INTRODUCTION

In view of the depletion of petroleum resources globally, and increasing concerns about global warming, there is growing interest in the development of biorefineries to serve as a sustainable alternative solution. In past decades, considerable effort and progress have been made in the microbial production of bulk chemicals, which hitherto could only be produced by chemical processes. Microbial production of 2,3-butanediol (2,3-BDO), also known as dimethylene glycol, is one such example. Potentially, 2,3-BDO is a valuable bulk chemical owing to its extensive industrial application in the manufacture of...
fuels, printing inks, synthetic perfumes, plasticizers, foods, and pharmaceuticals (Bialkowska, 2016). Since the microbial production of 2,3-BDO was first reported in 1906 (Harden & Walpole, 1906), various microbes including *Aeromonas* (Willets, 1984), *Bacillus* (Guragain, Chitta, Karanjikar, & Vadlanì, 2017), *Corynebacterium* (Yang et al., 2015), *Serratia* (Zhang et al., 2010), and *Enterobacter genera* (Jung, Ng, Song, Lee, & Oh, 2012) have been used for the production of 2,3-BDO through the fermentation process (Bialkowska, 2016). However, *Klebsiella* species, particularly *Klebsiella oxytoca* and *K. pneumoniae*, are the most competitive host organisms, and production of significant quantities (more than 100 g/L) have been achieved using them (Bialkowska, 2016; Ji, Huang, & Ouyang, 2011; Ma et al., 2009; Rhie et al., 2019).

As the demand for economic and eco-friendly production of 2,3-BDO increases, the development of microorganisms capable of utilizing sugars from cheaper biomass is attracting attention to reduce the costs of fermentation because raw materials used in the microbial fermentation process are a major part in the cost of 2,3-BDO production. Accordingly, a lot of research efforts have been devoted to the utilization of biomass-derived sugars for microbial production of 2,3-BDO and other chemicals in biorefinery industries (Guragain et al., 2017; Madhavan, Srivastava, Kondo, & Bisaria, 2012; Ranganathan et al., 2017; Rhie et al., 2019). Lignocellulosic residues from forests and agriculture are the primary carbohydrate source, and the use of various lignocellulosics like corn stover, sugarcane bagasse, and soybean hull have been pursued for the 2,3-BDO production using microorganisms (Cortivo, Machado, Hickert, Rossi, & Ayub, 2019; Um, Kim, Jung, Saratale, & Oh, 2017; Yang et al., 2016). However, these lignocellulosic hydrolysates contain mixed sugars including hexoses (glucose, galactose, and mannose) and pentoses (xylose and ribose), and most bacterial hosts including *Klebsiella* sp. exhibit inefficient consumption of sugars during the microbial fermentation process. One major reason for this inefficient sugar consumption is carbon catabolite repression (CCR), a well-known regulatory mechanism, by which the utilization of secondary sugar sources is delayed in the presence of a preferred substrate such as glucose (Ji, Nie, et al., 2011; Wu, Shen, Yuan, & Yan, 2016). In addition, the relatively slow consumption of other sugars after depletion of glucose (Jin, Laplaza, & Jeffries, 2004) significantly decreases the overall sugar consumption rate and limits achievable productivity in the bioprocess. Overcoming this delayed and slow consumption of mixed sugars is essential for the development of an efficient and economic bioprocess, particularly for the development of a consolidated bioprocess (Brethauer & Studer, 2014; Gao, Ploessl, & Shao, 2018).

Here, we sought to engineer *K. oxytoca* to utilize glucose and other sugars together in mixed sugars derived from biomass hydrolysates. For this purpose, we introduced a xylose transporter gene (*xylE*) from *Escherichia coli* to facilitate efficient xylose uptake as a carbon source, and the methylglyoxal synthase A (*mgsA*) gene was deleted for efficient sugar metabolism. In addition to genetic engineering, we performed adaptive laboratory evolution (ALE) for 90 days to further improve the ability of sugar consumption. The evolved strain (CHA006) was characterized to verify its superiority in sugar utilization by cultivating in the medium containing one or two sugars. Finally, we evaluated the evolved strain for cell growth, different types of sugar consumption abilities, and 2,3-BDO production by using sunflower and pine tree hydrolysates as major sugars for cultivation.

