ACS SyntheticBiology

Research Article

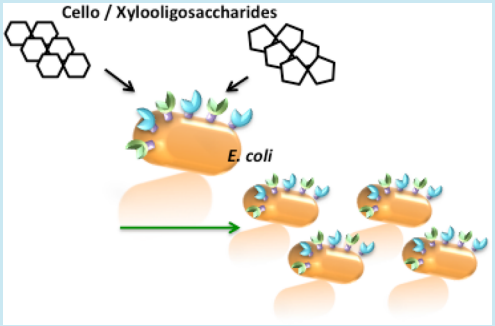
pubs.acs.org/synthbio

# Creation of Cellobiose and Xylooligosaccharides-Coutilizing *Escherichia coli* Displaying both β-Glucosidase and β-Xylosidase on Its Cell Surface

Tsutomu Tanaka, Yuuki Hirata, Mariko Nakano, Hitomi Kawabata, and Akihiko Kondo*

Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

**ABSTRACT:** We demonstrated direct utilization of xylooligosaccharides using β-xylosidase-displaying *Escherichia coli*. After screening active β-xylosidases, BSU17580 from *Bacillus subtilis* or Tfu1616 from *Thermobifida fusca* YX, were successfully displayed on the *E. coli* cell surface using Blc or HdeD as anchor proteins, and these transformants directly assimilated xylooligosaccharides as a carbon source. The final OD 600 in minimal medium containing 2% xylooligosaccharides was 1.09 (after 12 h of cultivation) and 1.30 (after 40 h of cultivation). We then constructed an *E. coli* strain displaying both β-glucosidase and β-xylosidase. β-glucosidase- and β-xylosidase-displaying *E. coli* was successfully grown on a 1% cellobiose and 1% xylooligosaccharides mixture, and the OD 600 was 1.76 after 10 h of cultivation, which was higher and reached faster than that grown on a glucose/xylose mixture (1.20 after 30 h of cultivation).




**KEYWORDS:** *E. coli, cell-surface display, β-xylosidase, β-glucosidase, xylooligosaccharides, cellobiose*

The utilization of biomass as a source of renewable, environmentally friendly energy and/or chemicals has attracted much attention because of the depletion of fossil fuels and increasing environmental problems. Starch-rich biomass derived mainly from corn is the main feedstock for biofuel production in North America, completely ignoring the massive amounts of ethanol produced from sugar cane in Brazil. As for the chemicals in general, most remain petroleum derivatives. As an alternative to starch-rich biomass, lignocellulosic biomass, which is one of the most abundant waste materials in the world, is regarded as a promising feedstock because it is abundant, inexpensive, and renewable and has favorable environmental properties.[1,2]

Lignocellulosic biomass contains not only glucose but also various other sugars. Xylose is the second most abundant sugar component of lignocellulose. Industrial effluents, such as lignocellulose hydrolysates, are composed of a mixture of mono- and oligomeric hexoses and pentoses; therefore, coutilization of several sugars in industrial effluents is essential for economically feasible productivity of biofuels and chemicals. Lignocellulose hydrolysates are mainly composed of cellooligosaccharides (glucose) and xylooligosaccharides (xylose); however, there are two major problems for efficient utilization of lignocellulosic biomass. One is that lignocellulosic biomass requires costly and complex hydrolyzing steps, such as pretreatment and/or lengthy cellulase treatment.[3−5] Efficient degradation of cellulose requires the synergistic action of the cellulolytic enzymes endoglucanases (EGs), cellobiohydrolases (CBHs), and β-glucosidases (BGLs). The cellulose is degradated by EG and CBH, resulting in cellobiose and some cellooligosaccharides, which can be converted to glucose by BGL. In the case of hemicellulosic materials such as xylan, several kinds of hemicellulases xylanases (XYNs) and beta-xylosidases (XYLs) are also required to produce monomeric pentoses.[6] Therefore, an efficient and cost-effective method for the degradation and fermentation of lignocellulosic biomass into commodity products is required. The other barrier to the successful utilization of mixed sugars in cellulosic hydrolysates is sequential utilization of xylose after glucose depletion because of carbon-catabolite repression (CCR).[6−8] In short, most microorganisms consume glucose preferentially to any other sugar, and whenever glucose is present in the fermentation medium, other carbon sources are used only when glucose is completely depleted.

*Escherichia coli* is a promising host for the production of a variety of useful compounds and can metabolize all major sugar monomers existing in plant biomass.[9,10] Metabolic engineering allows for the introduction of desirable pathways to produce target compounds, such as ethanol and other alcohols,[11] lactic acids and other organic acids,[12,13] and other compounds.[14,15] However, *E. coli* cannot assimilate both cellooligosaccharides and xylooligosaccharides, and it also suffers from sequential sugar utilization by CCR. Several approaches to alleviate glucose repression have been reported,[16−19] but coassimilation of xylose/glucose is poor when compared to fermentation using xylose as the sole carbon sugar.

**Received:** June 20, 2013
**Published:** October 24, 2013

Here, we constructed cellobiose and xylooligosaccharide-coassimilating *E. coli* strains by displaying both β-glucosidase and β-xylosidase. Usually, *E. coli* strains cannot assimilate cellooligosaccharides and xylooligosaccharides because of the lack of both BGL and XYL capable of hydrolyzing cellooligosaccharides into glucose and xylooligosaccharides into xylose, respectively. Providing cellobiose as a glucose source and xylooligosaccharide as a xylose source, the engineered *E. coli* was able to convert cellobiose to glucose and xylooligosaccharide to xylose on its cell surface and gradually consumed the produced glucose and xylose. This enables the glucose/xylose concentration outside the cell to be gradual, and it limited CCR. Using this approach, we successfully achieved efficient cellobiose/xylooligosaccharide coutilization.

