ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

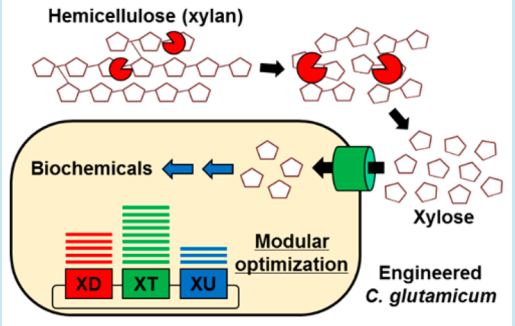
Modular Optimization of a Hemicellulose-Utilizing Pathway in *Corynebacterium glutamicum* for Consolidated Bioprocessing of Hemicellulosic Biomass

Sung Sun Yim,[†] Jae Woong Choi,[†] Se Hwa Lee,[†] and Ki Jun Jeong*[,†,‡]

[†]Department of Chemical and Biomolecular Engineering, BK21 Plus Program, [‡]Institute for the BioCentury, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

Supporting Information

ABSTRACT: Hemicellulose, which is the second most abundant polysaccharide in nature after cellulose, has the potential to become a major feedstock for microbial fermentation to produce various biofuels and chemicals. To utilize hemicellulose economically, it is necessary to develop a consolidated bioprocess (CBP), in which all processes from biomass degradation to the production of target products occur in a single bioreactor. Here, we report a modularly engineered *Corynebacterium glutamicum* strain suitable for CBP using hemicellulosic biomass (xylan) as a feedstock. The hemicellulose-utilizing pathway was divided into three distinct modules, and each module was separately optimized. In the module for xylose utilization, the expression level of the xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes was optimized with synthetic promoters of different strengths. Then, the module for xylose transport was engineered with combinatorial sets of synthetic promoters and heterologous transporters to achieve the fastest cell growth rate on xylose (0.372 h^{-1}). Next, the module for the enzymatic degradation of xylan to xylose was also engineered with different combinations of promoters and signal peptides to efficiently secrete both endoxylanase and xylosidase into the extracellular medium. Finally, each optimized module was integrated into a single plasmid to construct a highly efficient xylan-utilizing pathway. Subsequently, the direct production of lysine from xylan was successfully demonstrated with the engineered pathway. To the best of our knowledge, this is the first report of the development of a consolidated bioprocessing *C. glutamicum* strain for hemicellulosic biomass.



KEYWORDS: *Corynebacterium glutamicum, hemicellulose, consolidated bioprocess, synthetic promoter, modular engineering*

Lignocellulosic biomass, such as wood and agricultural crops, is the most abundant renewable feedstock in the biosphere, and conversion of abundant lignocellulosic biomass into fuels and various chemicals by microbial fermentation presents many important advantages, including energy security, price stability, reduced greenhouse gas emissions, etc. Among the components of lignocellulosic biomass, utilization of hemicellulose is worth investigating because it comprises more than 30% of lignocellulosic biomass and can be acquired as agricultural or industrial waste at low cost from nonedible residues of agricultural crops, paper pulp industries, etc.[1−3] Current industrial bioprocesses using hemicellulose as well as other lignocellulosic biomass as a feedstock normally consist of three distinct process stages: (i) production of enzymes for the hydrolysis of biomass, (ii) enzymatic hydrolysis of the biomass into fermentable monosaccharides, and (iii) fermentative production of valuable biochemicals or biofuels.[4,5] However, these separate stages of the bioprocess require substantial capital and time, thus preventing the industrial bioprocessing of lignocellulose from being economically feasible.

As an attractive solution to the disadvantages of conventional separated multiple-stage bioprocesses, a consolidated bioprocess (CBP), which combines all three processes into a single bioreactor, has been considered due to its potential for decreasing the costs associated with multiple unit operations and exogenous enzyme supplementation.[4] For this ideal bioprocess, a single strain has to possess all of the characteristics required for efficient bioconversion of lignocellulosic biomass into biochemicals. For this, two strategies can be considered when developing consolidated bioprocessing microorganisms: (i) engineer cellulolytic microorganisms to have biochemical production abilities or (ii) engineer noncellulolytic microorganisms to have biomass utilization abilities. Even though cellulolytic microorganisms (e.g., *Clostridium thermocellum*, *Clostridium cellulovorans*, *Thermobifida fusca*, etc.) have great potential to become a major consolidated bioprocessing platform, they are often hard to engineer due to a lack of background information and genetic tools.[6−8] Engineering noncellulolytic industrial microorganisms to provide them with the abilities to degrade and utilize biomass is more practicable because their genetic and metabolic backgrounds and engineering tools are well-established. Therefore, several noncellulolytic

Received: November 8, 2015
Published: January 25, 2016

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

microorganisms, mostly *Escherichia coli* and yeasts, have been engineered to perform consolidated bioprocessing of lignocellulosic biomass so far.[9−12]

Corynebacterium glutamicum, an industrial workhorse that has a broad product spectrum including various amino acids, biochemicals (diamines, diols, polymers, etc.), biofuels (ethanol, butanol, etc.), and recombinant proteins, can be considered a promising host for CBP because it possesses several ideal intrinsic attributes.[13−16] First, *C. glutamicum* is already an industrial workhorse, which means that it is very strong at producing various biochemicals on an industrial scale. Next, under oxygen-limited or growth-arrested conditions, *C. glutamicum* exhibits a significant resistance against inevitable fermentation-inhibiting byproducts from biomass pretreatments, such as organic acids, furans, and phenols, such that productivity can be retained up to 100% in the presence of these inhibitors.[17] Finally and most important, *C. glutamicum* can secrete a large amount of recombinant protein into the extracellular environment and can keep the secreted protein stable for long periods of cultivation due to its hardly detectable extracellular proteolytic activity.[16,18,19] This unique protein secretion characteristic has led to this microorganism becoming an industrial protein manufacturer (Corynex of Ajinomoto Co. in Japan), and it means that the protein secretion ability of *C. glutamicum* can also be applied to highly efficient biomass degradation by high-level secretion of hydrolases. On the basis of these characteristics, *C. glutamicum* has been engineered to utilize various lignocellulosic sugars, such as cellobiose and xylose, and also to produce various biochemicals from hydrolysates of biomass (rice straw, wheat brans, molasses, or xylan).[20−24] Recently, consolidated bioprocessing strains of *C. glutamicum* have also been reported that utilize corn starch and microalgal starch by simply expressing α-amylase to produce various biochemicals.[25−28] However, there have been no reports on a consolidated bioprocessing *C. glutamicum* strain utilizing hemicellulosic biomass yet, which requires strain engineering to introduce several heterologous enzymes and transporters for efficient xylose utilization and multiple hydrolase secretion systems for hemicellulose degradation into a single *C. glutamicum* strain.

Here, we report the engineering of *C. glutamicum* strains capable of consolidated bioprocessing of hemicellulose. For hemicellulose utilization, the entire pathway was designed using a total of five heterologous enzyme and transporter genes for *C. glutamicum*, which cannot utilize even xylose monomer as a carbon substrate (Figure 1). The synthetic pathway was then divided into three modules with distinct functions: (i) a xylose utilization (XU) module for the assimilation of xylose, (ii) a xylose transport (XT) module for xylose uptake from the culture medium, and (iii) a xylan degradation (XD) module for enzymatic degradation of xylan to xylose in the extracellular medium (Figure 1). To achieve the best performance for each module by fine-tuning of the gene expression, we examined various synthetic promoters and signal peptides.[29−31] Finally, each optimized module was integrated into a single plasmid to construct an efficient xylan-utilizing pathway in *C. glutamicum*. Subsequently, CBP for the production of a biochemical (lysine) from xylan as a feedstock was successfully demonstrated.

