing 20 g/l glycerol alone, and in glycerol supplemented with 2 g/l CPF, xylan or cellulose. Cells were collected in the mid-exponential phase (approximately 8–20 h after cell inoculation) of the batch fermentation. Error bars indicate the standard deviations of three parallel replicates.

confirm the specific enzymatic activities of the proteins encoded by *dhaB1* and *dhaT*, *C. butyricum* cells were grown in M1 medium containing 20 g/l glycerol alone, or supplemented with 2 g/l of CPF, xylan or cellulose. The GlyD and 1,3-PDDH activities were clearly higher (by factors of 1.7–4.4) in media containing CPF and xylan supplements than in media containing glycerol alone and cellulose supplement (Table 2). These results correspond with the transcriptional levels of the *dhaB1* and *dhaT* genes in *C. butyricum* supplemented with CPF or xylan. However, why the CPF and xylan supplements strongly induce GlyD and 1,3-PDDH in the reductive pathway remains unclear. High GlyD expression rapidly accumulates the toxic intermediate 3-hydroxypropionaldehyde (3-HPA) (36), and *C. butyricum* may express 1,3-PDDH to detoxify itself of 3-HPA. *C. butyricum* might possess a regulatory system that operates through the cell membrane, and activates glycerol catabolism in the presence of xylan. Alternatively, a set of putative σ and anti-σ factors including extracellular polysaccharide-sensing components has been identified in *C. thermocellum*, an anaerobic bacterium with a highly efficient cellulolytic system. These factors may participate in cellulosomal gene regulation (37,38). Nataf et al. (38) proposed a mechanism for the activation of alternate σ factors by extracellular polysaccharides. *C. butyricum* might possess a similar mechanism that activates the glycerol reduction pathway at the transcriptional level when extracellular polysaccharides (such as xylan) are present. On the other hand, the transcription levels and the GlyD and 1,3-PDDH activities in the supplemented media did not correspond with the 1,3-PD production yields (Table 1). Although the transcription levels and enzyme activities were higher in the CPF and xylan supplements than in cellulose supplement and glycerol alone, the enzymes involved in 1,3-PD production by *C. butyricum* will be strictly regulated by the intracellular redox conditions (7,11,36) (Fig. 3).

Effects of CPF and xylan supplementation on intracellular reducing equivalent Supplementation with CPF and xylan increases 1,3-PD production in *C. butyricum* by activating the glycerol reductive pathway. The reductive pathway first dehydrates glycerol to 3-HPA. The 3-HPA is then reduced to 1,3-PD by 1,3-PDDH, which regenerates oxidized NAD. High GlyD expression might enhance the dehydration of NADH by 1,3-PDDH to avoid 3-HPA accumulation (39). Thus, NADH availability is important for improving the glycerol metabolism in *C. butyricum*. To investigate the balance of the reducing power in supplemented glycerol medium, we assayed the NADH/NAD^+ ratios in cells grown for 8 and 14 h. The NADH availability was maintained slightly higher level in *C. butyricum* on grown on CPF- and xylan-supplemented media than on glycerol alone and the cellulose-supplemented medium for 8 h (Table 3), suggesting that NADH is smoothly regenerated in the cells by CPF or xylan supplement by 8 h. We speculate that high GlyD and 1,3-PDDH expression in the presence of CPF and xylan promotes the consumption of NADH regenerated by the glycerol oxidative pathway (Fig. 3). The glycerol oxidative pathway regenerates NADH from NAD^+, and is important for maintaining the redox balance of glycerol metabolism in *C. butyricum* (7,36,39) (Fig. 3). NAD is regenerated through the 1,3-PD, acetate and butylate formation pathways (7). The glycerol is first oxidized to dihydroxyacetone (DHA) by GDH with NAD^+ cofactor. This intermediate is then reduced to 1,3-PD while the oxidized NAD is regenerated (7,36). To check the situation of the oxidative pathway, we also measured the concentrations of the end-products (lactate, acetate and butyrate) in the CPF- and xylan-supplemented glycerol by HPLC. When CPF or xylan was added to the glycerol medium, the acetate and butyrate concentrations were 1.3–3.0 times lower than in glycerol alone and in cellulose-supplemented medium (Table 1). Similar results were reported in a glucose–glycerol culture of *C. butyricum* (7), which enhanced 1,3-PD production by decreasing the acetyl-CoA/CoA and butyl-CoA/CoA ratios and increasing the ATP/ADP ratio (7). This chemical environment might inhibit the NADH consumption, increasing the NADH availability for 1,3-PD production. Therefore, when the reduction pathway is activated by the CPF and xylan supplements, the oxidative pathway might be adjusted to supply NADH.