## RESULTS AND DISCUSSION

**Active Xylosidase (XYL) Expression Using *E. coli* as a Host and Growth on Xylooligosaccharides as a Carbon Source.** We cloned 36 kinds of xylosidases (XYLs) from various microorganisms, and the XYL activities were evaluated by plate assays using 4-methylumbelliferyl-β-D-xyloside as a substrate (data not shown). Two kinds of *E. coli* colonies carrying XYL genes Tfu1616 from *Thermobifida fusca* YX and BSU17580 from *Bacillus subtilis* showed bright fluorescence using 4-methylumbelliferyl-β-D-xyloside as a substrate (data not shown). These two kinds of xylosidases were displayed on the *E. coli* cell surface using HdeD or Blc as anchor proteins, which are suitable anchor proteins for *E. coli* cell-surface display.[20]

These XYL activities on the cell surface or in culture medium were quantitatively evaluated using pNPX as a substrate (Figure 1A). In the case of *E. coli* JCM20137, HdeD-Tfu1616 showed the highest XYL activity (104 U/OD 600/mL) followed by Blc-Tfu1616 (68 U/OD 600/mL) and HdeD-BSU17580 (31 U/OD 600/mL). Similarly, in the case of *E. coli* BW25113, HdeD-Tfu1616 showed higher activity (52 U/OD 600/mL) than Blc-Tfu1616 (27 U/OD 600/mL). There were neither XYL activity nor BGL of blank *E. coli* JCM20137 cells. In all strains, XYL activity on the cell surface was higher than that in the culture supernatant (under 0.0001 U/mL; data now shown), showing that XYL was successfully expressed on the cell surface and retained its enzymatic function through the anchor protein.

Using these XYL-displaying *E. coli* JCM20137 strains, cell growth on 2% xylooligosaccharides as the sole carbon source was evaluated (Figure 1B). Xylooligosaccharides containing about 60% xylobiose, 30% xylotriose, and 10% xylooligosaccharides were used. The concentrations of each xylooligosaccharides were determined by HPLC analysis. Interestingly, in the case of *E. coli* JCM20137, BSU17580-displaying *E. coli* showed a higher growth rate compared to Tfu1616-displaying strains even though the XYL activity of BSU17580 was lower than that of Tfu1616 (Figure 1A). The values of OD 600 after 12 h of cultivation were approximately 1.09 (Blc-BSU17580) and 0.96 (HdeD-BSU17580), which were more than 2-fold higher than those of the Tfu1616-displaying strains (0.42 of HdeD-Tfu1616 and 0.38 of Blc-Tfu1616). However, the final values of OD 600 after 40 h of cultivation were approximately the same: 1.48 (HdeD-Tfu1616), 1.30 (Blc-BSU17580), 1.28 (HdeD-Tfu1616), and 1.17 (Blc-Tfu1616). The strain carrying only the pHLA control plasmid did not have an increased OD 600 (less than 0.15). These results clearly demonstrate the creation of xylooligosaccharide-utilizing *E. coli* strains by displaying active β-xylosidase. In the case of *E. coli* BW25113 strains, lower growth was observed compared to *E. coli* JCM strains (data not shown), which is in accordance with our previous report.[20] These results show that BSU17580 is a suitable XYL for direct xylooligosaccharide assimilation in minimal medium using cell-surface display. In addition, the evaluation of growth on xylooligosaccharides as well as the evaluation of XYL activity is important for enhancing direct xylooligosaccharide assimilation.

**Cellooligosaccharide and Xylooligosaccharide-Coutilizing *E. coli* by Display of both BGL and XYL.** We created BGL-displaying *E. coli* strains by introducing a BGL-expression cassette into the *E. coli* genome. Figure 2A shows the cell-

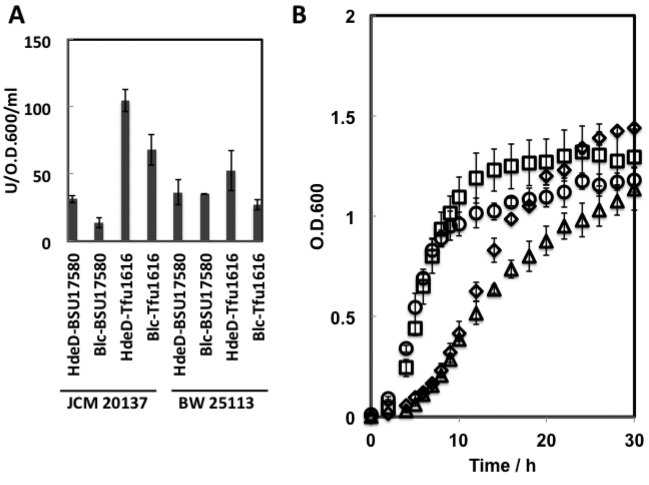



**Figure 1.** (A) XYL activity on the *E. coli* cell surface after 24 h of cultivation in LB medium. Each XYL was displayed on the cell surface using HdeD or Blc as an anchor protein. (B) Growth analysis of each XYL-displaying *E. coli* strain using xylooligosaccharides as the sole carbon source. The symbols shown are as follows: JCM/HdeD-BSU17580 (circles), JCM/Blc-BSU17580 (squares), JCM/HdeD-Tfu1616 (diamonds), and JCM/Blc-Tfu1616 (triangles). Data are averages from three independent experiments, and error bars represent standard deviation.