RESULTS

Optimization of Xylose Utilization (XU) Module. Because it lacks the *xylA* gene encoding xylose isomerase, the wild-type *C. glutamicum* strain cannot utilize xylose as a carbon

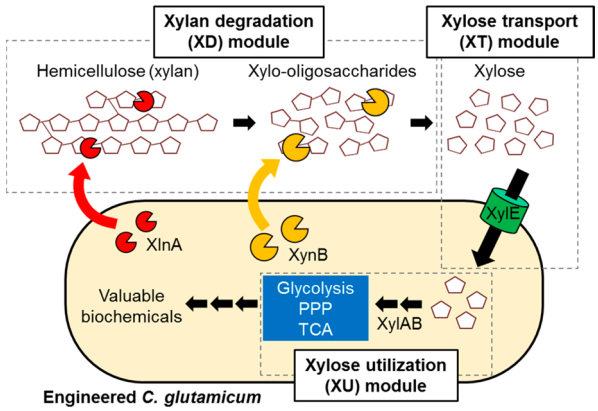


Figure 1. Schematic diagram of *C. glutamicum* engineering for the consolidated bioprocessing of hemicellulose (xylan). A xylan-utilizing pathway was divided into the three different modules: a xylan degradation (XD) module, a xylose transport (XT) module, and a xylose utilization (XU) module. XlnA, endoxylanase; XynB, xylosidase; XylE, xylose transporter; XylA, xylose isomerase; XylB, xylulokinase; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle.

source for cell growth.[32] To construct the xylose utilization (XU) module in *C. glutamicum*, the xylose isomerase pathway, composed of xylose isomerase (XylA) and xylulokinase (XylB), was chosen since the pathway does not require cofactors, such as NADPH and NAD^+, which are required and can result in cofactor imbalance in an oxidoreductase-based xylose metabolic pathway.[33] There have been several reports of the heterologous *xylA* and *xylB* genes being introduced into *C. glutamicum* to achieve xylose assimilation.[21,23,34] *E. coli xylA* and *xylB* were previously reported to be less efficient in terms of the cell growth rate than *xylA* from *X. campestris* and *xylB* from *C. glutamicum*.[23] However, in this study, we chose *E. coli xylA* and *xylB* for engineering the XU module because they have higher specific enzymatic activities and therefore greater potential when xylose uptake is further improved with a xylose transport system. When introducing these genes, it is important to optimize their expression because an expression level that is too low would not be sufficient for optimal cell growth, whereas one that is too high would waste too many cellular resources just to produce the enzymes. Therefore, we utilized synthetic promoters of different strengths, which we previously isolated by FACS screening a synthetic promoter library in *C. glutamicum*.[30] Among 20 isolated promoters, three different synthetic promoters (L26, I16, and H30) that have different strengths, low (L26), intermediate (I16), and high (H30), respectively, were chosen for the expression of the *E. coli xylA* and *xylB* genes in an operon (pXU1, pXU2, and pXU3 in Supporting Information, Figure S1). After cultivation in BHI complex medium for 48 h, the production level of each enzyme was analyzed. As shown in Figure 2A, the expression level of the *xylA* and *xylB* genes under the three synthetic promoters was highly correlated with the strength of the promoter. Among the three promoters, the strongest promoter (H30) exhibited a higher expression level of both genes compared to that of the intermediate strength promoter (I16), which also exhibited a relatively higher expression level compared to that of the low strength promoter (L26).

Next, the cells were cultivated in minimal medium containing 10 g/L of xylose as the sole carbon source, and cell growth rates were compared. Cells harboring pXU1 with the low strength promoter (L26) exhibited a relatively lower cell growth rate

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

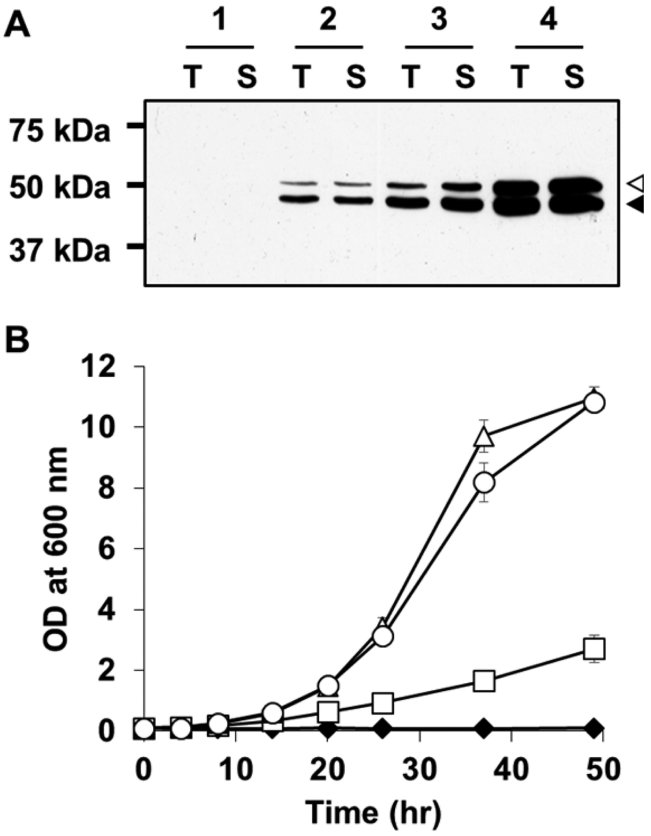


Figure 2. Optimization of the XU module. (A) Western blot analysis for the expression of the *xylA* and *xylB* genes under three different synthetic promoters. After cultivation, cell concentrations were normalized to an OD_{600nm} of 4, and the same volume (8 μL) of cell lysate was loaded in each lane. Lanes 1−4: *C. glutamicum* harboring pCES208, pXU1, pXU2, or pXU3, respectively. Black and white arrowheads indicate XylA (50.7 kDa) and XylB (53.6 kDa), respectively. T and S represent total and soluble lysates, respectively. (B) Time profiles of cell growth in minimal media containing xylose (10 g/L) as the sole carbon source. Symbols: ♦, *C. glutamicum* harboring pCES208; □, pXU1; △, pXU2; ○, pXU3. All error bars represent standard deviations of triplicate experiments.

(specific growth rate, μ = 0.099 ± 0.005 h^{-1}) compared to those of the other clones harboring pXU2 and pXU3 with the intermediate strength promoter (I16) and high strength promoter (H30), respectively (Figure 2B). Additionally, the cells harboring pXU2 exhibited slightly faster cell growth (specific growth rate, μ = 0.142 ± 0.008 h^{-1}) than that of the cells harboring pXU3 (μ = 0.138 ± 0.005 h^{-1}). From these results, we decided to use the intermediate strength synthetic promoter (I16) for the optimal expression of the *xylA* and *xylB* genes in the XU module.

Optimization of Xylose Transport (XT) Module. To further increase the cell growth rate on xylose, engineering a xylose uptake system can be a crucial step. Even though *C. glutamicum* has an unidentified transporter (IM_{xyl}) that can mediate xylose uptake, it has been reported to exhibit a very poor xylose consumption rate at low concentrations of xylose,[24,32,35] and this slow uptake of xylose at low concentrations can significantly affect the cell growth rate on xylan in CBP. To address this issue, we decided to introduce a xylose transport (XT) module in addition to the optimized XU module (pXU2). For heterologous transporters, AraE from *C. glutamicum* ATCC 31831 and *E. coli* K-12 (MG1655) were chosen first because the AraE transporters were previously reported to improve the uptake of pentoses (xylose and arabinose) in *C. glutamicum*.[24,36,37] In addition, we also examined XylE from *E. coli* MG1655 for the first time in *C. glutamicum*. The overexpression of a membrane protein in bacterial hosts can often negatively affect cell viability and growth rate,[37,38] so it is necessary to find the proper level of gene expression. Similar to the engineering of the XU module, we sought to optimize the expression of the XT module with three synthetic promoters (L10, I12, and H72) of different strengths. Therefore, a total of nine combinatorial XT modules composed of three synthetic promoters and three heterologous transporters were constructed, and each combination was added to the previously optimized XU module (pXU2), yielding the pXU2T series of cells (Supporting Information, Figure S1). Cells containing the XU and XT modules were cultivated in minimal media containing a low concentration of xylose (3.6 g/L), and the growth rate of each strain was monitored. In the case of AraE from *C. glutamicum* ATCC 31831, the cell growth rates increased as the promoter strength increased; however, the use of XylE from *E. coli* had the opposite result: the cell growth rates were inversely correlated with the strength of the promoter used (Figure 3A and Supporting Information Figure S2). In the case of AraE from *E. coli*, the highest growth rate was obtained with the intermediate strength promoter (I12) (Figure 3A and Supporting Information Figure S2). Among the nine combinatorial sets of transporter modules, cells harboring pXU2T7, in which the XylE transporter from *E. coli* was expressed with the low strength synthetic promoter (L10), had the highest cell growth rate for a low concentration of xylose (3.6 g/L). The growth rate of cells harboring pXU2T7 was further examined with two other controls, *C. glutamicum* (pXU2) with only the optimized XU module without the XT module and *C. glutamicum* (pCES208), in flask cultivation with both high (20 g/L) and low (3.6 g/L) concentrations of xylose. The specific growth rate of the cells with the XylE transporter (pXU2T7) reached 0.372 h^{-1}, which was the highest recorded cell growth rate on xylose in *C. glutamicum*. Moreover, it was 2.62-fold higher than that of pXU2 without the XylE transporter. The xylose concentration profiles also support this result. When the xylose transporter was introduced (pXU2T7), the cells exhibited a significant increase in the xylose consumption rate compared to that of the other controls for both the high and low concentrations of xylose (Figure 3C).

