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Engineering of *Corynebacterium glutamicum* for Consolidated Conversion of Hemicellulosic Biomass into Xylonic Acid

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Xylonic acid is a promising platform chemical with various applications in the fields of food, pharmaceuticals, and agriculture. However, in the current process, xylonic acid is mainly produced by the conversion of xylose, whose preparation requires substantial cost and time. Here, *Corynebacterium glutamicum* is engineered for the consolidated bioconversion of hemicellulosic biomass (xylan) into xylonic acid in a single cultivation. First, for the efficient conversion of xylose to xylonic acid, xylose dehydrogenase (Xdh) and xylonolactonase (XylC) from *Caulobacter crescentus* are evaluated together with a previously optimized xylose transporter module (XylE of *Escherichia coli*), and cells expressing *xdh* and *xylE* genes with an optimized expression system can produce xylonic acid from xylose with 100% conversion yield. Next, to directly process xylan as a substrate, an engineered xylan-degrading module is introduced, in which two xylan-degrading enzymes (endoxylanase and xylosidase) are secreted into the culture medium. The engineered *C. glutamicum* successfully produce 6.23 g L^{-1} of xylonic acid from 20 g L^{-1} of xylan. This is the first report on xylonic acid production in *C. glutamicum* and this robust system will contribute to development of an industrially relevant platform for production of xylonic acid from raw biomass.

1. Introduction

Xylonic acid is a five-carbon organic acid that can be acquired by oxidation of xylose, a very important alternative pentose sugar present in hemicellulosic biomass.[1] Xylonic acid is considered by the U.S. Department of Energy to be one of the 30 chemicals of highest value because it can be used in a variety of applications, including as a chelator, dispersant, clarifying agent, pH regulator, antibiotic, and health enhancer.[2] For the production of xylonic acid, there are two possible routes: i) chemical oxidation using platinum or gold catalysts or ii) enzymatic oxidation using xylose dehydrogenase and xylonolactonase. Compared to the chemical route, in which selectivity is relatively poor, enzymatic oxidation through microbial fermentation is considered an economically feasible and environmentally friendly approach for the production of xylonic acid at industrial scale. To date, bio-based production of xylonic acid has been accomplished in various microorganisms, including *Escherichia coli*, *Saccharomyces cerevisiae*, and *Kluyveromyces lactis*, by introducing xylose dehydrogenase or xylonolactonase genes from *Caulobacter crescentus* or *Trichoderma reesei*.[3–6] Even though these previous approaches produced considerable amounts of xylonic acid from xylose, with competitive productivities and production yields, they all used purified xylose as a substrate. To acquire xylose from hemicellulosic biomass, however, enzymatic hydrolysis or chemical hydrolysis is also required, and this process costs substantial capital and time.[7,8] In addition, most hosts used in previous reports utilize xylose as a carbon source for cell growth, so it was necessary to eliminate the xylose catabolic pathways in these hosts to minimize the loss of substrate.[3,4] To overcome current limitations in the production of xylonic acid, an alternative consolidated conversion process for the direct production of xylonic acid from degraded biomass (i.e., xylan) is desirable. The consolidated process would include, in a single bioreactor i) enzyme production and enzymatic degradation of biomass to hexose/pentose sugars and ii) microbial fermentation for production of targeted biochemicals. With this consolidated bioprocess, we expected a significant decrease in cost.[9,10]

Corynebacterium glutamicum is a gram-positive and non-sporulating bacterium that has been used traditionally for the production of L-amino acids, especially L-glutamate and L-lysine, and various biochemicals.[11–14] *C. glutamicum* is considered a promising host for consolidated bioprocessing because of the following ideal attributes: i) significant resistance against inevitable fermentation-inhibiting by-products, such as organic

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acids, furans, and phenols, which can form during biomass pretreatment processes[15]; ii) the strong ability to secrete recombinant proteins into culture medium, which is critical for the enzymatic degradation of extracellular biomass.[16,17] Recently, based on these characteristics, *C. glutamicum* has been successfully engineered to utilize various biomass, including corn starch, microalgae, and xylan, by simply expressing biomass-degrading enzymes.[18–22] In addition, unlike hosts used for xylonic acid production previously, *C. glutamicum* has no competing catabolic pathways for xylose,[23,24] so entire xylose substrate can be used to produce xylonic acid in *C. glutamicum*, without the need for further engineering to eliminate a xylose catabolic pathway. In addition, *C. glutamicum* is generally recognized as safe (GRAS); therefore, *C. glutamicum*-derived xylonic acid can be used for therapeutic and pharmaceutical applications, as shown in other cases of GRAS organisms.[12,16,25]

Here, we report the engineering of *C. glutamicum* for the consolidated conversion of hemicellulosic biomass (xylan) into xylonic acid. For this purpose, we sought to construct a module for the conversion of xylose to xylonic acid in the cytoplasm with xylose dehydrogenase (Xdh) and xylonolactonase (XylC), and the module was further combined with two previously developed modules: i) a xylose transport module composed of xylose transporter (XylE) and ii) a xylan degradation module for the enzymatic degradation of xylan into xylose in the medium with endoxylanase (XlnA) and xylosidase (XynB)[21] (**Figure 1**). To the end, consolidated conversion of xylan into xylonic acid without addition of any exogenous enzymes as well as purification of xylose, was successfully demonstrated in a single flask cultivation using the engineered *C. glutamicum*.