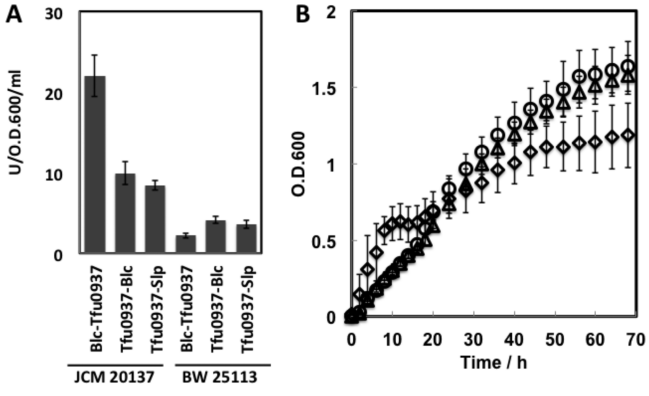



**Figure 2.** (A) BGL activity on the *E. coli* cell surface after 24 h of cultivation in LB medium. BGL genes were integrated into the *E. coli* genome, and each BGL was displayed on the cell surface using Blc or Slp as an anchor protein. (B) Growth analysis of each BGL-displaying *E. coli* JCM20137 strain using cellobiose as the sole carbon source. The symbols shown are as follows: JCM-Tfu0937(bgl)-Blc (circles), JCM-Blc-Tfu0937(bgl) (triangles), and JCM-Tfu0937(bgl)-Slp (diamonds). Data are averages from three independent experiments, and error bars represent standard deviation.


dx.doi.org/10.1021/sb400070q | *ACS Synth. Biol.* 2014, 3, 446−453

surface BGL activity of BGL-displaying *E. coli* strains. *E. coli* JCM-Blc-Tfu0937(bgl) showed the highest BGL activity (22 U/OD 600/mL) followed by JCM-Tfu0937(bgl)-Blc (9.9 U/OD 600/mL) and JCM-Tfu0937(bgl)-Slp (8.4 U/OD 600/mL). In the case of BW25113 strains, BGL activity was lower compared to JCM20113-derived strains (Figure 2A). Using these BGL-displaying *E. coli* strains, cell growth on 2% cellobiose as the sole carbon source was evaluated (Figure 2B). JCM-Tfu0937(bgl)-Blc and JCM-Blc-Tfu0937(bgl) showed similar growth rates, and the values of OD 600 after 35 h were 1.05 and 1.01, respectively. After 80 h of cultivation, the OD 600 reached 1.67 and 1.61, respectively. In the case of JCM-Tfu0937(bgl)-Slp, although high growth was observed during the initial phase after 10 h of cultivation, the final OD 600 value was 1.17 after 80 h of cultivation. In the case of BW25113-derived *E. coli* strains (BW-Tfu0937(bgl)-Blc, BW-Blc-Tfu0937(bgl), and BW-Tfu0937(bgl)-Slp, the values of OD 600 were less than 0.9 after 80 h of cultivation (data not shown); therefore, JCM-Tfu0937(bgl)-Blc, JCM-Blc-Tfu0937-(bgl), and JCM-Tfu0937(bgl)-Slp were used in the following experiments. One possible explanation is that the difference of the substrate (pNPG or cellobiose) might cause the difference between pNPG activity and cellobiose assimilation.

XYL-displaying plasmids were introduced into the BGL-displaying *E. coli* JCM20137 strains described above, and we measured growth on a mixture of 1% (w/v) cellobiose and 1% (w/v) xylooligosaccharides (Figure 3). In the case of JCM-

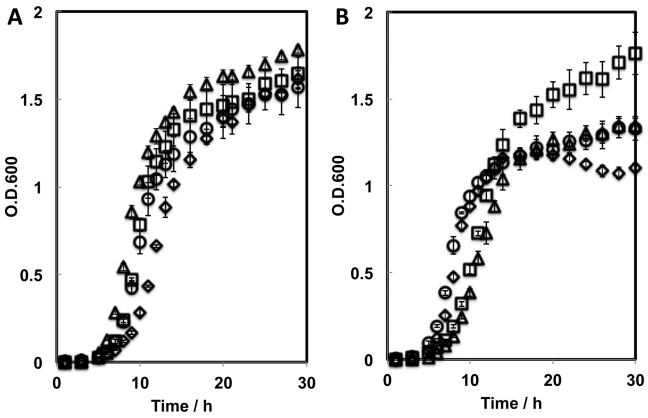



**Figure 3.** Growth analysis of both BGL- and XYL-displaying *E. coli* using 1% cellobiose and 1% xylooligosaccharides mixture (total of 2%) as a carbon source: (A) *E. coli* JCM-Tfu0937(bgl)-Blc strains and (B) JCM-Tfu0937(bgl)-Slp strains. The symbols shown are as follows: Blc-BSU17580(xyl) (squares), Blc-Tfu1616(xyl) (triangles), HdeD-Tfu1616(xyl) (diamonds), and HdeD-BSU17580(xyl) (circles). Data are averages from three independent experiments, and error bars represent standard deviation.

Tfu0937(bgl)-Blc strains harboring each XYL-displaying plasmid, *E. coli* JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616(xyl) showed almost the same growth rate as the others (Figure 3A). The OD 600 value was significantly increased after about an 8−10 h of cultivation, and the final OD 600 values were 1.76 for JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616(xyl), 1.63 for JCM-Tfu0937(bgl)-Blc/Blc-BSU17580(xyl), 1.61 for JCM-Tfu0937-(bgl)-Blc/Hded-Tfu1616(xyl), and 1.60 for JCM-Tfu0937-(bgl)-Blc/HdeD-BSU17580(xyl). In the case of JCM-Tfu0937-(bgl)-Slp strains harboring each XYL-displaying plasmid, *E. coli* JCM-Tfu0937(bgl)-Slp/HdeD-Tfu1616(xyl) showed a higher OD 600 value after 30 h of cultivation (1.76) compared to the others (Figure 3B). When JCM-Blc-Tfu0937(bgl) was used as the host strain, cell growth was slower and the final OD 600 value was less than 1.0 after 30 h of cultivation (data not shown).