Improvement of 1,3-PD production by CPF and xylan supplementation at high glycerol concentration High initial concentrations of glycerol are known to prevent *C. butyricum* growth and decrease the efficiency of its 1,3-PD biosynthesis (39). High glycerol concentration causes considerable osmotic stress, and additional stress is imposed by toxins and inhibitors (40). The optimal initial glycerol concentration was previously determined as 20 g/l (41,42). To confirm whether CPF and xylan supplementation improves 1,3-PD production and productivity at high glycerol concentrations, a fermentation test was carried out in M1 medium containing 60 g/l of glycerol supplemented with 2 g/l of CPF or xylan. When the M1 medium containing 60 g/l glycerol was supplemented with 2 g/l CPF and 2 g/l xylan, the maximum 1,3-PD productions were 10.2 ± 0.88 g/l and 9.82 ± 0.19 g/l, respectively, higher than in glycerol alone and in cellulose-supplemented medium (Table 1). Specifically, after 24-h fermentation in the presence of 60 g/l glycerol, the CPF and xylan supplements enhanced the 1,3-PD productivity to 0.41± 0.03 g/l/h and 0.42 ± 0.02 g/l/h, respectively (versus 0.31 ± 0.01 g/l/h and 0.30 ± 0.01 g/l/h in glycerol alone and in cellulose-supplemented glycerol, respectively). After the 24-h fermentation, the

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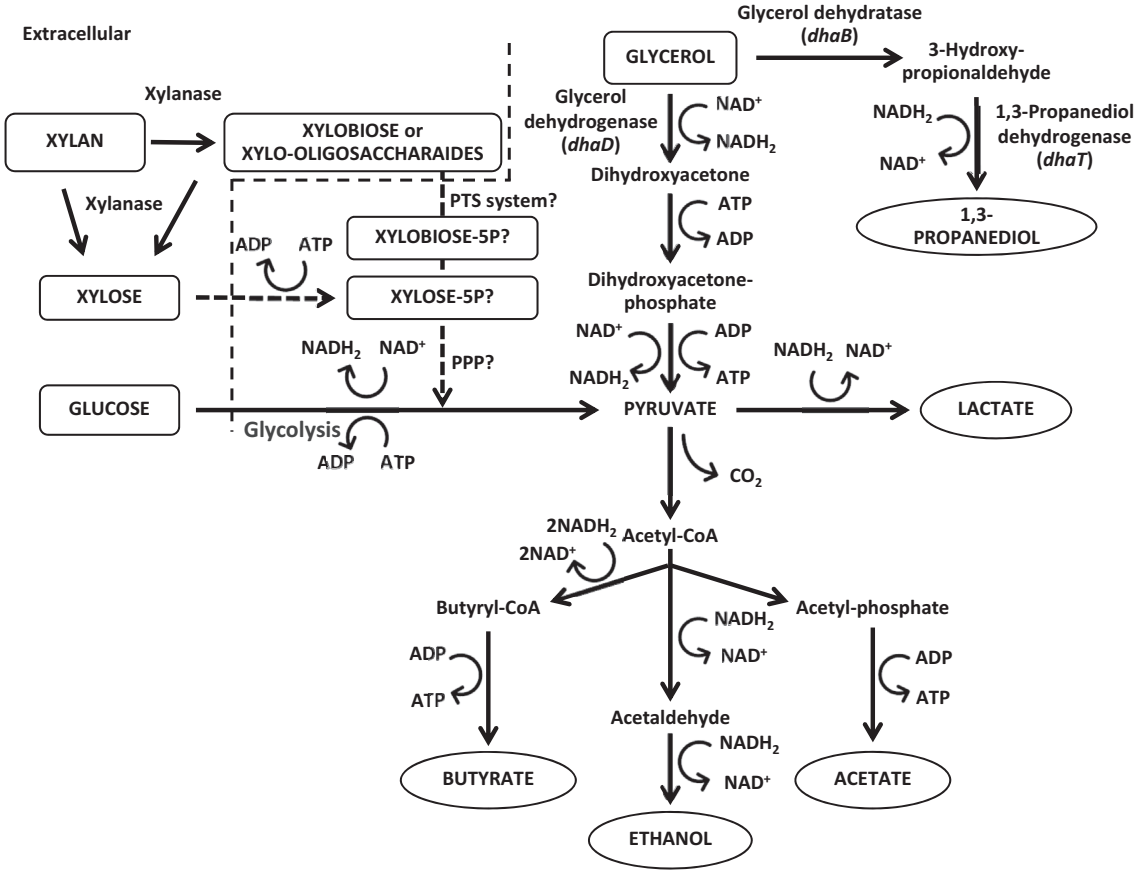