2. Experimental Section

2.1. Bacterial Strains and Plasmid Manipulation

The bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* XL1-Blue was used as a host for gene cloning and plasmid maintenance, and *C. glutamicum* ATCC 13032 was used as the main host for the production of xylonic acid. For gene expression in *C. glutamicum*, plasmid pCES208, an *E. coli-C. glutamicum* shuttle vector, was used as a backbone plasmid. The polymerase chain reaction (PCR) was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with PrimeSTAR HS polymerase (Takara Bio, Inc., Shiga, Japan). The nucleotide sequences of all primers used in this study are listed in **Table 2**.

To express the xylose dehydrogenase gene (*xdh*) from *C. crescentus*, *xdh* was amplified from pET28a-cxylB[4] by PCR with two primers, Xdh-F and Xdh-R. After digestion with *Bam*HI and *Not*I, the PCR product was cloned into pCG-H36A, which has a strong synthetic promoter (P_{H36}) and *rrnB*T1T2 terminator, yielding pX. To enhance expression of *xdh*, the bacteriophage T7 gene 10 RBS (ribosome binding site) and an N-terminal 6× His tag (HHHHHH) was ligated with *xdh* by PCR with two primers, UH-Xdh-F and Xdh-R. After digestion with *Bam*HI and *Not*I, the PCR product was cloned into pCES-L10-M18, pCES-I16-M18,

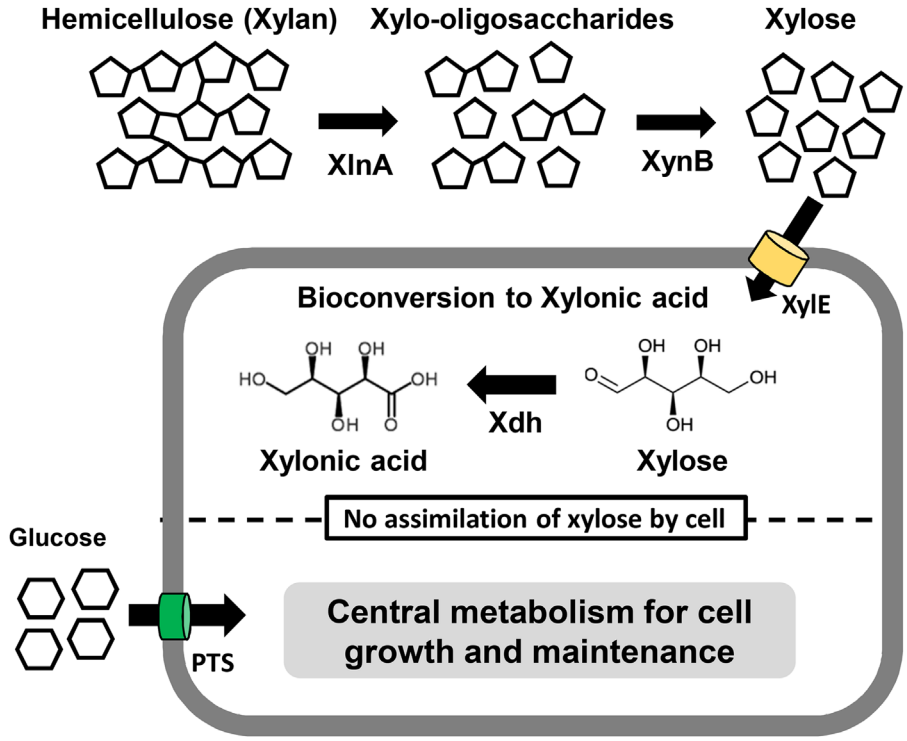

Figure 1. Schematic diagram of xylonic acid production directly from hemicellulose (xylan). Since xylose, which can be derived from xylan degradation, cannot be naturally metabolized by *C. glutamicum*, glucose and xylose will be utilized through two parallel routes, cell growth and maintenance route (bottom) and bioconversion route (top) for xylonic acid production, respectively.

Table 1. Bacterial strains and plasmids used in this study.

Strains	Relevant characteristics	Reference or source
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tet')]*	Stratagene[a)]
C. glutamicum	Wild-type	ATCC 13032
Plasmids		
pCES208	5.9 kb; *E. coli – C. glutamicum* shuttle vector; Km^r	[36]
pET28a-cxylB	pET28a derivative; P_{T7}, *xylB* (*xdh*) from *C. crescentus*	[4]
pUC5-cxylC	pUC57 derivative; *xylC* from *C. crescentus*	This study
pXU2T7	11 kb; pXU2 derivative; P_{L10}, *xylE* from *E. coli*	[21]
pXD5	9.5 kb; pCES208 derivative; P_{cg1514}, signal sequence of *cg1514*, *xlnA* from *S. coelicoloar* A3(2), signal sequence of *cgR0949*, *xynB* from *B. pumilus*	[21]
pX	6.8 kb; pCES208 derivative; P_{H36}, *xdh* from *C. crescentus*	This study
pUX	6.8 kb; pCES208 derivative; P_{H36}, T7 *g10* RBS, *xdh* from *C. crescentus* with N-terminal 6×His tag	This study
pUX-L10	pCES208 derivative; P_{L10}, T7 *g10* RBS, *xdh* from *C. crescentus* with N-terminal 6×His tag	This study
pUX-I16	pCES208 derivative; P_{I16}, T7 *g10* RBS, *xdh* from *C. crescentus* with N-terminal 6×His tag	This study
pUXC	7.8 kb; pUX derivative; T7 *g10* RBS, *xylC* from *C. crescentus* with N-terminal 6×His tag	This study
pUXE	8.4 kb; pUX derivative; P_{L10}, *xylE* from *E. coli*	This study
pUXE-I12	pUX derivative; P_{I12}, *xylE* from *E. coli*	This study
pUXE-H72	pUX derivative; P_{H72}, *xylE* from *E. coli*	This study
pUXED	12 kb; pUXE derivative; P*cg1514*, signal sequence of *cg1514*, *xlnA* from *S. coelicolor* A3(2), signal sequence of *cgR0949*, *xynB* from *B. pumilus*	This study