# MATERIALS AND METHODS

## 2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *K. oxytoca* KCTC1686 (consistent with ATCC8724) were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, South Korea). *E. coli* XL1-Blue was used for gene cloning and plasmid maintenance. Cells were cultivated in Luria–Bertani (LB) broth or semi-defined medium supplemented with glucose (5 g/L), xylose (5 g/L), yeast extract (5 g/L), (NH₄)₂SO₄ (6.6 g/L), K₂HPO₄ (8.7 g/L), KH₂PO₄ (6.8 g/L), MgSO₄·7H₂O (0.25 g/L), and a trace metal solution (2%, v/v). The trace metal solution consisted of FeSO₄·7H₂O (0.05 g/L), ZnSO₄·7H₂O (0.001 g/L), MnSO₄·H₂O (0.001 g/L), and CaCl₂·2H₂O (0.001 g/L). The concentration of each sugar was varied according to the experiment. LB medium was used only for the initial cultivation prior to ALE, and all other cultivations were conducted in semi-defined medium. After cultivation in a tube (5 ml) overnight, the culture was transferred into 50 ml medium in a 250 ml Erlenmeyer flask with an initial optical density (OD₆₀₀) of 0.05, and cultivated at 37°C and 200 rpm for 12–24 hr.

For the preparation of sunflower hydrolysate, the sunflower stalk was hydrolyzed by CelliC Tect3 enzyme (150 FPU/ml) at 50°C and 200 rpm for 72 hr (Kim, Oh, et al., 2016). The hydrolysate was filtered with a 0.2 μm membrane filter. The pine tree hydrolysates, which were obtained through concentrated acidic saccharification (Jang & Choi, 2018), were kindly supplied by GS Caltex Corporation. Each 20 g/L hydrolysate (about 10 ml sunflower stalk; about 6 ml pine tree) was added to 50 ml semi-defined medium in a 250 ml flask, and the cells were cultivated at 37°C and 200 rpm.

## 2.2 Integration of xylE gene into the chromosomal DNA

To integrate the *xylE* gene into the *mgsA* gene loci in the chromosomal DNA of *K. oxytoca*, pJH0901-containing...
expression systems for λ-Red recombinase and cre-loxP gene (Park et al., 2017) was used. *K. oxytoca* harboring pJH0901 was cultivated at 30°C in the LB medium supplemented with 100 μg/ml ampicillin, 50 μg/ml streptomycin, and 0.2% (w/v) L-arabinose (13.3 mM) for the induction of the λ-Red recombinase expression, and then competent cells for electroporation were prepared as a standard protocol (Jeong, Lee, Lee, & Chang, 1998). For homologous recombination between the chromosomal DNA and a linear DNA fragment at the mgsA gene loci, the gene to be replaced was constructed with an *xylE* gene from *E. coli* and a chloramphenicol resistance gene (CmR) flanked by two homologous arms against both ends of the mgsA gene loci. The CmR flanked by loxLE and loxRE was amplified from pECmuloxC by polymerase chain reaction (PCR) using F1-Cm and R1-Cm. A His6-tagged xylE gene under the control of the strong constitutive promoter BBa_J23118, was synthesized by PCR with F1-xylE, R1-xylE, F2-xylE, and R-mgsA HR2-xylE as primers. Both constructs (CmR and xylE gene) were linked by overlap extension PCR with F-mgsA HRI-Cm, R2-mgsA HR2-xylE, F-HR1, and R-HR2 primers. The PCR product (2 μg) was transferred into 100 μl of *K. oxytoca* competent cells by using electroporation at a voltage of 2.5 kV using 0.2 cm cuvettes and a MicroPulser (Bio-Rad). The transformed cells were then selected on an LB agar plate supplemented with 0.2% (w/v) arabinose, 100 μg/ml ampicillin, 8.75 μg/ml chloramphenicol, and 25 μg/ml streptomycin at 30°C. The integrated clones were confirmed by colony PCR with F-cm and R-xylE primers. The confirmed clones were cultured in LB broth supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside, 100 μg/ml ampicillin, and 25 μg/ml streptomycin at 30°C to remove the CmR gene by inducing Cre recombinase expression. The curing of pJH0901 was conducted by growing the cells at 37°C in LB broth supplemented with 100 μg/ml ampicillin because the plasmid has a temperature-sensitive origin of replication. All PCRs were carried out by using a C1000™ Thermal Cycler (Bio-Rad) with PrimeSTAR HS Polymerase (Takara). All primers used in this study are listed in Table S1.

### 2.3 Adaptive laboratory evolution

CHA004 cells were cultivated in semi-defined medium (50 ml) containing 20 g/L xylose in 250 ml Erlenmeyer flasks. The cultivation was performed at 0.05 of initial OD<sub>600</sub> in a shaking incubator at 37°C, 200 rpm, and at every 12 hr (early stationary phase); cells were transferred to fresh medium at the same initial cell density (0.05 of OD<sub>600</sub>). In total, 180 serial transfers for 90 days were performed, and cell growth rate and sugar consumption rate were compared on every 30 days.