FIG. 3. Schematic of the glycerol and glucose metabolic pathways of *C. butyricum* (7). The metabolic pathway of xylan was predicted from the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html) using the *C. butyricum* genome database. PTS and PPP refer to the phosphotransferase system and the pentose phosphate pathway, respectively.

C. butyricum cellular protein concentrations in the CPF- and xylan-supplemented glycerol were 2.21 ± 0.1 g/l and 2.12 ± 0.1 g/l, respectively, again higher than in glycerol alone (1.61 ± 0.02 g/l) and in cellulose-supplemented glycerol (1.60 ± 0.07 g/l). However, the maximum specific 1,3-PD productivities were similar in glycerol alone and in glycerol co-fermented with CPF, xylan, and cellulose (Table 1). Additionally, when CPF or xylan was added to the concentrated glycerol medium, the acetate and butyrate concentrations were lower than in concentrated glycerol alone and 20 g/l glycerol alone (Table 1). These results suggest that the CPF and xylan supplements accelerate the growth rate and 1,3-PD fermentation of *C. butyricum* I5-42. The rapid 1,3-PD production might arise through the smooth generation of NAD in the glycerol oxidative pathway.

1,3-PD is a toxic metabolite produced during glycerol fermentation by *Clostridium* (41,43). *Clostridium* might overcome the inhibition of its intracellular 1,3-PD concentration by modifying its membrane organization to increase its fluidity (44), and by inhibiting the membrane ATPase and transport mechanisms (45). To avoid the problems arising from high glycerol concentrations, some researchers have prepared fed-batch cultures (30,43,46). Xylan not only reduces the toxicity of 1,3-PD in C. *butyricum*, but also enhances the 1,3-PD production and productivity at high glycerol concentrations. How xylan improves the 1,3-PD production in *C. butyricum*, and the signal that induces the 1,3-PD formation pathway, remain unknown. Nevertheless, the proposed supplemental strategy might improve the efficiency of fed-batch cultures and other distinguished *Clostridium* strains.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2017.12.006.

TABLE 3. Enzymatic activities and intracellular reducing equivalents in cell extracts of *C. butyricum* I5-42 cultured in glycerol supplemented with CPF or CPF components.

Carbon sources	Specific activity (μmol/min/mg of protein)		$NADH/NAD^+$ ratio	
	Glycerol dehydratase	1,3-PD dehydrogenase	8 h	14 h
Glycerol	0.19 ± 0.06	0.77 ± 0.06	8.5	11.9
Glycerol + cellulose	0.14 ± 0.05	0.50 ± 0.06	6.6	12.3
Glycerol + CPF	0.22 ± 0.06	2.20 ± 0.12	9.5	8.1
Glycerol + xylan	0.24 ± 0.07	1.06 ± 0.12	10.3	8.3

Values are the averages and standard deviations of three independent experiments. The cells were grown for 8 or 14 h in glycerol medium supplemented with CPF, cellulose or xylan, and cell-free extracts were prepared for NADH and NAD^+ assay.