[a)] Stratagene, La Jolla, CA, USA.

and pCG-H36A to yield pUX-L10, pUX-I16, and pUX, respectively. To introduce the xylonolactonase (*xylC*) gene from *C. crescentus* (Gene ID: 7329903), *xylC* was synthesized by GenScript Co. (Piscataway, NJ, USA). Synthesized *xylC* was amplified by PCR with two primers, XylC-F and XylC-R, and after digestion with *Not*I, the PCR product was cloned into pUX to yield pUXC. To introduce the xylose transport system, a previously developed expression system for *E. coli* xylose transporter (*xylE*) gene was selected for use.[19] The whole expression system for *xylE* including low strength L10 (P_{L10}), intermediate strength I12 (P_{I12}), and strong strength H36 (P_{H36}) synthetic promoters and *lpp* terminator were isolated by digesting pXU2T7, pXU2T8, and pXU2T9 with *Nco*I, and the resulting fragments were cloned into the pUX to yield pUXE, pUXE-I12, and pUXE-H72, respectively. To introduce the xylan degradation system, endoxylanase (*xlnA*) from *Streptomyces coelicolor* A3(2) and xylosidase (*xynB*) from *Bacillus pumilus* were employed.[19] The whole expression system for *xlnA* and *xynB*, including the *C. glutamicum* ATCC 13032 *cg1514* native promoter and signal sequence for *xlnA* and *C. glutamicum* R *cgR0949* signal sequence for *xynB* was amplified by PCR using two primers, XD-F and XD-R. After digestion with *Not*I, the PCR product was cloned into pUXE, yielding pUXED. The schematic diagrams of all gene expression systems are shown in **Figure 2**.

2.2. Media and Cultivation Conditions

To prepare plasmids, *E. coli* was cultivated in Luria–Bertani (LB) media (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and NaCl, 10 g L^{-1}) at 37 °C with shaking at 200 rpm (shaking amplitude: 30 mm). *C. glutamicum* strains were cultivated in Brain Heart

Table 2. List of oligonucleotides used in this study.

Primers	Sequences (5′ to 3′)[a)]
Xdh-F	ATTAAT**GGATCC**ATGTCCTCAGCCATCTATCCC
Xdh-R	ATTAAT**GCGGCCGC**CTATCATTTGTCATCGTCATCTTTATAATCACGCCAGCCGGCGTCGATCCAGTA
UH-Xdh-F	ATTAAT**GGATCC**TCTAGATAACTTTAAGAAGGAGATATACATATGCATCACCATCACCATCATTCCTCAGCCATCTATCCC
XylC-F	ATTAAT**GCGGCCGC**TCTAGATAACTTTAAGAAGGAGATATACATATGCATCACCATCACCATCATACCGCTCAAGTCACTTGCGTAT
XylC-R	ATTAAT**GCGGCCGC**TTAGACAAGGCGGACCTCATGCTG
XD-F	ATTAAT**GCGGCCGC**AGCCTGACTAGCGGTGTTTAAG
XD-R	ATTAAT**GCGGCCGC**TCATTATTTGTCATCGTCATCTTTATAATCTTCG

[a)] Bold letters represent the sequences of restriction enzymes.