### 2.4 Other analytical methods

Growth of the bacterial cells was monitored by measuring the optical density of the culture at a wavelength of 600 nm (OD<sub>600</sub>) using a UV/VIS Spectrophotometer (Optizen Pop; Mecasys). To measure the concentrations of sugars and 2,3-BDO, the cells were pelleted by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was filtrated with a 0.22 μm syringe filter (Futecs), and analyzed using high performance liquid chromatography (HPLC). The HPLC system (Shimadzu) consisted of a pump (LC-20AD), an autosampler (SIL-30AC), a column oven (CTO-20A), and a refractive index detector (RID-10A). An Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad) was eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> as the mobile phase at a flow rate of 0.5 ml/min, operating at 65°C. A chromatographic software Lab solution (Shimadzu) was used for sample acquisition and...
For the analysis of four sugars (glucose, xylose, galactose, and mannose) in pine tree hydrolysate, carbohydrate analysis system (Dionex™ ICS-5000; Thermo Scientific) with Asahipak NH 2P-50 column was used. An 80% (v/v) acetonitrile was used as a mobile phase at a flow rate of 1 ml/min, and the oven temperature was maintained at 30°C.

3 | RESULTS

3.1 | Chromosomal integration of XylE

Previously, we analyzed sugar utilization ability in *K. oxytoca*, and found that among four major sugars, namely xylose, glucose, galactose, and mannose, xylose utilization ability was much lower than that of the other sugars (Kim et al., 2015). Therefore, to elevate mixed sugar utilization ability, we focused on engineering xylose utilization ability. For efficient xylose utilization, we first introduced the *xylE* gene of *E. coli*, and for gene integration, we chose the *mgsA* loci in the chromosome of *K. oxytoca*. MgsA, an enzyme initiating the methylglyoxal pathway as an alternative route of glycolysis, converts dihydroxyacetone-phosphate to methylglyoxal, which is known as an inhibitor of sugar metabolism (Tötemeyer, Booth, Nichols, Dunbar, & Booth, 1998; Zhu, Skraly, & Cameron, 2001). It is known that co-metabolism of multiple sugars in *E. coli* was accelerated by deletion of the *mgsA* gene (Yomano, York, Shanmugam, & Ingram, 2009). We also previously confirmed that disruption of the *mgsA* gene in *K. oxytoca* resulted in improved utilization of multiple sugars (glucose and galactose) and higher cell growth (Park et al., 2017). An *xylE* gene expression system under a synthetic BBa_J23118 promoter (Andersen et al., 1998), was integrated into the *mgsA* gene loci of *K. oxytoca* to generate *K. oxytoca* CHA004. To evaluate the effect of *xylE* expression on xylose consumption, cells were grown in a semi-defined medium supplemented with xylose (5 g/L) and glucose (5 g/L). As shown in Figure 1, it was clearly observed that the CHA004 strain consumed xylose much faster than the wild-type (WT) strain. After the depletion of glucose (6 hr), xylose began to be consumed and depleted in 14 hr, which is a typical CCR phenomenon. The CHA004 strain also showed rapid consumption of glucose, but xylose consumption began before the depletion of glucose (~6 hr). At 10 hr, all xylose was consumed, which was 4 hr faster than that of the WT strain. The xylose-specific consumption rate increased to 0.55 g L⁻¹ hr⁻¹, 1.4-fold higher than that of the WT strain (0.40 g L⁻¹ hr⁻¹; Table 2). Consequently, the overall sugar consumption rate in CHA004 strains (1.04 g L⁻¹ hr⁻¹) was also 1.4-fold higher than that of the WT strain (0.75 g L⁻¹ hr⁻¹). From these results, we concluded that chromosomal integration of heterologous *xylE* gene into the *mgsA* loci drives to improve xylose utilization in *K. oxytoca*.

3.2 | ALE of CHA004

To further enhance xylose consumption with CHA004 strain, we performed the ALE strategy, which is considered a powerful tool to acquire desired environmental phenotypes through natural improvement of the microorganism (Sandberg, Lloyd, Palsson, & Feist, 2017). ALE of CHA004 was performed in a medium containing xylose (20 g/L) as a sole carbon source for 90 days. As ALE progressed, cells were collected every 30 days (30, 60, and 90 days), and its performance in cell growth, sugar consumption, and 2,3-BDO production were determined by cultivation in a medium containing xylose and glucose (5 g/L each). Cell-specific growth rate (μ) gradually increased and finally reached 0.76 hr⁻¹ on day 90, which was 2.04-fold higher than that of the parent CHA004 strain (0.38 hr⁻¹; Figure S1; Table S2). The rate of xylose consumption also increased gradually from 0.53 g L⁻¹ hr⁻¹ (CHA004) to 4.28 ± 0.74 g L⁻¹ hr⁻¹ on day 90, which was 2.04-fold higher than that of the parent CHA004 strain (0.38 g L⁻¹ hr⁻¹; Figure S1; Table S2).
To verify the superiority of CHA006 in sugar utilization and 2,3-BDO production, cells were cultivated separately in two media containing xylose (20 g/L) or glucose (20 g/L). As shown earlier, the CHA006 strain showed remarkably faster depletion of xylose on day 90 than that of CHA004 (Figure S1). Although ALE was carried out in a medium containing xylose as the sole carbon source (without glucose), we also found that glucose consumption rate on day 90 was improved compared with CHA004 (Figure S1). As the consumption rate of both sugars increased, the yield and productivity of 2,3-BDO were improved (Table S2). After ALE for 90 days, cells were spread on LB agar plate. After evaluation of random clones, one best candidate (CHA006) was selected based on the levels of cell growth, sugar consumption, and 2,3-BDO production for further experiments.