Optimization of Xylan Degradation Module. The efficient hydrolysis of hemicellulose into fermentable sugars (mainly xylose) in the extracellular medium is the most important ability for a consolidated bioprocessing microorganism. For the enzymatic degradation of xylan into xylose, we chose the following two enzymes which can be well-expressed in *C. glutamicum*: (i) endoxylanase (XlnA) from *Streptomyces coelicolor* A3(2), which was abbreviated XynA in our previous report,[29] for the cleavage of the β-1,4-glycosidic bond in xylan to yield xylose and xylooligosaccharides and (ii) xylosidase (XynB) from *Bacillus pumilus*, which can mediate further hydrolysis of xylooligosaccharides to xylose.[29,39] For the engineering of the XD module with the two enzymes (XlnA and XynB), we tried to study the effect of xylosidase mainly because we already had some experience in high-level secretory production of XlnA.[29−31] For this study, therefore, we examined two issues, (i) localization of XynB (cytoplasm or extracellular medium) and (ii) secretory pathway for secretion

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

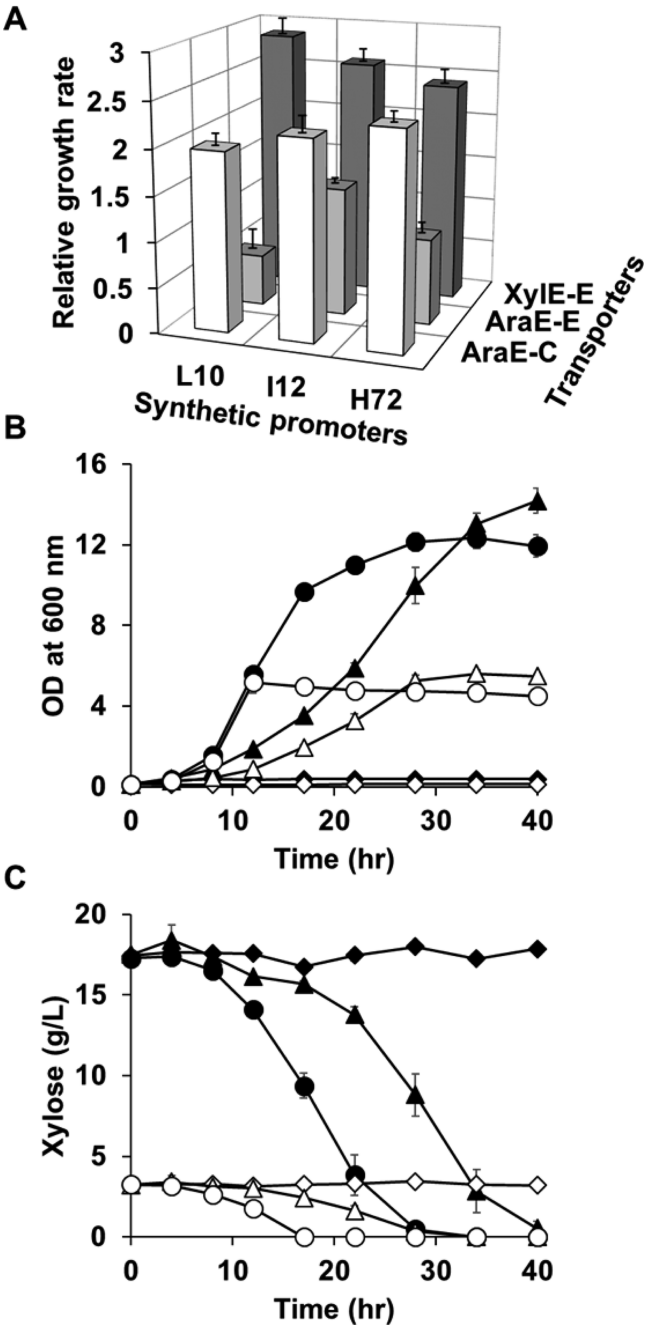


Figure 3. Optimization of the XT module. (A) Relative growth rate (fold change of cell growth rate compared to that of *C. glutamicum* harboring pXU2 in minimal media containing 3.6 g/L of xylose). AraE-C, AraE from *C. glutamicum* ATCC 31831; AraE-E, AraE from *E. coli* MG1655; XylE-E, XylE from *E. coli* MG1655. Bars are average value from triplicate experiments. (B) Time profiles of cell growth. (C) Sugar consumption profiles of *C. glutamicum* cells. Symbols: ◇, *C. glutamicum* harboring pCES208; Δ, pXU2; ○, pXU2T7. Open and closed symbols represent the cultivation in 3.6 and 20 g/L of xylose, respectively. All error bars represent standard deviations of triplicate experiments.

of XynB, while using a secretory expression system for XlnA that was fixed with the PorB signal peptide. Regarding the first issue (localization of XynB), we constructed pXD2 for the cytoplasmic production of XynB. Then, *C. glutamicum* cells harboring each pXD plasmid were cultivated in minimal media containing 10 g/L glucose and 10 g/L xylan at 30 °C. In this experiment, we used wild-type *C. glutamicum*, which cannot utilize xylose as a carbon source, and no genes related to xylose utilization and transport were included in the pXD plasmids. Therefore, enzymatic hydrolysis products (xylose and xylooligosaccharides) from xylan were expected to be present in the extracellular media, and the xylan degradation efficiencies by the XD modules could be clearly determined by measuring the concentration of xylose in the culture media. In all cultivations, cells grew well and showed similar growth patterns independent of xylan degradation (Supporting Information, Figure S3A). When the culture medium was analyzed to check the efficiency of xylan hydrolysis in each pXD construct, as shown in Figure 4A, the cytoplasmic expression of XynB

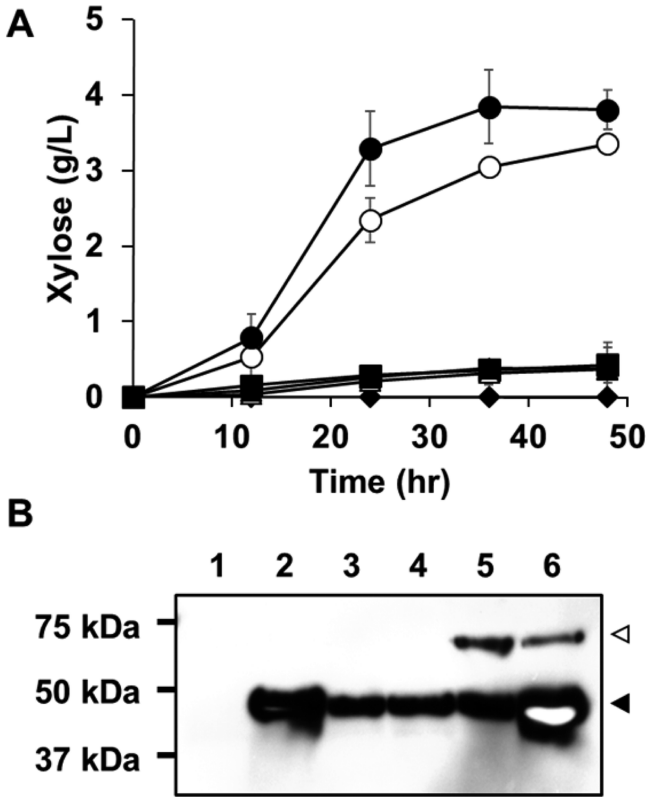


Figure 4. Optimization of the XD module. *C. glutamicum* cells with xylan degradation modules (without xylose utilization ability) were cultivated in minimal media containing 10 g/L of glucose and 10 g/L of xylan. (A) Time profiles of xylose concentration. Symbols: ♦, *C. glutamicum* harboring pCES208; ■, pXD1; Δ, pXD2; ▲, pXD3; ○, pXD4; ●, pXD5. All error bars represent standard deviations of triplicate experiments. (B) Western blot analysis for the secretion of XlnA and XynB into culture supernatant. Lanes 1−6: *C. glutamicum* harboring pCES208, pXD1, pXD2, pXD3, pXD4, or pXD5, respectively. Black and white arrowheads indicate XlnA (48 kDa) and XynB (62.2 kDa), respectively.