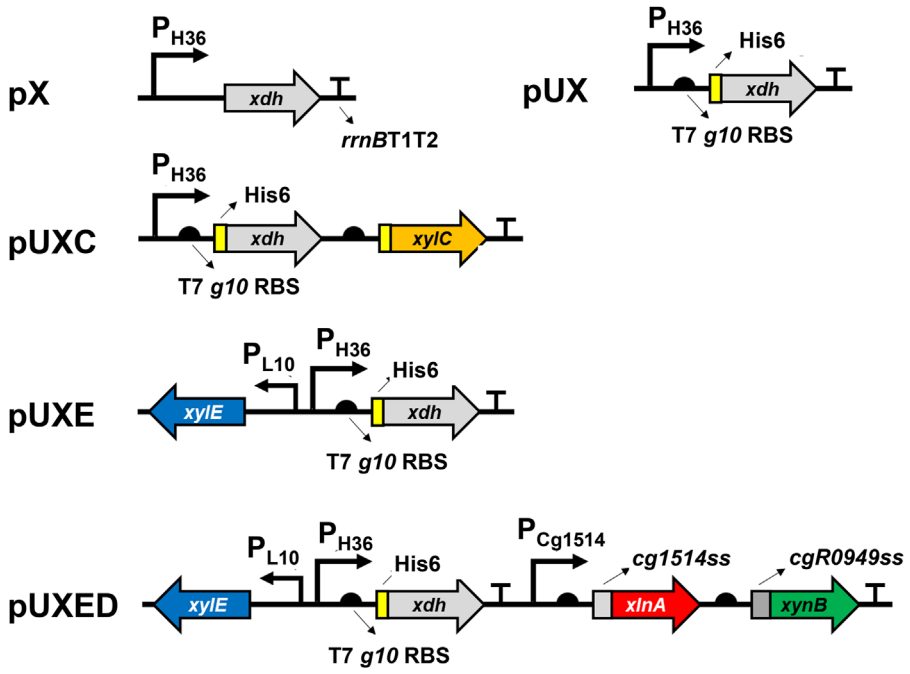

Figure 2. Schematic diagram of plasmids constructed in this study. P_{H36}, Strong synthetic promoter; P_{L10}, weak synthetic promoter; His6, hexahistidine tag; *cg1514*ss and *cgR0949*ss, signal sequences for protein secretion; *rrnB*T1T2, transcription terminator.

Infusion (BHI; Difco Laboratories, Detroit, MI, USA) and minimal medium (3 g L^{-1} of K_2HPO_4, 1 g L^{-1} of KH_2PO_4, 2 g L^{-1} of urea, 10 g L^{-1} of $(NH_4)_2SO_4$, 2 g L^{-1} of $MgSO_4$, 200 μg L^{-1} of biotin, 5 g L^{-1} of thiamine, 10 g L^{-1} of $MnSO_4$, 1 g L^{-1} of $ZnSO_4$, and 10 mg L^{-1} of $CaCl_2$) with variable concentrations of carbon sources as indicated. *C. glutamicum* cells were first inoculated into BHI media and grown at 30 °C for 24 h with shaking at 200 rpm (shaking amplitude: 30 mm). The fully grown cultures were then transferred at a concentration of 1/50 (for the experiment using xylose and glucose) or 1/20 (for the experiment using xylan and glucose) into 50 mL of minimal medium in 250-mL baffled flasks, and cells were cultivated for 48 h at 30 °C with shaking at 200 rpm (shaking amplitude: 30 mm). In all cultivations, kanamycin (Km, 25 μg L^{-1}) was added to the culture medium as the sole antibiotic. Beechwood xylan (Sigma–Aldrich, St. Louis, MO, USA), which mostly consists of xylose residues (>90%), was used for xylan degradation. In all cultivations, pH was monitored at the beginning and the end of the cultivation (Table S1, Supporting Information).

2.3. Protein Preparation and Analysis

After cultivation in shaking flasks, cells were harvested by centrifugation at 3341×*g* (≈6000 rpm) for 10 min at 4 °C. Cells were then washed twice with phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2PO_4, 1.4 mM KH_2PO_4, pH 7.2) and resuspended in the same buffer. Crude cell lysates were prepared by sonication (7 min at 40% pulse and 20% amplitude), and the extracts were centrifuged at 9279×*g* (≈10 000 rpm) for 10 min at 4 °C to yield soluble lysates in the supernatant. All fractionated protein samples were stored at −20 °C until further analysis. Proteins were analyzed by electrophoresis on a 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel.

2.4. Analysis of Sugars and Xylonic Acid Concentrations

To analyze sugar concentrations in the culture medium, culture supernatants were prepared by centrifugation at 15 682×*g* (≈13 000 rpm) for 10 min at 4 °C, followed by filtration of residual insoluble matters with a 0.45-μm syringe filter (Sartorius Stedim Biotech, Goettingen, Germany). The prepared samples were diluted 30-fold in distilled water and loaded onto a high performance liquid chromatograph (HPLC; Watters Breeze 2 system, Water Chromatography, Milford, MA, USA) equipped with UV/Vis (G1314B, Agilent, Wakefield, MA, USA) and refractive index (RI) detectors (Shodex RI-71, Tokyo, Japan). An Eclipse Plus C18 column (4.6 × 150 mm, Agilent) was used for the analysis. It was operated at 50 °C with a 1 mL min^{-1} flow rate in the mobile phase (5 mM H_2SO_4). When xylonic acid was present, xylose concentrations were estimated by subtraction of the xylonic acid peak (detected by UV) from the combined xylose and xylonic acid peak detected by RI.[26]

3. Results

3.1. Expression of *xdh* from *C. crescentus* for Xylonic acid Production

Because wild-type *C. glutamicum* ATCC 13032 cannot produce xylonic acid from xylose, *xdh* from *C. crescentus* was introduced to

construct a xylonic acid pathway in *C. glutamicum*. *C. crescentus xdh* was chosen because it was previously reported as having much higher enzymatic activity than other xylose dehydrogenases from *T. reesei* or pig liver.[2] A *xdh* gene was cloned into pCES208, in which *xdh* gene was expressed under the control of a strong constitutive promoter (P_{H36}).[27] In this construct (pX), however, *xdh* expression was not sufficiently high to be clearly detected by SDS–PAGE (**Figure 3**A). Irrespective of the promoter strength, the UTR (untranslated region) sequence between the promoter and start codon of the target gene can affect gene expression in bacterial hosts.[28] Therefore, to enhance *xdh* expression, an RBS of T7 gene 10, which is known to promote efficient gene expression,[29,30] was also introduced. With this construct (pUX), we found that xylose dehydrogenase production was significantly increased (Figure 3A).