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References

1. **Liu, H., Xu, Y., Zheng, Z., and Liu, D.:** 1,3-Propanediol and its copolymers: Research, development and industrialization, Biotechnol. J., **5**, 1137–1148 (2010).
2. **Lee, C. S., Aroua, M. K., Daud, W. M. A. W., Cognet, P., Pérès-Lucchese, Y., Fabre, P. L., Reynes, O., and Latapie, L.:** A review: conversion of bioglycerol into 1,3-propanediol via biological and chemical method, Renew, Sustain. Energy Rev., **42**, 963–972 (2015).

3. **Dobson, R., Gray, V., and Rumbold, K.:** Microbial utilization of crude glycerol for the production of value-added products, J. Ind. Microbiol. Biotechnol., **39**, 217–226 (2012).
4. **da Silva, G. P., Mack, M., and Contiero, J.:** Glycerol: a promising and abundant carbon source for industrial microbiology, Biotechnol. Adv., **27**, 30–39 (2009).
5. **Wischral, D., Zhang, J., Cheng, C., Lin, M., De Souza, L. M. G., Pessoa, F. L. P., Pereira, N., Jr., and Yang, S.-T.:** Production of 1,3-propanediol by *Clostridium beijerinckii* DSM 791 from crude glycerol and corn steep liquor: process optimization and metabolic engineering, Bioresour. Technol., **212**, 100–110 (2016).
6. **Chatzifragkou, A., Papanikolaou, S., Dietz, D., Doulgeraki, A., Nychas, G.-J., and Zeng, A.-P.:** Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process, Appl. Microbiol. Biotechnol., **91**, 101–112 (2011).
7. **Saint-Amans, S., Girbal, L., Andrade, J., Ahrens, K., and Soucaille, P.:** Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures, J. Bacteriol., **183**, 1748–1754 (2001).
8. **Kaur, G., Srivastava, A. K., and Chand, S.:** Determination of kinetic parameters of 1,3-propanediol fermentation by *Clostridium diolis* using statistically optimized medium, Bioprocess Biosyst. Eng., **35**, 1147–1156 (2012).
9. **Kaur, G., Srivastava, A. K., and Chand, S.:** Bioconversion of glycerol to 1,3-propanediol: a mathematical model-based nutrient feeding approach for high production using *Clostridium diolis*, Bioresour. Technol., **142**, 82–87 (2013).
10. **Yazdani, S. S. and Gonzalez, R.:** Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr. Opin. Biotechnol., **18**, 213–219 (2007).
11. **Abbad-Andaloussi, S., Amine, J., Gerard, P., and Petitdemange, H.:** Effect of glucose on glycerol metabolism by *Clostridium butyricum* DSM 5431, J. Appl. Microbiol., **84**, 515–522 (1998).
12. **Whelan, W. J. and Nasr, H.:** The amylase of *Clostridium butyricum*,, Biochem. J., **48**, 416–422 (1951).
13. **Xin, B., Wang, Y., Tao, F., Li, L., Ma, C., and Xu, P.:** Co-utilization of glycerol and lignocellulosic hydrolysates enhances anaerobic 1,3-propanediol production by *Clostridium diolis*, Sci. Rep., **6**, 19044 (2016).
14. **Jin, P., Li, S., Lu, S.-g., Zhu, J.-g., and Huang, H.:** Improved 1,3-propanediol production with hemicellulosic hydrolysates (corn straw) as cosubstrate: impact of degradation products on *Klebsiella pneumoniae* growth and 1,3-propanediol fermentation, Bioresour. Technol., **102**, 1815–1821 (2011).
15. **Apiwatanapiwat, W., Vaithanomsat, P., Tachaapaikoon, C., Ratanakhanokchai, K., and Kosugi, A.:** Effect of cassava pulp supplement on 1,3-propanediol production by *Clostridium butyricum*, J. Biotechnol., **230**, 44–46 (2016).
16. **Vaithanomsat, P., Kosugi, A., Apiwatanapiwat, W., Thanapase, W., Waeonukul, R., Tachaapaikoon, C., Pason, P., and Mori, Y.:** Efficient saccharification for non-treated cassava pulp by supplementation of *Clostridium thermocellum* cellulosome and *Thermoanaerobacter brockii* β-glucosidase, Bioresour. Technol., **132**, 383–386 (2013).