### 3.3 Characterization of the evolved strain (CHA006) through cultivation with a single sugar

| Sugar | Strain | Max. cell density (OD$_{600}$) | Consumption rate (g L$^{-1}$ hr$^{-1}$) | 2,3-BDO |
|-------|--------|-------------------------------|--------------------------------------|---------|
|       |        |                               | Xylose | Glucose | Galactose | Mannose | Titer (g/L) | Yield (g/g) | Productivity (g L$^{-1}$ hr$^{-1}$) |
| Xylose (20 g/L) | CHA004 | 5.19 ± 0.38 | 1.30 ± 0.00 | — | — | — | 5.59 ± 0.32 | 0.29 ± 0.02 | 0.37 ± 0.02 |
|           | CHA006 | 7.17 ± 0.13 | 1.95 ± 0.01 | — | — | — | 5.92 ± 0.06 | 0.31 ± 0.01 | 0.59 ± 0.01 |
| Glucose (20 g/L) | CHA004 | 4.99 ± 0.07 | — | 1.52 ± 0.02 | — | — | 6.18 ± 0.09 | 0.30 ± 0.00 | 0.48 ± 0.01 |
|           | CHA006 | 7.18 ± 0.09 | — | 2.40 ± 0.00 | — | — | 6.73 ± 0.12 | 0.31 ± 0.01 | 0.75 ± 0.01 |
| Galactose (20 g/L) | CHA004 | 5.28 ± 0.51 | — | — | 1.70 ± 0.07 | — | 5.41 ± 0.11 | 0.29 ± 0.00 | 0.49 ± 0.01 |
|           | CHA006 | 6.58 ± 0.28 | — | — | 1.88 ± 0.11 | — | 5.85 ± 0.08 | 0.30 ± 0.00 | 0.56 ± 0.03 |
| Mannose (20 g/L) | CHA004 | 5.47 ± 0.13 | — | — | — | 1.35 ± 0.01 | 4.97 ± 0.06 | 0.25 ± 0.00 | 0.33 ± 0.00 |
|           | CHA006 | 6.98 ± 0.03 | — | — | — | 2.06 ± 0.01 | 6.11 ± 0.01 | 0.30 ± 0.00 | 0.61 ± 0.00 |
| Xylose (10 g/L) & Glucose (10 g/L) | CHA004 | 5.63 ± 0.27 | 0.61 ± 0.00 | 0.94 ± 0.00 | — | — | 5.08 ± 0.13 | 0.25 ± 0.00 | 0.34 ± 0.01 |
|           | CHA006 | 7.08 ± 0.11 | 0.79 ± 0.00 | 1.72 ± 0.00 | — | — | 5.82 ± 0.08 | 0.29 ± 0.01 | 0.45 ± 0.01 |