To analyze xylonic acid production from xylose, cells harboring either pX or pUX were cultivated in minimal medium containing 10 g L^{-1} of glucose as a carbon source for cell growth and 20 g L^{-1} of xylose as a substrate for xylonic acid production. After flask cultivation, cell growth and xylonic acid production were analyzed. In all cultures, glucose as the sole carbon source was depleted in 10 h, and cells entered the stationary growth phase (**Figure 4**A and B). At this time (10 h), simultaneously, xylose began to be consumed and xylonic acid began to be produced (Figure 4C and D). Cells harboring pX or pUX exhibited similar levels of xylonic acid production yields (1.03 or 1.00 g g^{-1} xylose, respectively) (**Table 3**). However, compared with cells harboring pX, cells harboring pUX showed higher levels of *xdh* expression, faster consumption of xylose, and higher productivity (0.77 g L^{-1} h^{-1}) (Table 3). Because the conversion of xylose into xylonic acid by Xdh can be changed by the intracellular level of Xdh, we examined two more promoters which have different strength (low-strength P_{L10} and intermediate-strength P_{I16}) for the expression of *xdh* gene, and the expression level and productivity in each expression system was compared with those of strong P_{H36} promoter system (pUX). Both cells harboring pUX-L10 (low-strength P_{L10}) and pUX-I16 (intermediate-strength P_{I16}) showed almost similar growths as cells harboring pUX, and it was also clearly observed that the expression level of *xdh* gene was highly correlated with promoter strength: under the strong P_{H36} promoter, the expression level was much higher than those of intermediate and lower strength promoters (Figure S2, Supporting Information). We found that cells harboring pUX consumed xylose much faster than others, and about twofold higher productivity of xylonic acid (0.77 g L^{-1} h^{-1}) could be achieved compared with other systems, pUX-L10 (0.36 g L^{-1} h^{-1}) and pUX-I16 (0.39 g L^{-1} h^{-1}) (Figure S2, Supporting Information). From these comparison, the strong P_{H36} promoter was chosen for the expression of *xdh* gene.

3.2. Introduction of *xylC* from *C. crescentus*

We sought to further increase the hydrolysis rate of xylonolactone, which can be spontaneously converted to xylonic acid, by introducing *xylC* gene from *C. crescentus*.[3] To co-express *xylC* and *xdh*, *xylC* was cloned into the pUX plasmid to yield pUXC, in which *xylC* was placed in the downstream of *xdh* as an operon (Figure 2). After cultivation in a shake flask, *xdh* and *xylC* expressions were analyzed by SDS–PAGE, and the expressions of both genes were clearly confirmed (Figure 3B). Next, to measure the production of xylonic acid, cells were cultivated in the same minimal medium. Similar to the earlier cases (pX and pUX), glucose was completely consumed in 10 h, and xylose began to be consumed immediately (Figure 4). However, after 16 h of cultivation, xylose was no longer consumed, and xylonic acid production did not increase: the final concentration of xylonic acid was 5.86 g L^{-1}, and the production yield (0.29 g g^{-1} glucose) was also very low (Figure 4 and Table 3). Therefore, we decided not to employ *xylC*.

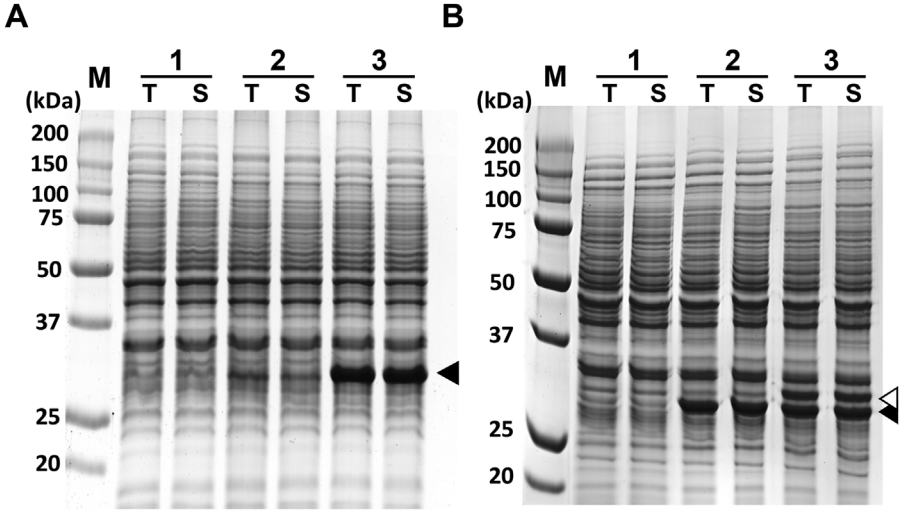

Figure 3. SDS–PAGE analysis for the expression of *xdh* and *xylC* from *C. crescentus* in *C. glutamicum*. A) Engineering of *xdh* expression. Lane 1-3: *C. glutamicum* harboring pCES208, pX, or pUX, respectively. B) Introduction of *xylC*. Lane 1–3: *C. glutamicum* harboring pCES208, pUX, or pUXC, respectively. Black and white arrowheads indicate Xdh (28.5 kDa) and XylC (32.4 kDa), respectively. Lane M represents the molecular weight markers (kDa). Lanes T and S represent total and soluble protein fractions, respectively.