(pXD1) did not reveal any difference compared to that without coexpression of XynB (pXD1). This result indicated that *C. glutamicum* cannot uptake xylooligosaccharide into the cell. Therefore, we next put further effort into secreting XynB into the extracellular medium by examining two secretory pathways (the PorB signal peptide[29] for the Sec-dependent pathway (pXD3) and the CgR0949 signal peptide[40,41] for the TAT-dependent pathway (pXD4)) for XynB secretion. Between these two systems, higher degradation was achieved in pXD4 in which XynB was secreted via the TAT-dependent CgR0949

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

signal peptide (Figure 4A). Finally, to further improve the secretion efficiency of XlnA, we exchanged the PorB signal peptide, which was used for the secretion of XlnA in pXD1−4, with the more efficient Sec-dependent signal peptide (Cg1514), which was recently isolated by our group (pXD5).[31] As a result, more xylose could be liberated from xylan by pXD5 than by pXD4 (Figure 4A). In the cultivation of the cells harboring pXD5, approximately 40% of the xylan was hydrolyzed into xylose (3.84 g/L) without xylobiose and xylotriose in the extracellular medium after 36 h of cultivation (Supporting Information, Figure S3). On the other hand, cells harboring pXD1, pXD2, or pXD3, in which XlnA was produced and XynB was not produced or secreted into the medium, hydrolyzed just a small amount of xylan fully into xylose (<0.4 g/L), and most of the enzymatic hydrolysis products were xylobiose and xylotriose (Supporting Information, Figure S3). Western blot analysis was also conducted to analyze the secretory production of XlnA and XynB into the extracellular medium, and it was clearly confirmed that XynB could be secreted only by pXD4 and pXD5 with the CgR0949 signal peptide and that the level of XlnA could be increased by the Cg1514 signal peptide in pXD5 compared to that with other pXD modules (Figure 4B). The enzymatic activities of XlnA and XynB in the culture supernatant were also analyzed, and the results coincided well with the Western blot data. As described above, we could optimize the XD module by testing only five constructs out of 18 possible combinations of two promoters and three kinds of signal peptides for each enzyme. From these results, we decided to use the XD5 module containing the *cg1514* promoter, Cg1514 signal peptide for XlnA secretion, and CgR0949 signal peptide for XynB secretion.

Integration of All Optimized Modules for Xylan Utilization as the Sole Carbon Source. After optimizing all of the modules, XU, XT, and XD, separately, each optimized module (expression systems for *xylA*, *xylB*, and *xylE* in pXU2T7 and for *xlnA* and *xynB* in pXD5) was integrated into a single plasmid to develop a xylan utilization system in a single strain. Two versions of the xylan utilization system were constructed: (i) pHCBP275, which contains all of the optimized modules, and (ii) pHCBP205, which contains the same optimized modules except for the XT module. Then, *C. glutamicum* was cultivated in minimal media containing xylan (20 g/L) as the sole carbon source. *C. glutamicum* harboring pHCBP275 exhibited faster cell growth on xylan than that of cells harboring pHCBP205, and *C. glutamicum* harboring pXU2T7 (without the XD module) or pCES208 did not grow on xylan (Figure 5A). Profiles of the total residual sugar concentration were also evaluated, and *C. glutamicum* harboring pHCBP275 exhibited faster sugar consumption than that of *C. glutamicum* harboring pHCBP205, whereas *C. glutamicum* harboring pXU2T7 or pCES208 did not utilize xylan as a sugar substrate (Figure 5B). After 48 h of cultivation in xylan containing minimal media, *C. glutamicum* harboring pHCBP275 utilized 58.7% of the xylan in the culture media. The hydrolysis efficiency of the pHCBP275 was much higher than that of the pXD5 alone (~40% in pXD5) (Figure 4A), even though the enzymatic activities of XlnA and XynB in the culture supernatant were slightly lower in the pHCBP275 than in the pXD5 (Table 1). This may be explained by reduced end-product inhibition of XlnA and XynB in pHCB275.[42] In the cultivation of *C. glutamicum* harboring pHCBP275, the xylose produced from xylan hydrolysis could be simultaneously consumed by the host, so xylose can be maintained at a low enough concentration that it does not inhibit the activities of both enzymes, which can also be one of the benefits of CBP.

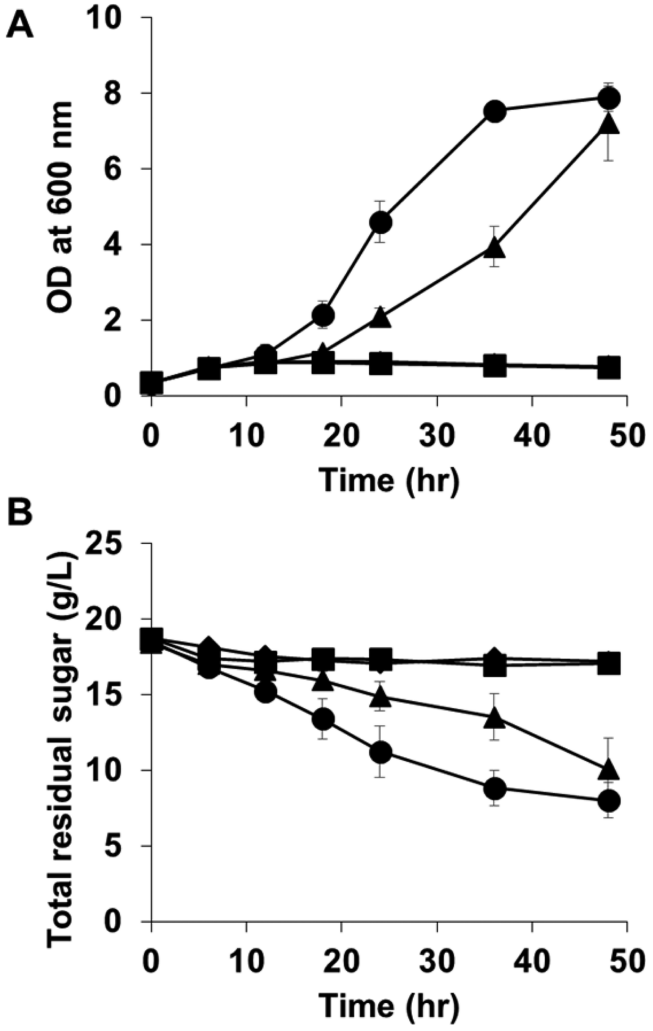


Figure 5. Assembling all of the optimized modules for the xylan-utilizing pathway. (A) Time profiles of cell growth in minimal media containing 20 g/L xylan as the sole carbon source. (B) Time profiles of total residual sugar concentration during the cultivations. Symbols: ♦, *C. glutamicum* harboring pCES208; ■, pXU2T7; ▲, pHCBP205; ●, pHCBP275. All error bars represent standard deviations of triplicate experiments.

Demonstration of a Consolidated Bioprocess Using Hemicellulose as Feedstock. To demonstrate the production of value-added biochemicals from hemicellulose (xylan), the optimized xylan utilization system (pHCBP275) was used for the production of lysine from xylan. For the purpose of producing lysine directly from xylan, pHCBP275 was introduced into the lysine-producing *C. glutamicum* strain KCTC 1857.[43,44] *C. glutamicum* KCTC 1857 harboring pHCBP275 was then cultivated in a semi-defined medium containing 0.5 g/L of yeast extract and 2% xylan as the carbon source. Since *C. glutamicum* KCTC 1857 cannot grow without additional homoserine and leucine,[43] we used a semi-defined medium that contained 0.5 g/L of yeast extract in the xylan containing minimal media. *C. glutamicum* KCTC 1857 harboring pHCBP275 grew well with xylan and produced lysine as high as 1.12 g/L. In contrast, *C. glutamicum* KCTC 1857 harboring pCES208 (no xylan utilization modules) did not continue to grow and produce lysine (Figure 6A). Profiles

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

Table 1. Activities of Secreted Endoxylanase (XlnA) and Xylosidase (XynB) in the Culture of *C. glutamicum* ATCC 13032 Harboring the pXD and pHCBP Series

plasmid	pXD1	pXD2	pXD3	pXD4	pXD5	pHCBP205	pHCBP275
XlnA activity (U/L)	390.22	184.26	172.67	197.04	410.13	383.38	318.89
XynB activity (U/L)	n.d.[a]	n.d.	n.d.	8.44	10.54	5.93	3.72

[a]n.d., not detectable.