As a control for the cellobiose/xylooligosaccharides mixture, growth on a glucose/xylose mixture is shown in Figure 4. BGL-

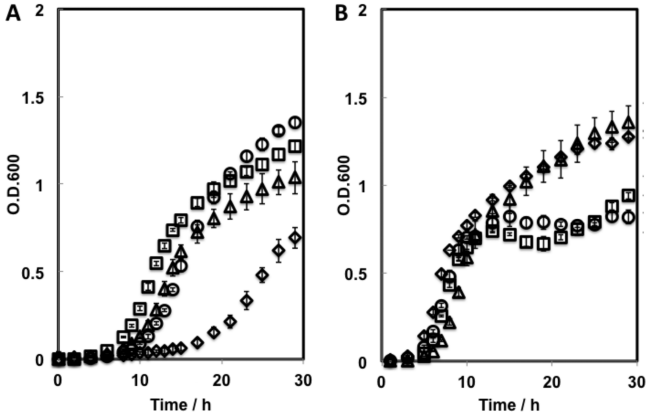



**Figure 4.** Growth analysis of both BGL- and XYL-displaying *E. coli* using 1% glucose and 1% xylose mixture (total of 2%) as a carbon source: (A) *E. coli* JCM-Tfu0937(bgl)-Blc strains and (B) JCM-Tfu0937(bgl)-Slp strains. The symbols shown are as follows: Blc-BSU17580(xyl) (squares), Blc-Tfu1616(xyl) (triangles), HdeD-Tfu1616(xyl) (diamonds), and HdeD-BSU17580(xyl) (circles). Data are averages from three independent experiments, and error bars represent standard deviation.

and XYL-displaying *E. coli* showed slower growth compared to the cellobiose/xylooligosaccharides mixture. The final OD 600 values were less than 1.5 after 30 h of cultivation, notably, 0.69 for JCM-Tfu0937(bgl)-Blc/HdeD-Tfu1616(xyl). The OD 600 values of the other strains were 1.20 for JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616(xyl), 1.21 for JCM-Tfu0937(bgl)-Blc/Blc-BSU17580(xyl), and 1.35 for JCM-Tfu0937(bgl)-Blc/HdeD-BSU17580(xyl). In the case of JCM-Tfu0937(bgl)-Slp strains, the OD 600 values were also lower compared to those from cellobiose/xylooligosaccharides cultivation (Figure 3). The OD 600 values were 1.28 for JCM-Tfu0937(bgl)-Slp/HdeD-Tfu1616(xyl), 1.36 for JCM-Tfu0937(bgl)-Slp/Blc-Tfu1616-(xyl), 0.94 for JCM-Tfu0937(bgl)-Slp/Blc-BSU17580(xyl), and 0.82 for JCM-Tfu0937(bgl)-Slp/HdeD-BSU17580(xyl).

Figure 5 shows time courses of sugar concentration for JCM-Tfu0937(bgl), JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616(xyl), and JCM-Tfu0937(bgl)-Blc/Blc-BSU17580(xyl). When cells were grown on cellobiose/xylooligosaccharides mixture, cellobiose was gradually decreased, and about 7 g/L of cellobiose was consumed after 48 h (Figure 5A−C). Xylooligosaccharides were retained in the case of JCM-Tfu0937(bgl), (Figure 5A); however, successful assimilation of xylooligosaccharides was achieved when the BGL/XYL-displaying *E.coli* strain was used (Figure 5B,C). Interestingly, a small amount of glucose and xylose were detected in the medium, suggesting that improvement in the glucose and/or xylose uptake ability could increase the oligosaccharides-assimilating ability of *E. coli*.

Using both BGL- and XYL-displaying strains, coassimilation of cellobiose/xylooligosaccharides was demonstrated (Figure 3). Interestingly, the cell growth rate was slightly improved compared to when xylooligosaccharides or cellobiose were used as sole carbon sources (Figures 1 and 2). When a 1% cellobiose/1% xylose mixture or a 1% glucose/1% cellobiose


dx.doi.org/10.1021/sb400070q | *ACS Synth. Biol.* 2014, 3, 446−453

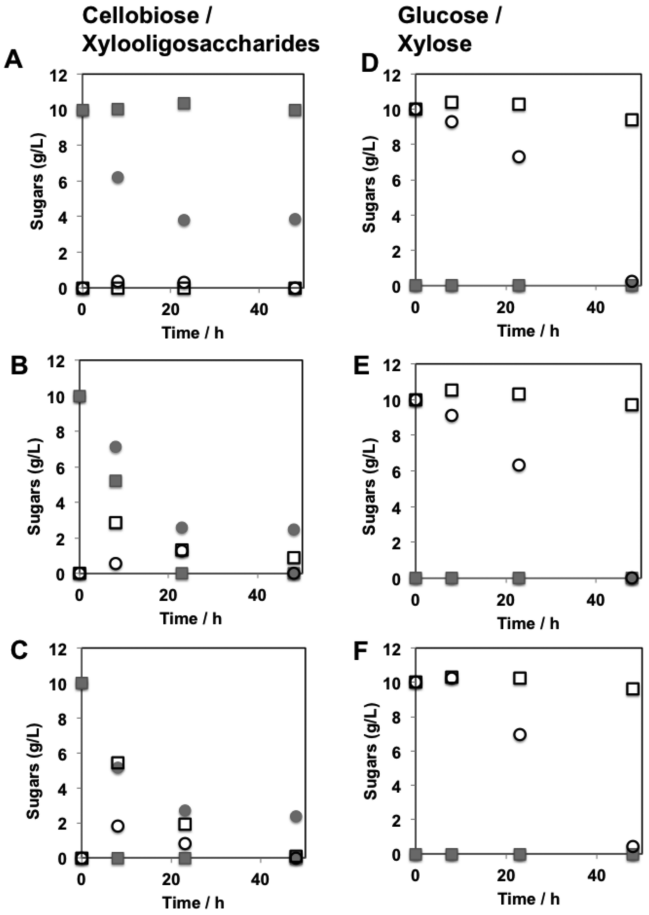



**Figure 5.** Sugar concentrations of both BGL- and XYL-displaying *E. coli* using a 1% cellobiose and xylooligosaccharides mixture (A–C) or a 1% glucose and 1% xylose mixture (D–F) as a carbon source. (A, D) *E. coli* JCM-Tfu0937(bgl)-Blc, (B, D) JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616(xyl), and (C, F) JCM-Tfu0937(bgl)-Blc/Blc-Bsu17580(xyl). The symbols shown are as follows: cellobiose (closed circles), glucose (open circles), xylooligosaccharides (closed squares), and xylose (open squares). Data are averages from three independent experiments, and error bars represent standard deviation.