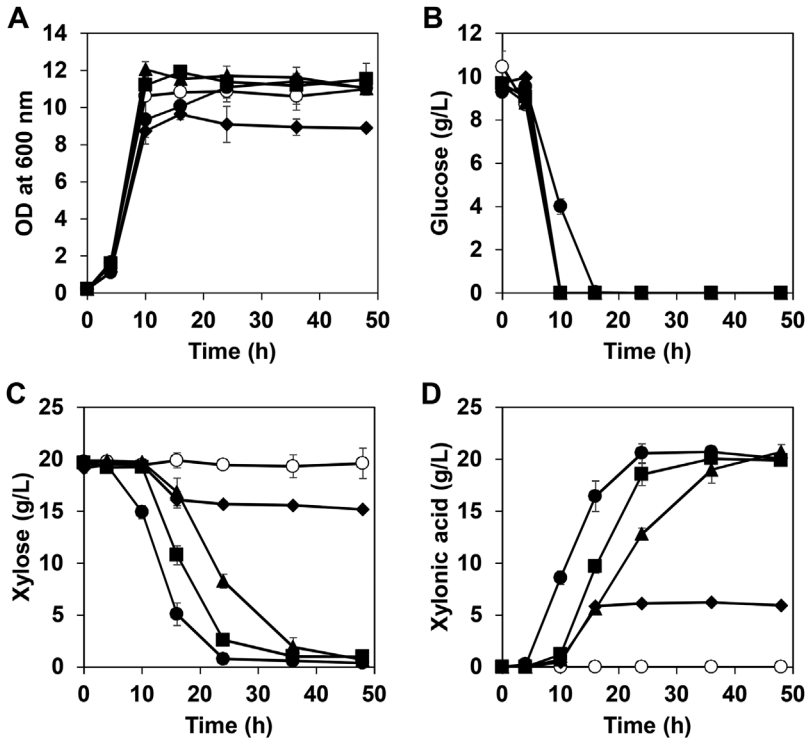

Figure 4. Production of xylonic acid from xylose. Time profiles of A) cell growth in optical density (OD) at 600 nm, B) glucose concentration (g L^{-1}), C) xylose concentration (g L^{-1}), and D) xylonic acid concentration (g L^{-1}). Symbols: open circle (○), *C. glutamicum* harboring pCES208; closed triangle (▲), pX; closed square (■), pUX; closed diamond (◆), pUXC; closed circle (●), pUXE. Results are the mean of duplicate experiments and error bars indicate standard deviations.

3.3. Introduction of the Xylose Transporter for Enhanced Uptake of Xylose

After we confirmed the negative effect of XylC, xylose uptake was chosen as the next target for engineering. Even though wild-type *C. glutamicum* is capable of xylose uptake, it does not have a xylose catabolic pathway. Therefore, this bacterium exhibits a very slow xylose uptake rate, particularly at low concentrations of xylose.[31] This slow xylose uptake can have a critical effect on the overall conversion of xylose into xylonic acid. To address this issue and to increase xylonic acid production, the previously engineered xylose transport module was introduced into the pUX plasmid to yield pUXE, in which the expression of the *E. coli* XylE transporter gene was controlled by a weak synthetic promoter (P_{L10})[21] (Figure 2). Cells harboring pUXE were cultivated in minimal media containing 10 g L^{-1} of glucose and 20 g L^{-1} of xylose, and sugar consumption and xylonic acid production were compared with those of other constructs. Cells producing the XylE transporter exhibited a little slower glucose consumption, but faster consumption of xylose (Figure 4). Production of xylonic acid began much earlier, and the maximum yield (20.71 g L^{-1}) could be achieved much earlier (24 h). Xylonic acid productivity (1.02 g L^{-1} h^{-1}) was also significantly increased relative to that of cell harboring pUX (0.77 g L^{-1} h^{-1}) (Figure 4). In previous work,[21] we examined three different synthetic promoters (low-strength P_{L10}, intermediate-strength P_{I12} and strong P_{H72}) for the expression of XylE transporter, and we found that the expression level of *xylE* gene can give a significant effect on the xylose consumption. In this study, we also examined the same synthetic promoters (P_{L10}, P_{I12}, and P_{H72}) to find the optimal expression level of *xylE* gene, and xylose consumption and xylonic acid productivities were compared. Similar to previous study,[21] it was clearly observed that cells harboring pUXE (weak P_{L10} promoter) consumed xylose more rapid and exhibited 1.4–1.6-fold higher productivities of xylonic acid (1.02 g L^{-1} h^{-1}) compared with those of pUXE-I12 (0.74 g L^{-1} h^{-1}) and pUXE-H72 (0.65 g L^{-1} h^{-1}) (Figure S3, Supporting Information). Taken all above results, we concluded that the use of strong promoter (P_{H36}) for *xdh* gene expression and weak promoter (P_{L10}) for *xylE* gene expression can be the optimal system for the xylonic acid production from xylose and we decided to use this optimized expression system for the following development of consolidated conversion.