Abbreviation: 2,3-BDO, 2,3-butanediol.
improved characteristics compared with the parental CHA004 strain in both the xylose or glucose media (Figure 2). In the xylose medium, CHA006 showed much faster cell growth than CHA004; CHA006 rapidly reached the stationary phase (9 hr) 5 hr earlier than CHA004 (14 hr), and its maximum cell density (7.17 at OD_{600}) was also much higher than CHA004 (5.19 at OD_{600}; Figure 2a; Table 3), which means higher yield of biomass (Y_{X/S}). CHA006 exhibited superior consumption of xylose compared with CHA004. Xylose was completely consumed within 10 hr, which was about 4–5 hr earlier than CHA004 (Figure 2b), and xylose consumption rate (1.95 g L^{-1} hr^{-1}) was also increased by 1.5-fold over CHA004 (1.30 g L^{-1} hr^{-1}; Table 3). Productivity of 2,3-BDO (0.59 g L^{-1} hr^{-1}) by CHA006 was also 1.6-fold higher than that of CHA004 (0.37 g L^{-1} hr^{-1}), which may be attributed to efficient sugar consumption as an adaptive evolutionary effect (Figure 2c; Table 3). Similarly, even from the results of the glucose medium, CHA006 showed rapid cell growth (Figure 2d) and faster glucose consumption rate (60% increase) compared with CHA004 (Figure 2e; Table 3). CHA006 also exhibited higher productivity of 2,3-BDO (0.75 g L^{-1} hr^{-1}) than CHA004 (0.48 g L^{-1} hr^{-1}) in the glucose medium. During CHA006 cultivation in xylose or glucose media, the titer of 2,3-BDO reached the maximum value at 10 or 9 hr, respectively, and then rapidly decreased (Figure 2c,f). The last step in the 2,3-BDO biosynthesis is the conversion of acetoin to 2,3-BDO, which is reversibly mediated by acetoin reductase (Yang, Rathnasingh, Lee, & Seung, 2014). We analyzed the titer of acetoin, and observed that it increased rapidly immediately after reaching the maximum titer of 2,3-BDO (at 10 hr or 9 hr), which indicates the reverse synthesis of acetoin from 2,3-BDO after depletion of the carbon source (Figure S2). In the cultivation of CHA004, acetoin also accumulated after reaching the maximum titer of 2,3-BDO at 13 hr after the depletion of the carbon source.

In addition to xylose or glucose as a single carbon source, we also evaluated the utilization of galactose or mannose in CHA006. Cells were cultivated in the media containing 20 g/L galactose or mannose, and all parameters namely cell growth, sugar consumption, and 2,3-BDO productivity were analyzed. Similar to the earlier evaluations for xylose or glucose, CHA006 also showed improved performance for galactose or mannose utilization (Figure 3). Particularly, CHA006 showed much improved utilization of mannose; CHA006 depleted mannose in 10 hr, which was 5 hr faster than CHA004 and produced 2,3-BDO to the maximum titer at 10 hr with 85% increased productivity compared to CHA004 (Figure 3f; Table 3). In summary, we concluded that the evolved CHA006 strain successfully acquired superior ability in the utilization of major sugars, and it can be a potential host for the utilization of mixed sugars.

3.4 Characterization of CHA006 through cultivation with two sugars

Next, we investigated the performance of CHA006 in a medium supplemented with two sugars (10 g/L xylose and
10 g/L glucose) to confirm the evolutionary effect on the utilization of mixed sugars. Similar to cultivation in single sugar, CHA006 showed much faster cell growth and higher cell density (7.08 of OD600) than CHA004 (5.63 of OD600; Figure 4a). In both strains, glucose was consumed first, and then xylose was utilized after the depletion of glucose. CHA006 depleted glucose completely within 6 hr, and xylose was consumed immediately, and depleted within 13 hr (Figure 4b,c). In contrast, CHA004 showed slower consumption of both sugars; glucose was completely depleted within 11 hr while xylose was consumed immediately and depleted within 17 hr (Figure 4b,c). Although CCR was not eliminated in CHA006, glucose and xylose consumption rates (1.72 and 0.79 g L⁻¹ hr⁻¹, respectively) in CHA006 were much higher than those in CHA004 (0.94 and 0.61 g L⁻¹ hr⁻¹, respectively; Table 3). It was also clearly observed that all 2,3-BDO profiles like titer, yield, and productivity were improved remarkably in CHA006 (Figure 4d; Table 3). By rapid consumption of both sugars in CHA006, 2,3-BDO production began early, and reached the maximum concentration (5.82 g/L) within 13 hr, and then gradually decreased. The productivity of 2,3-BDO (0.45 g L⁻¹ hr⁻¹) in CHA006 was 1.32-fold higher than that of CHA004 (0.34 g L⁻¹ hr⁻¹; Table 3).

3.5 | Production of 2,3-BDO by cultivation with lignocellulosic biomass hydrolysates