17. **Khempaka, S., Molee, W., and Guillaume, M.:** Dried cassava pulp as an alternative feedstuff for broilers: effect on growth performance, carcass traits, digestive organs, and nutrient digestibility, J. Appl. Poultry Res., **18**, 487–493 (2009).
18. **Apiwatanapiwat, W., Vaithanomsat, P., Ushiwaka, S., Morimitsu, K., Machida, M., Thanapase, W., Murata, Y., and Kosugi, A.:** A new pretreatment using ammonia gas absorption fiber expansion for saccharification of cassava pulp, Biomass Convers. Biorefinery, **6**, 181–188 (2016).
19. **Kumar, A. K. and Sharma, S.:** Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review, Bioresour. Bioprocess., **4**, 7 (2017).
20. **Zhang, H.-J., Fan, X.-G., Qiu, X.-L., Zhang, Q.-X., Wang, W.-Y., Li, S.-X., Deng, L.-H., Koffas, M. A. G., Wei, D.-S., and Yuan, Q.-P.:** A novel cleaning process for industrial production of xylose in pilot scale from corncob by using screw-steam-explosive extruder, Bioprocess Biosyst. Eng., **37**, 2425–2436 (2014).
21. **Fan, X., Cheng, G., Zhang, H., Li, M., Wang, S., and Yuan, Q.:** Effects of acid impregnated steam explosion process on xylose recovery and enzymatic conversion of cellulose in corncob, Carbohydr. Polym., **114**, 21–26 (2014).
22. **Rasmussen, H., Sørensen, H. R., and Meyer, A. S.:** Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms, Carbohydr. Res., **385**, 45–57 (2014).
23. **Palmqvist, E. and Hahn-Hägerdal, B.:** Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, Bioresour. Technol., **74**, 25–33 (2000).
24. **Deng, L., Mori, Y., Sermsathanaswadi, J., Apiwatanapiwat, W., and Kosugi, A.:** Cellulose hydrolysis ability of a *Clostridium thermocellum* cellulosome containing small-size scaffolding protein CipA, J. Biotechnol., **212**, 144–152 (2015).
25. **Boenigk, R., Bowien, S., and Gottschalk, G.:** Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*, Appl. Microbiol. Biotechnol., **38**, 453–457 (1993).
26. **Toraya, T., Krodel, E., Mildvan, A. S., and Abeles, R. H.:** Role of peripheral side chains of vitamin B12 coenzymes in the reaction catalyzed by dioldehydrase, Biochemistry, **18**, 417–426 (1979).
27. **Okada, H. and Shinmyo, A.:** Xylanase of *Bacillus pumilus*, pp. 632–637, in: Wood, W. A. and Kellogg, S. T. (Eds.), Methods in Enzymology, vol. 160. Academic Press, Amsterdam (1988).
28. **Pason, P., Kosugi, A., Waeonukul, R., Tachaapaikoon, C., Ratanakhanokchai, K., Arai, T., Murata, Y., Nakajima, J., and Mori, Y.:** Purification and characterization of a multienzyme complex produced by *Paenibacillus curdlanolyticus* B-6, Appl. Microbiol. Biotechnol., **85**, 573–580 (2010).
29. **Kosugi, A., Kondo, A., Ueda, M., Murata, Y., Vaithanomsat, P., Thanapase, W., Arai, T., and Mori, Y.:** Production of ethanol from cassava pulp via fermentation with a surface-engineered yeast strain displaying glucoamylase, Renew. Energy, **34**, 1354–1358 (2009).
30. **Wilkens, E., Ringel, A. K., Hortig, D., Willke, T., and Vorlop, K.-D.:** High-level production of 1,3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a, Appl. Microbiol. Biotechnol., **93**, 1057–1063 (2012).
31. **Lo, Y.-C., Lu, W.-C., Chen, C.-Y., and Chang, J.-S.:** Dark fermentative hydrogen production from enzymatic hydrolysate of xylan and pretreated rice straw by *Clostridium butyricum* CGS5, Bioresour. Technol., **101**, 5885–5891 (2010).