mixture were used as carbon sources, repression of cell growth was also observed (data not shown), which may have been partially caused by CCR. One possible explanation for this difference is that supplying monomeric sugars (i.e., glucose and xylose) gradually to *E. coli* cells avoids CCR and achieves efficient utilization of mixed sugars.

Most previous work done to develop strains capable of sugar coutilization focused on glucose and xylose.[16] Recently, engineered pathways for production of fatty acid-derived molecules from hemicelluloses or glucose were introduced in *E. coli*.[21] There are three main approaches for this: engineering microorganisms to avoid CCR,[23,24] cellobiose/xylose coutilization using cellobiose-assimilating engineered microorganisms,[25−27] and binary culture using glucose-assimilating microorganisms and xylose-assimilating microorganisms.[22,28] Our approach, cellobiose/xylooligosaccharide coutilization using a BGL- and XYL-displaying strain, is an additional approach to utilize biomass containing several oligomeric sugars efficiently. In addition, this approach can reduce biomass degradation costs resulting from partially hydrolyzed lignocellulosic biomass containing cello/xylooligosaccharides that are used directly without the addition of $\beta$-glucosidase and/or $\beta$-xylosidase.

We developed both BGL- and XYL-displaying *E. coli* and successfully demonstrated direct growth on a cellobiose/xylooligosaccharide mixture. Our approach will be useful for the production of biofuels and/or biochemicals from lignocellulosic biomass.

## METHODS

**Strains and Media.** *E. coli* strains BW25113 (National Institute of Genetics, Japan) and JCM20137 (Japan Collection of Microorganisms, RIKEN BRC, which is participating in the National BioResource Project of the MEXT, Japan) were used as host strains. *E. coli* NovaBlue (Novagen, San Diego, CA, USA) and LB media were used for plasmid construction. Minimum medium containing 2% carbon source(s) (0.6% $Na_2HPO_4$, 0.3% $K_2HPO_4$, 0.05% NaCl, 0.1% $NH_4Cl$, 1 mM $MgSO_4$, 0.001% thiamine, and 0.1 mM $CaCl_2$) was used for growth analysis. As a carbon source, glucose (Nacalai Tesque, Inc., Kyoto, Japan), xylose (Nacalai Tesque, Inc.), cellobiose (Sigma-Aldrich, St. Louis, MO, USA), and xylooligosaccharides (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used. All strains are summarized in Table 1.

**Plasmids Construction.** Plasmids and primers are summarized in Table 1. The plasmids for XYL expression on the cell surface using the Blc or HdeD anchor protein[20] were constructed as follows. The gene encoding Tfu1616 from *T. fusca* YX was amplified by PCR using *T. fusca* YX genomic DNA (ATCC no. BAA-629D-5) as a template with the following primers: NotI_Tfu1616_F and Tfu1616_R. The amplified fragment was digested with NotI/XhoI and ligated into plasmid Blc-Tfu0937-pHLA, HdeD-Tfu0937-pHLA, or Slp-Tfu0937-pHLA.[20] The resultant plasmids were named Blc-Tfu1616-pHLA, HdeD-Tfu1616-pHLA, and Slp-Tfu1616-pHLA, respectively. The gene encoding BSU17580 from *B. subtilis* 168 was amplified by PCR using *B. subtilis* genomic DNA (NBRC no. 13719G) as a template with the following primers: NotI_BSU17580_F and BSU17580_R. The amplified fragment was digested with NotI/XhoI and ligated into plasmid Blc-Tfu0937-pHLA, HdeD-Tfu0937-pHLA, or Slp-Tfu0937-pHLA.[20] The resultant plasmids were named Blc-BSU17580-pHLA, HdeD-BSU17580-pHLA, and Slp-BSU17580-pHLA, respectively.

The plasmids for genomic integration of BGL displayed on the cell surface were constructed as follows. The gene encoding the HCE promoter region, Tfu0937 ($\beta$-glucosidase), and anchor protein was amplified by PCR with the following primers: pZSint_EcoRI_HCEp_F and pZSint_XbaI_R. Plasmids named Blc-Tfu0937-pHLA, Tfu0937-Slp-pHLA, and Tfu0937-Blc-pHLA[20] were used as templates. Each amplified fragment was ligated into the EcoRI/XbaI site of plasmid pZS4int-laci (EXPRESSYS) using an In-Fusion HD kit (TAKARABIO, Inc., Shiga, Japan) according to the manufacturer's instructions. The resultant plasmids were named pZS4int-Blc-Tfu0937, pZS4int-Tfu0937-Blc, and pZS4int-Tfu0937-slp, respectively. These plasmids were digested with SpeI/AvrII, and the fragments were introduced into the *E. coli* JCM20137 or BW25113 genome, according to manufacturer's instructions. The resultant strains were named JCM-Blc-Tfu0937(bgl), JCM-Tfu0937(bgl)-Slp, JCM-Tfu0937(bgl)-Blc, BW-Blc-Tfu0937(bgl), BW-Tfu0937(bgl)-Slp, and BW-Tfu0937(bgl)-Blc, respectively.