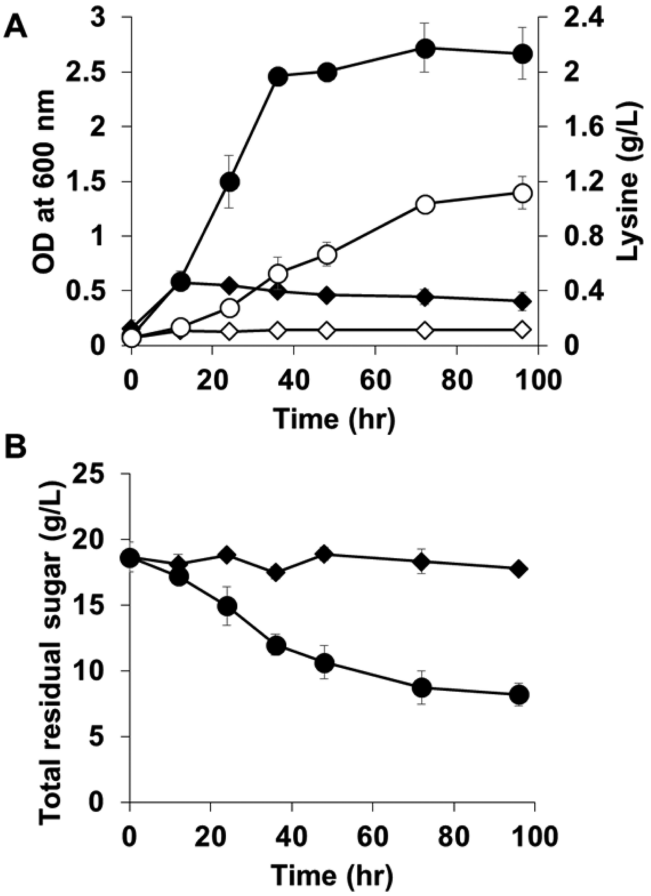


Figure 6. Direct production of lysine from xylan. Cells were cultivated in semi-defined media containing 0.5 g/L of yeast extract and 20 g/L of xylan. (A) Time profiles of cell (*C. glutamicum* KCTC 1857) growth and lysine production. Symbols: ♦, cell growth of pCES208, ●, cell growth of pHCBP275; ◇, lysine conc. of pCES208; ○, lysine conc. of pHCBP275. (B) Time profiles of total residual sugar concentration during the cultivations. Symbols: ♦, pCES208; ●, pHCBP275.

of the total residual sugar concentration were also evaluated, and *C. glutamicum* KCTC 1857 harboring pHCBP275 showed xylan consumption, whereas *C. glutamicum* KCTC 1857 harboring pCES208 did not utilize xylan as a sugar substrate (Figure 6B). Compared with glucose as the carbon source, it is well-known that the use of xylose as the sole carbon source can strongly affect intracellular metabolite pools and product formation performance.[21,34] Therefore, further metabolic engineering for carbon flux redirection would improve the lysine production yield from xylan.

DISCUSSION

Biobased processes for the production of value-added biochemicals from renewable biomass are an attractive alternative to traditional petrochemical processes. A consolidated bioprocess (CBP) featuring the production of biomass-degrading enzymes, enzymatic hydrolysis of biomass to utilizable sugars, and microbial fermentation in one reactor has been suggested as an ideal process, with outstanding potential for lower costs and higher efficiency than those of conventional multireactor processes.[4] To realize this potential, it is necessary to engineer a CBP-enabled microorganism that can utilize biomass as a carbon source. Engineering this type of strain requires the expression of multiple genes in a single host, and the expression level of all of the constructed genes used should be optimized toward higher performance in cell growth and the production of the desired products. In this study, we successfully developed a CBP-enabled *C. glutamicum* strain using hemicellulose (xylan) as the sole carbon source for the production of value-added biochemicals. In the engineering of this strain, we sought not to only introduce a total of five heterologous genes for three distinct modules (XD, XT, and XU) but also to optimize the gene expression level in each module. To fine-tune the gene expression, we used synthetic promoters of different strengths that were previously developed by our group.[30] The potential of this modular engineering strategy with synthetic promoters for fine-tuning gene expression was demonstrated by achieving the fastest cell growth rate (0.37 h^{-1}) on xylose, which is even comparable to the cell growth rate on glucose (~0.4 h^{-1}) (Figure 3). As shown here, the use of a stronger promoter could result in higher levels of gene expression, but better performance of the cells was not always guaranteed. In the optimization of the XU module, the use of the intermediate strength promoter (I16) resulted in a similar cell growth rate as that with the high strength promoter (H30) (Figure 2). Furthermore, in the optimization of the XT module, the use of the lower strength promoter (L10) for XylE transporter expression resulted in a much higher cell growth rate than those of the other stronger promoters (I12 and H72) (Figure 3).

Through the ideal combination of promoter strength and signal peptide, the xylan degradation module (XD) was also optimized to hydrolyze approximately 40% of the xylan present in the culture medium fully into xylose without any xylobiose or xylotriose present (Figure 4). For the secretory production of recombinant proteins in *C. glutamicum*, two secretory pathways (Sec- and TAT-dependent pathways) have mainly been used, and it is well-known that the secretion efficiency of target proteins is highly dependent on the secretion pathway.[31] In this study, we examined three different signal peptides, PorB, Cg1514, and CgR0949, which mediate Sec-dependent (PorB and Cg1514) and Tat-dependent (CgR0949) secretion. As shown in Figure 4, XynB was secreted well into the medium with the Tat-dependent CgR0949 signal peptide (pXD4 and pXD5), whereas XynB was not detected in the culture medium when the Sec-dependent PorB signal was used (pXD3). Generally, the Tat-pathway is useful for the secretion of folded proteins rather than unfolded proteins and, thus, the Tat-dependent pathway could be a preferable pathway for many cytosolic proteins, which are easily folded in the cytoplasm. A XynB (β-xylosidase) used in this study was from *B. pumilus*, and

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

it is also known that XynB is a cytosolic protein in that host.[45] In contrast, XlnA (endoxylanse) from *S. coelicolor* A3(2) is natively a secretory protein that is folded after secretion into the culture medium,[31] so XlnA was secreted well into the medium through the Sec-dependent pathway with the PorB and Cg1514 signal peptides.

Even though our effort in the construction of an efficient hemicellulose-utilizing pathway in *C. glutamicum* was able to significantly improve the performance of the entire heterologous pathway for efficient cell growth on xylan as the sole carbon source, there is still room to improve the efficiency of hemicellulose utilization in the industrial process. Because only 58.7% of the xylan present in the culture supernatant was converted to xylose and the cell growth rate on xylan was slower than that on xylose, further metabolic engineering targets for improved hemicellulose utilization might aim at enhancing the xylan degradation module. First, there is a problem with the optimal temperature for the endoxylanase and xylosidase used in this study. Optimal temperatures for these hydrolases are higher than 40 °C.[46] That high of a temperature, however, is not appropriate for the cultivation of *C. glutamicum* because its optimal growth is at 30 °C. Thus, more suitable endoxylanases and xylosidases with optimal enzymatic activities near 30 °C need to be explored, or we can consider the use of a *Corynebacterium efficiens* strain, which can grow above 40 °C.[47] Also, we can consider the transport of xylooligosaccharides as well as xylose for more rapid supplementation of the carbon source into the host. Recently, a xyloside ABC transporter of *Corynebacterium alkanolyticum* was functionally characterized in *C. glutamicum* for the uptake of xylooligosaccharides,[48] and this kind of transporter might be useful in constructing a more efficient xylan degradation module in *C. glutamicum*. In addition, to improve the growth rate and lysine production further, we also need to optimize the catabolic pathway of xylose, including the glycolysis/pentose phosphate pathways and TCA cycle as well as lysine biosynthesis pathways. For that engineering, we can use our synthetic promoters. All possible expression systems of the related genes can be constructed by the combinatorial assembly of each expression module with synthetic promoters, and the best strain can be isolated by screening a combinatorial library. Engineering of an arabinose utilization system in *C. glutamicum* would also be helpful for complete utilization of hemicellulose, since arabinose is the second most abundant component of hemicellulosic biomass.