Table 3. Summary of xylonic acid production in the engineered *C. glutamicum*.

Plasmid	Xylonic acid concentration (g L^{-1})	Xylonic acid productivity (g L^{-1} h^{-1})	Xylonic acid production yield (g g^{-1} xylose)
pCES208	n.d.	n.d.	n.d.
pX	20.69	0.53	1.03
pUX	20.04	0.77	1.00
pUXC	5.86	0.37	0.29
pUXE	20.71	1.02	1.04

3.4. Consolidated Conversion of Hemicellulose into Xylonic Acid

To further increase the economic feasibility of the xylonic acid production process, we sought to develop a consolidated bioprocessing system for the direct bioconversion of hemicellulosic biomass (xylan) into xylonic acid. Wild-type *C. glutamicum* cells have no xylan hydrolysis activity; therefore, for enzymatic hydrolysis of xylan, two enzymes needed to be introduced: i) endoxylanase, for hydrolysis of xylan to xylooligosaccharides and ii) xylosidase, for further hydrolysis of xylooligosaccharides to xylose. In addition, both enzymes needed to be secreted into the culture medium for the degradation of xylan, which cannot be transported into cells. For this purpose, we introduced an engineered xylan degradation module with endoxylanase (*xlnA*) from *S. coelicolor* A3(2) and xylosidase (*xynB*) from *B. pumilus*.[21] In this construct (pUXED), the both genes were controlled by the *cg1514* native promoter, and two

different signal peptides (Cg1514 and CgR0949) were employed for the secretion of each enzyme, respectively (Figure 2). *C. glutamicum* cells harboring pUXED were cultivated in minimal medium containing 10 g L^{-1} glucose and 20 g L^{-1} xylan (without xylose), and xylonic acid production was analyzed. All examined cells showed similar cell growth (11–13 of OD_{600}) and glucose consumption (depletion of glucose in 12 h) (**Figure 5**A and B). In *C. glutamicum* harboring pUXED, secretory productions of both enzymes (XlnA and XynB) into culture medium were clearly confirmed by SDS-PAGE and Western blot (Figure S1, Supporting Information). Using xylan as a substrate, *C. glutamicum* harboring pUXED successfully produced xylonic acid, with the concentration of xylonic acid increasing to 6.23 g L^{-1} in 48 h, whereas *C. glutamicum* harboring pUXE or pCES208 did not produce any xylonic acid (Figure 5). These data clearly indicate that the xylan in the culture medium was successfully degraded by enzymes produced by *C. glutamicum* harboring pUXED, and the resulting sugar (xylose) was transported into *C. glutamicum* and consequently, converted into xylonic acid.

4. Discussion

Until now, fully purified sugar substrates such as glucose, xylose, or arabinose could mostly be used by microorganisms for sugar acid production.[4,32,33] In this work, we successfully engineered *C. glutamicum* to produce xylonic acid from xylose and also from hemicellulosic biomass, which can be used in a consolidated bioprocess. For the xylonic acid production from xylose, we constructed two modules: i) conversion module from xylose to xylonic acid by expression of *xdh* gene and ii) xylose transport module by expression of *xylE* gene, and in both modules, gene expression levels were optimized through examining three different strength promoters. Using the optimized modules employing the strong P_{H36} promoter for *xdh* gene expression and the weak P_{L10} for *xylE* gene expression, xylonic acid was successfully produced with a maximum yield of xylonic acid from xylose (≈100%) and high productivity (1.02 g L^{-1} h^{-1}). These results of the xylonic acid productivity and yield are quite competitive compared to previous results using other hosts: *E. coli* (1.09 g L^{-1} h^{-1}, 0.98 g g^{-1} xylose), *S. cerevisiae* (0.44 g L^{-1} h^{-1}, 0.8 g g^{-1} xylose), and *K. lactis* (0.16 g L^{-1} h^{-1}, 0.6 g g^{-1} xylose).[2,4,5] As we mentioned in the introduction section, *C. glutamicum* does not have a xylose catabolic pathway, so all of the substrate could be converted to xylonic acid, and the maximum conversion yield (100%) could be achieved without further engineering of cells. When xylose was supplied as a substrate, cells harboring pUXE showed a delayed consumption of glucose (Figure 4B). We do not assume the delayed consumption was caused by a cell burden because cell grew well as other strains (Figure 4A). Still, we do not know the reason for this phenomenon, but as one possible reason, we consider the introduction of transporter (XylE). By introducing XylE transporter, the consumption of xylose was significantly improved compared with other strains (Figure 4C), but the rapid influx of xylose into the cell might have caused the delayed consumption of glucose due to the simultaneous transport of xylose and glucose. In contrast, when xylan was used as a substrate instead of xylose, same cell (pUXE) did not show any delayed consumption of glucose (Figure 5B). In this experiment, xylose could be supplied after degradation of xylan, so the xylose supplementation was limited compared with the earlier cultivation using xylose substrate (Figure 4). The relatively slow influx of xylose might not cause the delayed consumption of glucose. We also tried additional expression of xylonolactonase from *C. cresentus* but *C. glutamicum* suddenly stopped consuming xylose to produce xylonic acid with the gene. In yeast, a strong loss of vitality in xylonolactonase-expressing cells was reported.[2] In that report, *S. cerevisiae* expressing both xylose dehydrogenase and xylonolactonase genes from *C. crescentus* initially produced more xylonic acid at an early phase of cultivation than cells expressing