All XYL-expression plasmids were introduced into *E. coli* JCM-Tfu0937(bgl)-Slp or JCM-Tfu0937(bgl)-Blc by electroporation using a Gene Pulser (Bio-Rad Laboratories, Hercules,


dx.doi.org/10.1021/sb400070q | *ACS Synth. Biol.* 2014, 3, 446−453

**Table 1. Strains, Plasmids, and Oligonucleotide Primers Used in This Study**

| strain, plasmid, or primer | relevant phenotype and description or sequence (5′−3′) | source or ref |
|---|---|---|
| Strains | | |
| *Escherichia coli* | | |
| NovaBlue | *endA1 hsdR17* (*rK12*$^{-}$ *mK12*$^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB*$^{+}$ *lacI*$^{q}$ZΔM15::Tn*10* (Tet$^{r}$)]; host for DNA manipulation | Novagen |
| BW25113 | *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lambda-, rph-1, Δ(rhaD-rhaB)568, hsdR514* | National Institute of Genetics, Japan |
| JCM20137 | | Japan Collection of Microorganisms, RIKEN BRC |
| XYL-displaying strains | | |
| JCM/Blc-BSU17580 | JCM20137 carrying a vector for XYL BSU17580 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of BSU17580 | |
| JCM/HdeD-BSU17580 | JCM20137 carrying a vector for XYL BSU17580 expression using HdeD anchor protein; the C-terminus of HdeD was fused to the N-terminus of BSU17580 | |
| JCM/Blc-Tfu1616 | JCM20137 carrying a vector for XYL Tfu1616 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of Tfu1616 | |
| JCM/HdeD-Tfu1616 | JCM20137 carrying a vector for XYL Tfu1616 expression using HdeD anchor protein; the C-terminus of HdeD was fused to the N-terminus of Tfu1616 | |
| BGL-displaying strains | | |
| JCM-Blc-Tfu0937(bgl) | JCM20137; HCE promoter, Blc-Tfu0937, and terminator region are integrated into its genome | this study |
| JCM-Tfu0937(bgl)-Slp | JCM20137; HCE promoter, Tfu0937-Slp, and terminator region are integrated into its genome | this study |
| JCM-Tfu0937(bgl)-Blc | JCM20137; HCE promoter, Tfu0937-Blc, and terminator region are integrated into its genome | this study |
| BW-Blc-Tfu0937(bgl) | BW25113; HCE promoter, Blc-Tfu0937, and terminator region are integrated into its genome | this study |
| BW-Tfu0937(bgl)-Slp | BW25113; HCE promoter, Tfu0937-Slp, and terminator region are integrated into its genome | this study |
| BW-Tfu0937(bgl)-Blc | BW25113; HCE promoter, Tfu0937-Blc, and terminator region are integrated into its genome | this study |
| Both BGL- and XYL-displaying strains | | |
| JCM-Tfu0937(bgl)-Blc/Blc-BSU17580 (xyl) | JCM-Tfu0937(bgl)-Blc carrying a vector for BSU17580 expression using Blc anchor protein | this study |
| JCM-Tfu0937(bgl)-Blc/HdeD-BSU17580 (xyl) | JCM-Tfu0937(bgl)-Blc carrying a vector for BSU17580 expression using HdeD anchor protein | this study |
| JCM-Tfu0937(bgl)-Blc/Blc-Tfu1616 (xyl) | JCM-Tfu0937(bgl)-Blc carrying a vector for Tfu1616 expression using Blc anchor protein | this study |
| JCM-Tfu0937(bgl)-Blc/HdeD-Tfu1616 (xyl) | JCM-Tfu0937(bgl)-Blc carrying a vector for Tfu1616 expression using HdeD anchor protein | this study |
| JCM-Tfu0937(bgl)-Slp/Blc-BSU17580 (xyl) | JCM-Tfu0937(bgl)-Slp carrying a vector for BSU17580 expression using Blc anchor protein | this study |
| JCM-Tfu0937(bgl)-Slp/HdeD-BSU17580 (xyl) | JCM-Tfu0937(bgl)-Slp carrying a vector for BSU17580 expression using HdeD anchor protein | this study |
| JCM-Tfu0937(bgl)-Slp/Blc-Tfu1616 (xyl) | JCM-Tfu0937(bgl)-Slp carrying a vector for Tfu1616 expression using Blc anchor protein | this study |
| JCM-Tfu0937(bgl)-Slp/HdeD-Tfu1616 (xyl) | JCM-Tfu0937(bgl)-Slp carrying a vector for Tfu1616 expression using HdeD anchor protein | this study |
| Genomic DNA | | |
| *Thermobifida fusca* | YX (ATCC no. BAA-629D-5) | ATCC |
| *Bacillus subtilis* | Type strain (NBRC no. 13719G) | NBRC |
| Plasmids | | |
| Blc-Tfu0937-pHLA | Vector for Tfu0937 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of Tfu0937 | 20 |
| Tfu0937-Slp-pHLA | Vector for Tfu0937 expression using Slp anchor protein; the N-terminus of Blc was fused to the C-terminus of Tfu0937 | 20 |
| Tfu0937-Blc-pHLA | Vector for Tfu0937 expression using Blc anchor protein; the N-terminus of Blc was fused to the C-terminus of Tfu0937 | 20 |