Finally, we examined the production of 2,3-BDO by CHA006 using two biomass hydrolysates derived from sunflower stalk and pine tree. Sunflower is one of the most abundant agricultural crop residues in the world. Its straw is a promising second generation feedstock for biofuel production such as ethanol, hydrogen, and lignin biopolymers, due to its abundance (3–7 tons dry biomass of sunflower straw per hectare of the cultivation; Antonopoulou, Vayenas, & Lyberatos, 2016; Kim, Yu, & Lee, 2016). Pine, a softwood, is also one of the renewable biomass that can be used for the production of advanced biofuels, energy, and value-added chemicals such as ethanol and butanol (Li et al., 2018; Vaid, Nargotra, & Bajaj, 2018). In this study, a pine tree trunk-derived hydrolysate was used. We confirmed the sugar composition of two hydrolysates; the hydrolysate from sunflower stalk contained glucose and xylose as the major sugars in a ratio of 3.6:1, and the hydrolysate from pine tree contained glucose, xylose, galactose, and mannose as the major sugars in a ratio of 9:1:3:1:3.9. First, cells were cultivated in the medium containing sunflower stalk hydrolysate containing 20 g/L mixed sugars (xylose and glucose). As shown in Figure 5, CHA006 showed relatively faster depletion of xylose (14 hr) and glucose (8 hr) compared with the WT (18 hr for xylose and 12 hr for glucose), and CHA004 (16 hr for xylose and 12 hr for glucose). Particularly, CHA006 showed about 1.6-fold higher glucose consumption rate (2.14 g L⁻¹ hr⁻¹) than that of the others (Table 4). In CHA006, 2,3-BDO production reached the maximum titer (6.11 g/L) at 8 hr, which was much faster than that of CHA004 (5.82 g/L) at 13 hr. In contrast, CHA004 showed slower consumption of both sugars; glucose was completely depleted within 11 hr while xylose was consumed immediately and depleted within 17 hr (Figure 4b,c). Although CCR was not eliminated in CHA006, glucose and xylose consumption rates (1.72 and 0.79 g L⁻¹ hr⁻¹, respectively) in CHA006 were much higher than those in CHA004 (0.94 and 0.61 g L⁻¹ hr⁻¹, respectively; Table 3). It was also clearly observed that all 2,3-BDO profiles like titer, yield, and productivity were improved remarkably in CHA006 (Figure 4d; Table 3). By rapid consumption of both sugars in CHA006, 2,3-BDO production began early, and reached the maximum concentration (5.82 g/L) within 13 hr, and then gradually decreased. The productivity of 2,3-BDO (0.45 g L⁻¹ hr⁻¹) in CHA006 was 1.32-fold higher than that of CHA004 (0.34 g L⁻¹ hr⁻¹; Table 3).

### Figure 5

Time profiles of (a) cell growth, (b, c) sugar concentration, and (d) production of 2,3-butanol (2,3-BDO) in wild-type (WT), CHA004, and CHA006 during cultivation with sunflower stalk hydrolysate containing 20 g/L mixed sugars (xylose and glucose). Symbols: ●, WT; ●, CHA004; □, CHA006. Error bars represent the standard deviation of duplicate experiments.

### Table 4

Comparison of cell growth and sugar consumption rate of WT and engineered strains (CHA004 and CHA006) during cultivation in sunflower or pine tree hydrolysates

| Hydrolysates | Strain | Max. cell density (OD₆₀₀) | Sugar consumption rate (g L⁻¹ hr⁻¹) |
|--------------|--------|---------------------------|-----------------------------------|
|              |        |                           | Glucose | Xylose | Galactose | Mannose | Overall   |
| Sunflower    | WT     | 7.17 ± 0.01                | 1.54 ± 0.01 | 0.27 ± 0.00 | —         | —         | 1.27 ± 0.01 |
|              | CHA004 | 5.05 ± 0.13                | 1.35 ± 0.04 | 0.30 ± 0.00 | —         | —         | 1.42 ± 0.02 |
|              | CHA006 | 7.92 ± 0.23                | 2.14 ± 0.00 | 0.34 ± 0.00 | —         | —         | 1.45 ± 0.02 |
| Pine tree    | WT     | 3.89 ± 0.10                | 1.04 ± 0.01 | 0.05 ± 0.02 | 0.09 ± 0.00 | 0.24 ± 0.00 | 0.83 ± 0.03 |
|              | CHA004 | 3.38 ± 0.31                | 0.84 ± 0.00 | 0.11 ± 0.02 | 0.04 ± 0.01 | 0.24 ± 0.00 | 1.01 ± 0.00 |
|              | CHA006 | 6.52 ± 0.31                | 1.57 ± 0.00 | 0.17 ± 0.00 | 0.18 ± 0.00 | 0.63 ± 0.00 | 1.76 ± 0.01 |

Abbreviation: WT, wild-type.
Next, WT, CHA004, and CHA006 were cultivated in the media containing 20 g/L pine tree hydrolysate which contained glucose (12.5 g/L), mannose (5 g/L), xylose (2 g/L), and galactose (1.4 g/L), and the changes in cell growth, sugar consumption, and 2,3-BDO production were compared. Similar to the results obtained with sunflower hydrolysates (Figure 5), CHA006 showed greatly improved cell growth than the WT and CHA004 strains, and achieved almost two-fold higher cell density (6.52 of OD_{600}) than the other strains (3.38–3.89 of OD_{600}; Figure 6a). As we confirmed during cultivation with each sugar (Figures 2 and 3), CHA006 showed much higher consumption of all four sugars (particularly galactose and mannose), and the overall sugar consumption rate in CHA006 increased by 1.75- and 2.12-fold compared with CHA004 and WT strains, respectively (Figure 6b–e; Table 4). Due to the faster rate of sugar consumption, the productivity of 2,3-BDO in CHA006 (0.73 g L^{-1} hr^{-1}) also increased remarkably (more than threefold) compared with those of CHA004 and WT (0.23–0.24 g L^{-1} hr^{-1}; Figure 6f; Table 5).