32. **Postma, P. W., Lengeler, J. W., and Jacobson, G. R.:** Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria, Microbiol. Rev., **57**, 543–594 (1993).
33. **Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., and Yang, S.:** Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose, Appl. Environ. Microbiol., **77**, 7886–7895 (2011).
34. **Mitchell, W. J.:** The phosphotransferase system in solventogenic clostridia, J. Mol. Microbiol. Biotechnol., **25**, 129–142 (2015).
35. **Patni, N. J. and Alexander, J. K.:** Catabolism of fructose and mannitol in *Clostridium thermocellum*: presence of phosphoenolpyruvate: fructose phosphotransferase, fructose l-phosphate kinase, phosphoenolpyruvate: mannitol phosphotransferase, and mannitol l-phosphate dehydrogenase in cell extracts, J. Bacteriol., **105**, 226–231 (1971).
36. **Gungormusler-Yilmaz, M., Shamshurin, D., Grigoryan, M., Taillefer, M., Spicer, V., Krokhin, O. V., Sparling, R., and Levin, D. B.:** Reduced catabolic protein expression in *Clostridium butyricum* DSM 10702 correlate with reduced 1,3-propanediol synthesis at high glycerol loading, AMB Express, **4** (2014). 63–63.
37. **Kahel-Raifer, H., Jindou, S., Bahari, L., Nataf, Y., Shoham, Y., Bayer, E. A., Borovok, I., and Lamed, R.:** The unique set of putative membrane-associated anti-σ factors in *Clostridium thermocellum* suggests a novel extracellular carbohydrate-sensing mechanism involved in gene regulation, FEMS Microbiol. Lett., **308**, 84–93 (2010).
38. **Nataf, Y., Bahari, L., Kahel-Raifer, H., Borovok, I., Lamed, R., Bayer, E. A., Sonenshein, A. L., and Shoham, Y.:** *Clostridium thermocellum* cellulosomal genes are regulated by extracytoplasmic polysaccharides via alternative sigma factors, Proc. Natl. Acad. Sci. USA, **107**, 18646–18651 (2010).
39. **Kubiak, P., Leja, K., Myszka, K., Celińska, E., Spychała, M., Szymanowska-Powałowska, D., Czaczyk, K., and Grajek, W.:** Physiological predisposition of various *Clostridium* species to synthetize 1,3-propanediol from glycerol, Process Biochem., **47**, 1308–1319 (2012).
40. **Colin, T., Bories, A., and Moulin, G.:** Inhibition of *Clostridium butyricum* by 1,3-propanediol and diols during glycerol fermentation, Appl. Microbiol. Biotechnol., **54**, 201–205 (2000).
41. **Biebl, H.:** Fermentation of glycerol by *Clostridium pasteurianum* - batch and continuous culture studies, J. Ind. Microbiol. Biotechnol., **27**, 18–26 (2001).
42. **Günzel, B., Yonsel, S., and Deckwer, W.-D.:** Fermentative production of 1,3-propanediol from glycerol by *Clostridium butyricum* up to a scale of $2m^3$, Appl. Microbiol. Biotechnol., **36**, 289–294 (1991).
43. **Papanikolaou, S., Ruiz-Sanchez, P., Pariset, B., Blanchard, F., and Fick, M.:** High production of 1,3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain, J. Biotechnol., **77**, 191–208 (2000).
44. **Jain, M. K., Gleeson, J., Upreti, A., and Upreti, G. C.:** Intrinsic perturbing ability of alkanols in lipid bilayers, Biochim. Biophys. Acta Biomembr., **509**, 1–8 (1978).
45. **Shimizu, T. and Katsura, T.:** Steady-state kinetic study on the inhibition of the adenosinetriphosphatase activity of dynein from *Tetrahymena* cilia by glycerol, J. Biochem., **103**, 99–105 (1988).
46. **Reimann, Abbad, A., Biebl, and Petitdemange:** 1,3-Propanediol formation with product-tolerant mutants of *Clostridium butyricum* DSM 5431 in continuous culture: productivity, carbon and electron flow, J. Appl. Microbiol., **84**, 1125–1130 (1998).

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