Conclusions. We succeeded in engineering a CBP-enabled *C. glutamicum* strain for the production of value-added biochemicals from hemicellulose. Until now, there have been a few reports about the utilization of lignocellulosic biomass for the production of value-added chemicals in *C. glutamicum*.[22,34] However, in those reports, biomass hydrolysates, which were prepared in separate processes, were used for the fermentation of *C. glutamicum*, and to the best of our knowledge, this is the first report for the production of value-added biochemicals from hemicellulosic biomass in a single bioreactor by *C. glutamicum*. Furthermore, in this study, *C. glutamicum* was engineered using novel genetic tools (synthetic promoters and signal sequences) previously developed by our group to tune gene expression for optimal performance. As a result, we could demonstrate convincingly that our synthetic biology approach of engineering *C. glutamicum* can yield greatly improved performance for desired metabolic pathways. As shown here, we believe that our modular engineering strategy can greatly contribute to the further engineering of *C. glutamicum* and that the engineered *C. glutamicum* will serve as a potential platform for the consolidated bioprocessing of biomass for use in bioindustry.

METHODS

Bacterial Strains and Plasmid Manipulation. The bacterial strains and plasmids used in this study are listed in Table 2. *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 optimization of hemicellulose utilization systems. *C. glutamicum* KCTC 1857 was used for lysine production.[43,44] The plasmid pCES208, an *E. coli*−*C. glutamicum* shuttle vector, was used as the main plasmid for gene expression in the *C. glutamicum* strain. The detailed procedures for the construction of all plasmids are described in the Supporting Information, and schematic diagrams of the gene expression systems are also shown in the Supporting Information, Figure S1. Polymerase chain reaction (PCR) was performed with the 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 Supporting Information, Table S1. After constructing the plasmids in an *E. coli* host, *C. glutamicum* was transformed with each plasmid via electroporation using a Gene Pulser (Bio-Rad).

Media and Cultivation Conditions. For plasmid preparation, *E. coli* was cultivated in Luria−Bertani (LB) broth (tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L) at 37 °C. For cultivation of *C. glutamicum* strains, brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA), minimal media (3 g/L of K_2HPO_4, 1 g/L of KH_2PO_4, 2 g/L of urea, 10 g/L of $(NH_4)_2SO_4$, 2 g/L of $MgSO_4$, 200 μg/L of biotin, 5 mg/L of thiamine, 10 mg/L of calcium pantothenate, 10 mg/L of $FeSO_4$, 1 mg/L of $MnSO_4$, 1 mg/L of $ZnSO_4$, and 10 mg/L of $CaCl_2$), or semi-defined media (0.5 g/L of yeast extract added in minimal medium) were used with variable concentrations of sugar carbon sources as indicated. For growth measurements, *C. glutamicum* were inoculated into BHI media and grown at 30 °C for 24 h. The fully grown cultures were then transferred at 1/50 (for the monosaccharide experiment, either xylose or glucose) or 1/20 (for the polysaccharide experiment, xylan) into 50 mL of minimal media in 250 mL baffled flasks. In all cultivations, kanamycin (Km, 25 μg/mL) was added to the culture medium as the sole antibiotic. For xylan, beechwood xylan (Sigma-Aldrich, St. Louis, MO) was used, which consists mostly of xylose residues (>90%).

Bioscreen C (Growth Curves USA, Piscataway, NJ, USA) was used to measure the growth rates of the strains carrying the xylose transport modules. For the experimental conditions, Bioscreen C was set to maintain 30 °C with a medium level of continuous shaking and to measure the optical density at 600 nm every 15 min. Four microliters of the fully grown cultures of *C. glutamicum* cells in BHI media was transferred into 200 μL of minimal media containing 3.6 g/L of xylose as the sole carbon source per well.

Protein Preparation and Analysis. After cell cultivation in shaking flasks for 48 h, cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The 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 total lysates of the cells were prepared by sonication (7 min at 40% pulse and 20%

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

Table 2. Bacterial Strains and Plasmids Used in This Study

strain	relevant characteristics	ref or source
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZ△M15 Tn10 (Tet′)]*	Stratagene[a]
C. glutamicum	Wild type	ATCC 13032
C. glutamicum	Lysine producing strain (ATCC 21543), Requirement for homoserine and leucine	KCTC 1857
plasmids	**relevant characteristics**	**ref or source**
pCES208	*E. coli*−*C. glutamicum* shuttle vector; Km^r	53
pASJ104	pCES208 derivative; P_{porB}, signal sequence of *porB*, *xln A* from *S. coelicoloar* A3(2)	29
pXU1	pCES208 derivative; P_{L26}, *xylB* and *xylA* from *E. coli*	this study
pXU2	pCES208 derivative; P_{I16}, *xylB* and *xylA* from *E. coli*	this study
pXU3	pCES208 derivative; P_{H30}, *xylB* and *xylA* from *E. coli*	this study
pXU2T1	pXU2 derivative; P_{L10}, *araE* from *C. glutamicum* ATCC 31831	this study
pXU2T2	pXU2 derivative; P_{I12}, *araE* from *C. glutamicum* ATCC 31831	this study
pXU2T3	pXU2 derivative; P_{H72}, *araE* from *C. glutamicum* ATCC 31831	this study
pXU2T4	pXU2 derivative; P_{L10}, *araE* from *E. coli*	this study
pXU2T5	pXU2 derivative; P_{I12}, *araE* from *E. coli*	this study
pXU2T6	pXU2 derivative; P_{H72}, *araE* from *E. coli*	this study
pXU2T7	pXU2 derivative; P_{L10}, *xylE* from *E. coli*	this study
pXU2T8	pXU2 derivative; P_{I12}, *xylE* from *E. coli*	this study
pXU2T9	pXU2 derivative; P_{H72}, *xylE* from *E. coli*	this study
pXD1	pASJ104 (termed here pXD1)	29
pXD2	pCES208 derivative; P_{porB}, signal sequence of *porB*, *xln A* from *S. coelicoloar* A3(2), *xynB* from *B. pumilus*	this study
pXD3	pCES208 derivative; P_{porB}, signal sequence of *porB*, *xln A* from *S. coelicoloar* A3(2), signal sequence of *porB*, *xynB* from *B. pumilus*	this study
pXD4	pCES208 derivative; P_{porB}, signal sequence of *porB*, *xln A* from *S. coelicoloar* A3(2), signal sequence of *cgR0949*, *xynB* from *B. pumilus*	this study
pXD5	pCES208 derivative; P_{cg1514}, signal sequence of *cg1514*, *xln A* from *S. coelicoloar* A3(2), signal sequence of *cgR0949*, *xynB* from *B. pumilus*	this study
pHCBP200	pCES208 derivative; P_{I16}, *xylB* and *xylA* from *E. coli*	this study
pHCBP205	pHCBP200 derivative; P_{cg1514}, signal sequence of *cg1514*, *xln A* from *S. coelicoloar* A3(2), signal sequence of *cgR0949*, *xynB* from *B. pumilus*	this study
pHCBP275	pHCBP205 derivative; P_{L10}, *xylE* from *E. coli*	this study

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

amplitude), and the extracts were centrifuged at 10000 rpm for 10 min at 4 °C to yield soluble lysates. Extracellular proteins were prepared using the acetone precipitation method.[49] After centrifugation, the culture supernatant was vigorously mixed with two volumes of cold acetone and incubated at −20 °C for 60 min. The protein samples were then precipitated by centrifugation at 13000 rpm for 30 min at 4 °C, and the pellets were resuspended in PBS. The protein samples were stored at −20 °C until further analysis.