### DISCUSSION

In this work, to produce 2,3-BDO using mixed sugars derived from inexpensive biomass, we engineered *K. oxytoca* by integrating the *xylE* gene expression system and ALE. Although we performed ALE in a medium containing xylose only, the engineered CHA006 showed much improved abilities in the utilization of xylose as well as glucose, galactose, and mannose, which are major sugars in most lignocellulosic biomass. However, we found the typical CCR phenomena was still observed when CHA006 was cultivated in the media containing glucose with other sugars. CCR by preferable glucose cause the reduction of overall sugar uptake in the cultivation, which is the most important issue in the utilization of multiple sugars (Kim et al., 2015). By the engineering (deletion or modification) of key components such as phosphoenolpyruvate-dependent phosphotransferase system (PTSGlc), CCR could be eliminated, and glucose and other sugars were utilized simultaneously (Ji, Nie, et al., 2011; Park et al., 2017). However, in many cases, engineering of PTSGlc caused the decrease of glucose consumption rate, so the overall sugar consumption rate was not improved significantly (Nichols, Dien, & Bothast, 2001; Sandberg et al., 2017). Although CCR was not eliminated in the present study, CHA006 showed relatively weak CCR compared to WT strain, and it was clearly observed that consumption rates for glucose was

| Hydrolysates | Strain | 2,3-BDO Titer (g/L) | Yield (g/g) | Productivity (g L^{-1} hr^{-1}) |
|--------------|--------|---------------------|------------|-------------------------------|
| Sunflower    | WT     | 5.48 ± 0.01         | 0.25 ± 0.00 | 0.32 ± 0.00                   |
|              | CHA004 | 6.14 ± 0.08         | 0.29 ± 0.00 | 0.41 ± 0.01                   |
|              | CHA006 | 6.11 ± 0.26         | 0.34 ± 0.02 | 0.76 ± 0.03                   |
| Pine tree    | WT     | 4.33 ± 0.12         | 0.23 ± 0.01 | 0.24 ± 0.08                   |
|              | CHA004 | 4.06 ± 0.13         | 0.22 ± 0.03 | 0.23 ± 0.05                   |
|              | CHA006 | 5.82 ± 0.07         | 0.30 ± 0.00 | 0.73 ± 0.01                   |

Abbreviations: 2,3-BDO, 2,3-butanediol; WT, wild-type.

**FIGURE 6** Time profiles of (a) cell growth, (b–e) sugar concentration, and (f) production of 2,3-butane diol (2,3-BDO) in wild-type (WT), CHA004 and CHA006 during cultivation with pine tree hydrolysate containing 20 g/L mixed sugars (xylose, glucose, galactose, and mannose). Symbols: ○, WT; ●, CHA004; ■, CHA006. Error bars represent the standard deviation of duplicate experiments.
maintained or a little improved in the cultivation with a sole glucose as well as multiple sugars. Under this high glucose consumption, cells grew better and utilized other sugars more efficiently. So, the overall sugar consumption rate and 2,3-BDO productivity were also increased in the cultivation with multiple sugars (Figures 4–6), which indicates that *K. oxytoca* CHA006 was successfully engineered and can be a potential host for the utilization of multiple sugars. Still, it is not clear yet how CHA006 strain can consume mixed sugars more efficiently after ALE in xylose media. Recently, Wang et al. (2018) reported that *Corynebacterium glutamicum* evolved in a sole glucose media also acquired the improved properties in the utilization of other sugars including sucrose, fructose, and xylose. Through omics studies (whole genome resequencing, transcriptomic analysis, and metabolic flux analysis), they revealed that two mutations in transcriptional regulators (GntR1 and RamA) enhanced the central metabolism flux, which leads to the improvement of multiple sugar utilization and cell growth. In the present work, to fully understand the evolved mechanism in CHA006 strain, it is also necessary to collect more data through omics studies (e.g., full genome sequencing for identification of key mutations, transcriptome and proteome analysis for gene expression, and metabolome analysis for metabolic flux), which studies will be performed in future.