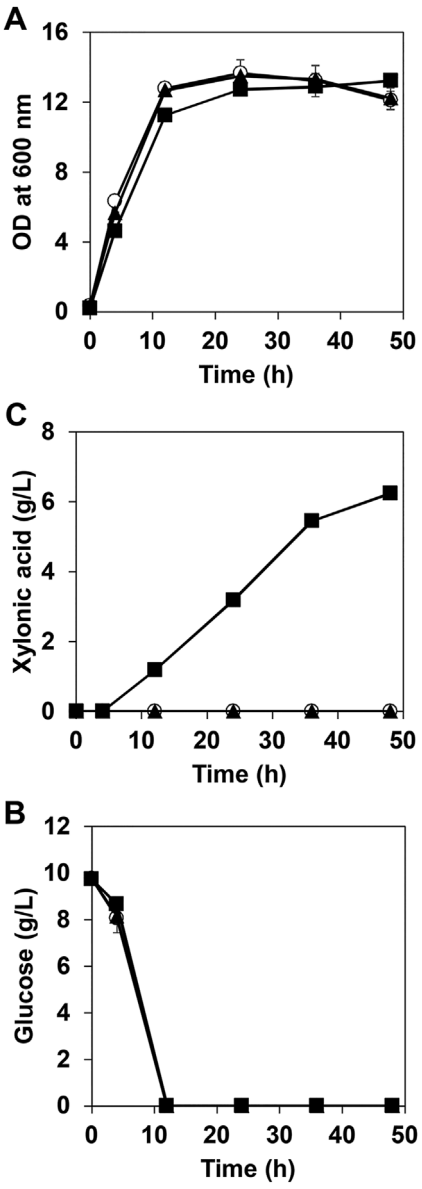

Figure 5. Production of xylonic acid from xylan. Time profiles of A) cell growth in optical density (OD) at 600 nm, B) glucose concentration (g L^{-1}), and C) xylonic acid concentration (g L^{-1}). Symbols: open circle (○), *C. glutamicum* harboring pCES208; closed triangle (▲), pUXE; closed square (■), pUXED. Results are the mean of duplicate experiments and error bars indicate standard deviations.

only the xylose dehydrogenase gene, but the percentage of metabolically active cells decreased much faster in the cells with both genes, resulting in less xylonic acid production. This suggests that xylonic acid may be more toxic than xylonolactone, and gradual hydrolysis of intracellular lactone into the acid may be advantageous to *C. glutamicum* cells.

By employing a xylan degradation module (pUXED), consolidated bioconversion was demonstrated and xylonic acid concentrations as high as 6.23 g L^{-1} were successfully produced from 20 g L^{-1} of xylan. Compared to the conversion of xylose to xylonic acid, the lower efficiency of consolidated conversion from xylan might be due to the incomplete degradation of xylan to xylose. Since optimal temperature of *C. glutamicum* is near 30 °C and optimal temperatures for the xylan hydrolases are higher than 40 °C, it is not easy to degrade biomass efficiently with *C. glutamicum*. By employing a more active enzyme at lower temperature, and by further optimizing culture conditions, the efficiency and productivity of the degradation can be improved, and a more cost-effective consolidated bioconversion system can be developed at industrial-scale. The final construct, pUXED has relatively long size (≈12 kb) which may cause the metabolic burden in the hosts, but we could not find any impaired cell growth during the cultivation (Figure 5). In addition to the plasmid size, several other factors including the gene sequence, length of transcript (mRNA), and copy number can be considered. In pUXED, the plasmid copy number is relatively low (approx. 5–10 copies), and each expression system was optimized with constitutive promoters (P_{H36}, P_{L10}, and P_{Cg1514}) to prevent the overexpression of each gene, which may give much less metabolic loads on the host cell. In addition to the constitutive promoters, the use of inducible promoters (P_{trc}, P_{lac}, etc.) can be considered for the tight control of gene expression in *C. glutamicum*. However, the inducible promoters require an expensive inducer such as IPTG and laborious process for optimization of induction, and the regulation of gene expression is not controlled as tight as in *E. coli* due to the low permeability of IPTG into *C. glutamicum*.[34] Instead of IPTG-inducible promoter, auto-inducible promoters which can tightly regulate gene expression at specific cell growth phase without inducer,[35] would also be useful in the industrial-scale process.

To the best of our knowledge, this is the first report on xylonic acid production in *C. glutamicum*, as well as the first report on the production of xylonic acid from xylan by a consolidated bioprocess. Although we did not confirm the applicability to the real-world xylan substrate, we believe this robust *C. glutamicum* system could be a starting point to develop an industrially relevant platform for production of xylonic acid from raw biomass.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

consolidated bioprocess, *Corynebacterium glutamicum*, hemicellulose, xylonic acid

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