dx.doi.org/10.1021/sb400070q | ACS Synth. Biol. 2014, 3, 446−453

**Table 1. continued**

| strain, plasmid, or primer | relevant phenotype and description or sequence (5′−3′) | source or ref |
|---|---|---|
| Plasmids | | |
| Blc-BSU17580-pHLA | Vector for BSU17580 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of BSU17580 | this study |
| Slp-BSU17580-pHLA | Vector for BSU17580 expression using Slp anchor protein; the C-terminus of Slp was fused to the N-terminus of BSU17580 | this study |
| HdeD-BSU17580-pHLA | Vector for BSU17580 expression using HdeD anchor protein; the C-terminus of HdeD was fused to the N-terminus of BSU17580 | this study |
| Blc-Tfu1616-pHLA | Vector for Tfu1616 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of Tfu1616 | this study |
| Slp- Tfu1616-pHLA | Vector for Tfu1616 expression using Slp anchor protein; the C-terminus of Slp was fused to the N-terminus of Tfu1616 | this study |
| HdeD-Tfu1616-pHLA | Vector for Tfu1616 expression using HdeD anchor protein; the C-terminus of HdeD was fused to the N-terminus of Tfu1616 | this study |
| Blc-BSU17580-pHLA | Vector for BSU17580 expression using Blc anchor protein; the C-terminus of Blc was fused to the N-terminus of BSU17580 | this study |
| Slp-BSU17580-pHLA | Vector for BSU17580 expression using Slp anchor protein; the C-terminus of Blc was fused to the N-terminus of BSU17580 | this study |
| HdeD-Tfu0937-pHLA | Vector for Tfu0937 expression using HdeD anchor protein; the C-terminus of Blc was fused to the N-terminus of Tfu0937 | this study |
| Tfu0937- Blc-pHLA | Vector for Tfu0937 expression using Blc anchor protein; the N-terminus of Blc was fused to the C-terminus of Tfu0937 | this study |
| Tfu0937- Slp-pHLA | Vector for Tfu0937 expression using Slp anchor protein; the N-terminus of Blc was fused to the C-terminus of Tfu0937 | this study |
| Tfu0937- HdeD-pHLA | Vector for Tfu0937 expression using HdeD anchor protein; the N-terminus of Blc was fused to the C-terminus of Tfu0937 | this study |
| pZS4int-laci | Vectors for chromosomal Integration | EXPRESSYS |
| pZS4int-Blc-Tfu0937 | Vectors for chromosomal Integration of HCE promoter, Blc-Tfu0937, and terminator region | This study |
| pZS4int-Tfu0937-Blc | Vectors for chromosomal Integration of HCE promoter, Tfu0937-Blc, and terminator region | This study |
| pZS4int-Tfu0937-slp | Vectors for chromosomal Integration of HCE promoter, Tfu0937-Slp, and terminator region | This study |
| Oligonucleotide Primers | | |
| NotI_BSU17580_F | AGCTGCGGCCGCTATGAAGATTACCAATCCCGTACTTAAAGGATTCAATCCCG | |
| BSU17580_R | TTTAAAGCTTCTCGAGTTATTTTTCTTTATAACGAAAATATCTAAAGTCGGCCGGAATATG | |
| NotI_Tfu1616_F | AGCTGCGGCCGCTATGACGTCTCCCCAAGTCACGTCCTCCCCGTCTCGTGAGG | |
| Tfu1616_R | TTTAAAGCTTCTCGAGTCAGGAGGGGGACTGAGGCCGGTAGGTGCAGAAGTCGAAGTCGGC | |
| pZS4int_EcoRI_HCEp_F | ATCCCGCCCCGAATTTTGATCTCTCCTTCACAGATTCCCAATCTCTTGTTAAATAACGAAAAAGC | |
| pZSint_XbaI_R | TACTCAGCTATCTAGGCGTTCTGATTTAATCTGTATCAGGCTGAAAATCTTCTCTCATCCGCCAAAACAGCC | |



dx.doi.org/10.1021/sb400070q | ACS Synth. Biol. 2014, 3, 446−453

CA, USA) according to the manufacturer's instructions. The resultant strains are summarized in Table 1.

**Evaluation of XYL or BGL Activity.** XYL activity was screened by a plate assay as follows. *E. coli* strain NovaBlue carrying XYL-expression plasmids was seeded on LB agar plates containing 100 μg/mL of ampicillin. After overnight incubation at 37 °C, 0.75% soft agar containing 0.034% 4-methylumbelliferyl-β-D-xyloside (Sigma-Aldrich) in 50 mM acetate buffer at pH 5 was spread on the plate. After another 1 h incubation at 37 °C, the fluorescence of the colonies was observed using a FAS-III gel imager (Toyobo, Osaka, Japan).

To evaluate the XYL or BGL activity quantitatively, activity was measured in 50 mM sodium acetate buffer (pH 5.0) at 37 °C with 2 mM *p*-nitrophenyl-β-D-xylopyranoside (pNPX) or *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (Nacalai Tesque, Inc., Kyoto, Japan) as the substrate. After a 24 h of cultivation in LB medium, the cells were collected by centrifugation. The cells were washed with PBS three times and resuspended in the reaction buffer. The OD 600 of the reaction buffer was adjusted to 0.0375. The amount of released *p*-nitrophenol was determined by measuring the absorbance at 400 nm. One unit of β-xylosidase or β-glucosidase activity was defined as the amount of enzyme producing 1 μmol/min *p*-nitrophenol at 37 °C and pH 5.0.

**Growth Analysis.** Cell growth with cellobiose was evaluated using minimum medium containing 2% carbon source(s) at 37 °C. The initial OD 600 was adjusted to 0.01. The OD 600 was then monitored using a Biophotorecorder (Advantech, Tokyo, Japan).

## AUTHOR INFORMATION


### Corresponding Author

*Tel./Fax: +81-78-803-6196. E-mail: akondo@kobe-u.ac.jp.


### Author Contributions

T.T., Y.H., M.N., and H.K. performed the experiments and analyzed the data. T.T. wrote the paper. A.K. supervised the project.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS


This work was supported mainly by the Development of Preparatory Basic Bioenergy Technologies of the New Energy and Industrial Technology Development Organization (NEDO), Tokyo, and partially supported by The Noguchi Institute and the Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.