All protein samples were analyzed by electrophoresis on a 12% (w/v) SDS-PAGE gel. For the immunodetection of the FLAG tag or 6-His tag fused protein, a monoclonal anti-His antibody−horseradish peroxidase (HRP) conjugate or anti-FLAG M2 antibody−horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) was used, respectively. The ECL kit (Amersham ECL prime Western blotting detection reagent, GE Healthcare Bio-Science AB, Uppsala, Sweden) was used for signal detection. Activities of secreted endoxylanase (XlnA) and xylosidase (XynB) (U/L) in the culture medium of *C. glutamicum* ATCC 13032 harboring each plasmid were measured at 48 h after cultivation in minimal media containing 10 g/L of glucose and 10 g/L xylan. The reducing end sugars already present in the culture supernatant were removed by dialysis using a 7000 MWCO dialysis tube (Pierce Biotechnology, Rockford, IL, USA) against 2 L of PBS for 24 h with three buffer exchanges. The activity of XlnA was assayed by the 3,5-dinitrosalicylic acid (DNS) method as described previously.[50] The activity of XynB was assayed with *p*-nitrophenyl-*β*-D-xylopyranoside substrate (pNPX) as described previously.[51] One unit of enzyme activity was defined as the amount of enzyme required to release 1 *μ*mol of product (xylose from xylan or *p*-nitrophenol from *p*-nitrophenyl-*β*-xylopyranoside) per minute at 30 °C.

Sugar Concentration Analysis. To analyze the sugar concentration in the culture medium, the culture supernatant was prepared by centrifugation at 13000 rpm for 10 min at 4 °C followed by filtration of residual insoluble matters with a 0.45 *μ*m syringe filter (Sartorius Stedim Biotech, Goettingen, Germany). The prepared sample was diluted 10-fold in distilled water and loaded onto a high-performance liquid chromatography system (HPLC, Waters Breeze 2 system, Water Chromatography, Milford, MA, USA) equipped with UV/vis (G1314B, Agilent, Wakefield, MA, USA) and refractive index 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 flow rate for the mobile phase (5 mM H_2SO_4). For the analysis of total residual sugar concentration in the xylan containing media, culture supernatant was prepared by centrifugation at 3000 rpm for 5 min, and the total sugar concentration in the culture supernatant was determined with the phenol−sulfuric acid method.[11,52]

Analysis of Lysine in the Culture Medium. For the analysis of L-lysine concentration, the culture supernatant was prepared by centrifugation of the culture medium at 13000 rpm for 10 min at 4 °C followed by filtration of the residual insoluble components with a 0.45 *μ*m syringe filter. The prepared culture supernatant sample was diluted 10-fold in distilled water, derivatized by *ortho*-phthalaldehyde-3-mercaptopropionic acid (OPA), and loaded onto an HPLC system (LC-20 AD, CTO-20A, SPD-20A; Shimadzu, Japan) equipped with a Zorbax Eclipse amino acid analysis (AAA) column (4.6 × 150 mm 3.5 *μ*m; Agilent). The separation of samples was attained according to the manufacturer's instructions using a binary nonlinear gradient with mobile phase A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 0.5 mM NaN_3, pH 8.2) and

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

mobile phase B (acetonitrile/MeOH/H_2O = 45:45:10 v/v/v). The elution conditions were as follows: equilibration, 1.9 min at 100% A; gradient, 16.2 min at 0−57% B and 3.7 min at 57−100% B; and cleaning, 4 min at 100% B. The column temperature was maintained at 40 °C. Samples were detected at 338 nm. The standard curve for L-lysine was determined using the same procedure for four standard solutions (0.125, 0.25, 0.5, and 1 g/L of L-lysine).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00228.

Method for plasmid manipulation, list of primers used in PCR experiments (Table S1), schematic diagrams of plasmids constructed in this study (Figure S1), optimization of xylose transport module (Figure S2), and optimization of xylan degradation module (Figure S3) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +82-42-350-3934. Fax: +82-42-350-3910. E-mail: kjjeong@kaist.ac.kr.

Author Contributions

K.J.J. and S.S.Y. designed the research; S.S.Y., J.W.C., and S.H.L. performed the experiments; K.J.J., S.S.Y., and J.W.C. analyzed the data; and K.J.J. and S.S.Y. wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Intelligent Synthetic Biology Center of the Global Frontier Project (grant no. 2014M3A6A8066443) and the National Research Foundation of Korea (grant no. NRF-2015R1A2A2A01007674) funded by the Ministry of Science, ICT, and Future Planning (MSIP).

■ REFERENCES

(1) Deutschmann, R., and Dekker, R. F. (2012) From plant biomass to bio-based chemicals: latest developments in xylan research. *Biotechnol. Adv. 30*, 1627−1640.

(2) Gupta, A., and Verma, J. P. (2015) Sustainable bio-ethanol production from agro-residues: A review. *Renewable Sustainable Energy Rev. 41*, 550−567.

(3) Saini, J. K., Saini, R., and Tewari, L. (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production. *3 Biotech 5*, 337−353.

(4) Lynd, L. R., van Zyl, W. H., McBride, J. E., and Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: an update. *Curr. Opin. Biotechnol. 16*, 577−583.

(5) Mathews, S. L., Pawlak, J., and Grunden, A. M. (2015) Bacterial biodegradation and bioconversion of industrial lignocellulosic streams. *Appl. Microbiol. Biotechnol. 99*, 2939−2954.

(6) Argyros, D. A., Tripathi, S. A., Barrett, T. F., Rogers, S. R., Feinberg, L. F., Olson, D. G., Foden, J. M., Miller, B. B., Lynd, L. R., Hogsett, D. A., and Caiazza, N. C. (2011) High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. *Appl. Environ. Microbiol. 77*, 8288−8294.

(7) Deng, Y., and Fong, S. S. (2011) Metabolic engineering of *Thermobifida fusca* for direct aerobic bioconversion of untreated lignocellulosic biomass to 1-propanol. *Metab. Eng. 13*, 570−577.

(8) Gronenberg, L. S., Marcheschi, R. J., and Liao, J. C. (2013) Next generation biofuel engineering in prokaryotes. *Curr. Opin. Chem. Biol. 17*, 462−471.

(9) Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., Lee, T. S., Tullman-Ercek, D., Voigt, C. A., Simmons, B. A., and Keasling, J. D. (2011) Synthesis of three advanced biofuels from ionic liquid-preteated switchgrass using engineered *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A. 108*, 19949−19954.

(10) Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S. B., and Keasling, J. D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature 463*, 559−562.

(11) Sun, J., Wen, F., Si, T., Xu, J. H., and Zhao, H. (2012) Direct conversion of xylan to ethanol by recombinant *Saccharomyces cerevisiae* strains displaying an engineered minihemicellulosome. *Appl. Environ. Microbiol. 78*, 3837−3845.

(12) Zheng, Z., Chen, T., Zhao, M., Wang, Z., and Zhao, X. (2012) Engineering *Escherichia coli* for succinate production from hemicellulose via consolidated bioprocessing. *Microb. Cell Fact. 11*, 37.

(13) Becker, J., and Wittmann, C. (2012) Bio-based production of chemicals, materials and fuels - *Corynebacterium glutamicum* as versatile cell factory. *Curr. Opin. Biotechnol. 23*, 631−640.

(14) Inui, M., Kawaguchi, H., Murakami, S., Vertès, A. A., and Yukawa, H. (2004) Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J. Mol. Microbiol. Biotechnol. 8*, 243−254.

(15) Park, S. H., Kim, H. U., Kim, T. Y., Park, J. S., Kim, S. S., and Lee, S. Y. (2014) Metabolic engineering of *Corynebacterium glutamicum* for L-arginine production. *Nat. Commun. 5*, 4618.

(16) Yim, S. S., An, S. J., Choi, J. W., Ryu, A. J., and Jeong, K. J. (2014) High-level secretory production of recombinant single-chain variable fragment (scFv) in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol. 98*, 273−284.

(17) Sakai, S., Tsuchida, Y., Okino, S., Ichihashi, O., Kawaguchi, H., Watanabe, T., Inui, M., and Yukawa, H. (2007) Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested *Corynebacterium glutamicum* R. *Appl. Environ. Microbiol. 73*, 2349−2353.

(18) Billman-Jacobe, H., Wang, L., Kortt, A., Stewart, D., and Radford, A. (1995) Expression and secretion of heterologous proteases by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 61*, 1610−1613.

(19) Vertès, A. A. (2013) Protein secretion systems of *Corynebacterium glutamicum*. In *Corynebacterium glutamicum: biology and biotechnology* (Yukawa, H., and Inui, M., Eds.) pp 351−389, Springer, Berlin.

(20) Adachi, N., Takahashi, C., Ono-Murota, N., Yamaguchi, R., Tanaka, T., and Kondo, A. (2013) Direct L-lysine production from cellobiose by *Corynebacterium glutamicum* displaying beta-glucosidase on its cell surface. *Appl. Microbiol. Biotechnol. 97*, 7165−7172.

(21) Buschke, N., Becker, J., Schäfer, R., Kiefer, P., Biedendieck, R., and Wittmann, C. (2013) Systems metabolic engineering of xylose-utilizing *Corynebacterium glutamicum* for production of 1,5-diaminopentane. *Biotechnol. J. 8*, 557−570.

(22) Gopinath, V., Meiswinkel, T. M., Wendisch, V. F., and Nampoothiri, K. M. (2011) Amino acid production from rice straw and wheat bran hydrolysates by recombinant pentose-utilizing *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol. 92*, 985−996.

(23) Meiswinkel, T. M., Gopinath, V., Lindner, S. N., Nampoothiri, K. M., and Wendisch, V. F. (2013) Accelerated pentose utilization by *Corynebacterium glutamicum* for accelerated production of lysine, glutamate, ornithine and putrescine. *Microb. Biotechnol. 6*, 131−140.

(24) Sasaki, M., Jojima, T., Kawaguchi, H., Inui, M., and Yukawa, H. (2009) Engineering of pentose transport in *Corynebacterium glutamicum* to improve simultaneous utilization of mixed sugars. *Appl. Microbiol. Biotechnol. 85*, 105−115.

(25) Lee, J., Sim, S. J., Bott, M., Um, Y. S., Oh, M. K., and Woo, H. M. (2014) Succinate production from CO2-grown microalgal biomass

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343

as carbon source using engineered *Corynebacterium glutamicum* through consolidated bioprocessing. *Sci. Rep. 4*, 5819.
(26) Seibold, G., Auchter, M., Berens, S., Kalinowski, J., and Eikmanns, B. J. (2006) Utilization of soluble starch by a recombinant *Corynebacterium glutamicum* strain: Growth and lysine production. *J. Biotechnol. 124*, 381−391.
(27) Tateno, T., Fukuda, H., and Kondo, A. (2007) Direct production of L-lysine from raw corn starch by *Corynebacterium glutamicum* secreting *Streptococcus bovis* alpha-amylase using *cspB* promoter and signal sequence. *Appl. Microbiol. Biotechnol. 77*, 533−541.
(28) Tateno, T., Okada, Y., Tsuchidate, T., Tanaka, T., Fukuda, H., and Kondo, A. (2009) Direct production of cadaverine from soluble starch using *Corynebacterium glutamicum* coexpressing alpha-amylase and lysine decarboxylase. *Appl. Microbiol. Biotechnol. 82*, 115−121.
(29) An, S. J., Yim, S. S., and Jeong, K. J. (2013) Development of a secretion system for the production of heterologous proteins in *Corynebacterium glutamicum* using the Porin B signal peptide. *Protein Expression Purif. 89*, 251−257.
(30) Yim, S. S., An, S. J., Kang, M., Lee, J., and Jeong, K. J. (2013) Isolation of fully synthetic promoters for high-level gene expression in *Corynebacterium glutamicum*. *Biotechnol. Bioeng. 110*, 2959−2969.
(31) Yim, S. S., Choi, J. W., Lee, R. J., Lee, Y. J., Lee, S. H., Kim, S. Y., and Jeong, K. J. (2016) Development of a new platform for enhanced secretory production of recombinant proteins in *Corynebacterium glutamicum*. *Biotechnol. Bioeng. 113*, 163−172.
(32) Kawaguchi, H., Vertès, A. A., Okino, S., Inui, M., and Yukawa, H. (2006) Engineering of a xylose metabolic pathway in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 72*, 3418−3428.
(33) Lee, S. M., Jellison, T., and Alper, H. S. (2012) Directed evolutioin of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol. 78*, 5708−5716.
(34) Buschke, N., Schröder, H., and Wittmann, C. (2011) Metabolic engineering of *Corynebacterium glutamicum* for production of 1,5-diaminopentane from hemicellulose. *Biotechnol. J. 6*, 306−317.
(35) Blombach, B., and Seibold, G. M. (2010) Carbohydrate metabolism in *Corynebacterium glutamicum* and its applications for the metabolic engineering of L-lysine production strains. *Appl. Microbiol. Biotechnol. 86*, 1313−1322.
(36) Kawaguchi, H., Sasaki, M., Vertès, A. A., Inui, M., and Yukawa, H. (2009) Identification and functional analysis of the gene cluster for L-arabinose utilization in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 75*, 3419−3429.
(37) Zhang, Y., Shang, X., Lai, S., Zhang, G., Liang, Y., and Wen, T. (2012) Development and application of an arabinose-inducible expression system by facilitating inducer uptake in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 78*, 5831−5838.
(38) Hartner, F. S., Ruth, C., Langenegger, D., Johnson, S. N., Hyka, P., Lin-Cereghino, G. P., Lin-Cereghino, J., Kovar, K., Cregg, J. M., and Glieder, A. (2008) Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. *Nucleic Acids Res. 36*, e76.
(39) Kumar, R., Singh, S., and Singh, O. V. (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *J. Ind. Microbiol. Biotechnol. 35*, 377−391.
(40) Teramoto, H., Watanabe, K., Suzuki, N., Inui, M., and Yukawa, H. (2011) High yield secretion of heterologous proteins in *Corynebacterium glutamicum* using its own Tat-type signal sequence. *Appl. Microbiol. Biotechnol. 91*, 677−687.
(41) Watanabe, K., Teramoto, H., Suzuki, N., Inui, M., and Yukawa, H. (2013) Influence of SigB inactivation on *Corynebacterium glutamicum* protein secretion. *Appl. Microbiol. Biotechnol. 97*, 4917−4926.
(42) Olofsson, K., Bertilsson, M., and Lidén, G. (2008) A short review on SSF − an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. Biofuels 1*, 7.
(43) Nakayama, K., and Araki, K. (1973) Process for producing L-lysine, Patent US3708395A, Jan 2.
(44) Wittmann, C., Kim, H. M., and Heinzle, E. (2004) Metabolic network analysis of lysine producing *Corynebacterium glutamicum* at a miniaturized scale. *Biotechnol. Bioeng. 87*, 1−6.
(45) Moriyama, H., Fukusaki, E., Cabrera Crespo, J., Shinmyo, A., and Okada, H. (1987) Structure and expression of genes coding for xylan-degrading enzymes of *Bacillus pumilus*. *Eur. J. Biochem. 166*, 539−545.
(46) Xu, W. Z., Shima, Y., Negoro, S., and Urabe, I. (1991) Sequence and properties of beta-xylosidase from *Bacillus pumilus* IPO. Contradiction of the previous nucleotide sequence. *Eur. J. Biochem. 202*, 1197−1203.
(47) Nishio, Y., Nakamura, Y., Kawarabayasi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K., and Gojobori, T. (2003) Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. *Genome Res. 13*, 1572−1579.
(48) Watanabe, A., Hiraga, K., Suda, M., Yukawa, H., and Inui, M. (2015) Functional characterization of *Corynebacterium alkanolyticum* β-xylosidase and xyloside ABC transporter in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 81*, 4173−4183.
(49) Jiang, L., He, L., and Fountoulakis, M. (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A 1023*, 317−320.
(50) Jeong, K. J., Lee, P. C., Park, I. Y., Kim, M. S., and Kim, S. C. (1998) Molecular cloning and characterization of an endoxylanase gene of *Bacillus* sp. in *Escherichia coli*. *Enzyme Microb. Technol. 22*, 599−605.
(51) Banka, A. L., Guralp, S. A., and Gulari, E. (2014) Secretory expression and characterization of two hemicellulases, xylanase, and β-xylosidase, isolated from *Bacillus subtilis* M015. *Appl. Biochem. Biotechnol. 174*, 2702−2710.
(52) DuBois, M., Gilles, K., Hamilton, J., Rebers, P., and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem. 28*, 350−356.
(53) Park, J. U., Jo, J. H., Kim, Y. J., Chung, S. S., Lee, J. H., and Lee, H. H. (2008) Construction of heat-inducible expression vector of *Corynebacterium glutamicum* and *C. ammoniagenes*: Fusion of lambda operator with promoters isolated from *C. ammoniagenes*. *J. Microbiol. Biotechnol. 18*, 639−647.

DOI: 10.1021/acssynbio.5b00228
ACS Synth. Biol. 2016, 5, 334−343