## REFERENCES


(1) Chandel, A. K., and Singh, O. V. (2011) Weedy lignocellulosic feedstock and microbial metabolic engineering: advancing the generation of 'Biofuel'. *Appl. Microbiol. Biotechnol. 89*, 1289−12303.

(2) Elkins, J. G., Raman, B., and Keller, M. (2010) Engineered microbial systems for enhanced conversion of lignocellulosic biomass. *Curr. Opin. Biotechnol. 21*, 657−662.

(3) Geddes, C. C., Nieves, I. U., and Ingram, L. O. (2011) Advances in ethanol production. *Curr. Opin. Biotechnol. 22*, 312−319.

(4) Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007) Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science 315*, 804−807.

(5) Lübken, M., Gehring, T., and Wichern, M. (2010) Microbiological fermentation of lignocellulosic biomass: Current state and prospects of mathematical modeling. *Appl. Microbiol. Biotechnol. 85*, 1643−1652.

(6) Polizeli, M. L., Rizzatti, A. C., Monti, R., Terenzi, H. F., Jorge, J. A., and Amorim, D. S. (2005) Xylanases from fungi: Properties and industrial applications. *Appl. Microbiol. Biotechnol. 67*, 577−591.

(7) Hanly, T. J., Urello, M., and Henson, M. A. (2012) Dynamic flux balance modeling of *S. cerevisiae* and *E. coli* co-cultures for efficient consumption of glucose/xylose mixtures. *Appl. Microbiol. Biotechnol. 93*, 2529−2541.

(8) Kim, J. H., Block, D. E., and Mills, D. A. (2010) Simultaneous consumption of pentose and hexose sugars: An optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. *Appl. Microbiol. Biotechnol. 88*, 1077−1085.

(9) Alterthum, F., and Ingram, L. O. (1989) Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. *Appl. Environ. Microbiol. 55*, 1943−1948.

(10) Trinh, C. T., and Srienc, F. (2009) Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. *Appl. Environ. Microbiol. 75*, 6696−6705.

(11) Atsumi, S., Hanai, T., and Liao, J. C. (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature 451*, 86−89.

(12) Mazumdar, S., Clomburg, J. M., and Gonzalez, R. (2010) *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. *Appl. Environ. Microbiol. 76*, 4327−4336.

(13) Zhang, X., Shanmugam, K. T., and Ingram, L. O. (2010) Fermentation of glycerol to succinate by metabolically engineered strains of *Escherichia coli*. *Appl. Environ. Microbiol. 76*, 2397−2401.

(14) Koma, D., Yamanaka, H., Moriyoshi, K., Ohmoto, T., and Sakai, K. (2012) Production of aromatic compounds by metabolically engineered *Escherichia coli* with an expanded shikimate pathway. *Appl. Environ. Microbiol. 78*, 6203−6216.

(15) 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*, 37-1−37-11.

(16) Chen, Y. (2011) Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review. *J. Ind. Microbiol. Biotechnol. 38*, 581−597.

(17) Dien, B. S., Nichols, N. N., and Bothast, R. J. (2002) Fermentation of sugar mixtures using *Escherichia coli* catabolite repression mutants engineered for production of L-lactic acid. *J. Ind. Microbiol. Biotechnol. 29*, 221−227.

(18) Eiteman, M. A., Lee, S. A., Altman, R., and Altman, E. (2009) A substrate-selective co-fermentation strategy with *Escherichia coli* produces lactate by simultaneously consuming xylose and glucose. *Biotechnol. Bioeng. 102*, 822−827.

(19) Vinuselvi, P., and Lee, S. K. (2012) Engineered *Escherichia coli* capable of co-utilization of cellobiose and xylose. *Enzyme Microb. Technol. 50*, 1−4.

(20) Tanaka, T., Kawabata, H., Ogino, C., and Kondo, A. (2011) Creation of a cellooligosaccharide-assimilating *Escherichia coli* strain by displaying active beta-glucosidase on the cell surface via a novel anchor protein. *Appl. Environ. Microbiol. 77*, 6265−6270.

(21) Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D. (2012) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature 463*, 559−562.

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

(23) Joshua, C. J., Dahl, R., Benke, P. I., and Keasling, J. D. (2011) Absence of diauxie during simultaneous utilization of glucose and xylose by *Sulfolobus acidocaldarius*. *J. Bacteriol. 193*, 1293−1301.

(24) Li, R., Chen, Q., Wang, P. G., and Qi, Q. (2007) A novel-designed *Escherichia coli* for the production of various polyhydroxyalkanoates from inexpensive substrate mixture. *Appl. Microbiol. Biotechnol. 75*, 1103−1109.
(25) Aeling, K. A., Salmon, K. A., Laplaza, J. M., Li, L., Headman, J. R., Hutagalung, A. H., and Picataggio, S. (2012) Co-fermentation of xylose and cellobiose by an engineered *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol. 39*, 1597−1604.
(26) Long, T. M., Su, Y. K., Headman, J., Higbee, A., Willis, L. B., and Jeffries, T. W. (2012) Cofermentation of glucose, xylose, and cellobiose by the beetle-associated yeast *Spathaspora passalidarum*. *Appl. Environ. Microbiol. 78*, 5492−5500.
(27) Oh, E. J., Ha, S. J., Rin, K. S., Lee, W. H., Galazka, J. M., Cate, J. H., and Jin, Y. S. (2013) Enhanced xylitol production through simultaneous co-utilization of cellobiose and xylose by engineered *Saccharomyces cerevisiae*. *Metab. Eng. 15*, 226−234.
(28) Shin, H. D., McClendon, S., Vo, T., and Chen, R. R. (2010) *Escherichia coli* binary culture engineered for direct fermentation of hemicellulose to a biofuel. *Appl. Environ. Microbiol. 76*, 8150−8159.