Using two hydrolysates of biomass from sunflower and pine tree, we demonstrated that CHA006 strain has much more superiority in sugar utilization than the parental and WT strains (Figures 5 and 6). During the cultivation in each hydrolysate, the engineered cells showed a little difference in performance; particularly utilization of xylose in the three strains. In the sunflower hydrolysate, all strains showed a little difference in xylose consumption, but in the pine tree hydrolysate, WT strain showed much delayed and slower consumption of xylose (Figures 5 and 6). Regarding these differences, we first considered the different ratios of the sugars in each hydrolysate. Pine tree hydrolysate contained two more sugars (galactose and mannose) in addition to xylose and glucose, and it was previously confirmed that *K. oxytoca* WT preferred galactose and mannose than xylose (Kim et al., 2015). Therefore, WT cells could not consume xylose before depletion of the three sugars (16–20 hr), which caused much delay, and resulted in the incomplete consumption of xylose as compared to the other strains (CHA004 and CHA006). Second, we considered the presence of toxic compounds and unknown byproducts in each hydrolysate. In the use of lignocellulosic hydrolysates for fermentation, one major issue is the contamination of toxic compounds such as acetic acid, furfural, 4-hydroxybenzoic acid, phenol, and 5-hydroxymethyl furfural (5-HMF), which are generated during acid treatment for biomass hydrolysis (Almeida, Bertilsson, Gorwa-Grauslund, Gorsich, & Lidén, 2009). These toxic compounds inhibit protein synthesis (Eiteman & Altman, 2006) or function of several key enzymes including pyruvate dehydrogenase and alcohol dehydrogenase (Modig, Liden, & Taherzadeh, 2002), which cause severe inhibitory effect on cell growth and product production. Wu et al. reported the effect of three toxic compounds (acetic acid, furfural, and 5-HMF) on the growth of *K. oxytoca* and 2,3-BDO production: the 2,3-BDO yield was sensitive to acetic acid concentration, and elimination of furfural in the hydrolysate improved growth of *K. oxytoca* and 2,3-BDO production (Wu et al., 2013). To achieve higher production, a detoxification process such as carbon adsorption is generally required (Li et al., 2016). In the present work, sunflower stalk and pine tree hydrolysates were prepared through enzymatic and concentrated acidic saccharification process, respectively, and those toxic inhibitors were not eliminated before being utilized for cultivation. The exact composition of toxic compounds in each hydrolysate is not determined yet, but enzymatic hydrolysis generally generates much less toxic compounds compared with acid treatment, which means that the use of sunflower hydrolysate may provide a more preferable condition to the host, showing higher cell growth than in pine tree hydrolysate (Figures 5a and 6a).

In addition to xylose, we also noted the consumption of galactose. When cells were cultivated with galactose as a sole carbon source, CHA006 exhibited just a little higher consumption of galactose compared to CHA004 (Figure 3b). On the other hand, in cultivation with pine tree hydrolysate, CHA006 exhibited much faster consumption of galactose (depletion in 8 hr) than CHA004 (depletion in 21 hr; Figure 6d). For this change in galactose consumption, we consider a different cell density caused by a different sugar consumption during cultivation in hydrolysates. In the early stage (0–8 hr), CHA006 grew much faster than CHA004 due to rapid consumption of glucose and mannose (Figure 6c,e), and then the increased cells could drive much better utilization of other sugars (xylose and galactose) compared to CHA004. During cultivation in a single galactose medium (Figure 3), both CHA004 and CHA006 consumed galactose in similar rates during the early stage, which did not make any difference in cell growth, so overall consumption rate of galactose was not different. These results clearly indicate the beneficial property of CHA006 strain in the utilization of hydrolysates containing mixed sugars.

In conclusion, by *xylE* integration and ALE, *K. oxytoca* was successfully engineered to utilize multiple sugars derived from biomass hydrolysates and, with the engineered CHA006 strain, higher cell growth and 2,3-BDO productivity could be achieved. Until now, there have been several reports showing efficient consumption of two, three, or biomass hydrolysates, but to the best of our knowledge, this
study is the first report that presents a detailed improved profiles (max. cell density, sugar consumption rate, and metabolite production) for each sugar under the cultivation with four sugars. As demonstrated with real biomass hydrolysates, the engineered CHA006 can be a potential host for the economic production of 2,3-BDO from various renewable biomass hydrolysates. The sugar utilization ability in CHA006 will be further improved by identifying key mutations acquired by using ALE, which can be done through extensive omics analysis including full genome sequencing, RNA-Seq-based transcriptome analysis, and metabolomics. We believe that acquiring these information can give more understanding about sugar utilization in microbial hosts. Based on those information, CHA006 will be further engineered and a much efficient and economic biorefinery process can be developed. Particularly, the engineered K. oxytoca has great potential in fed-batch mode, which supplies nutrient feeding solutions during fermentation. Through efficient consumption of multiple sugars, the engineered cells can grow rapidly to a much higher cell density without accumulation of unused sugars. In this way, the time for cultivation can be remarkably reduced and much higher productivity can be achieved.

ACKNOWLEDGEMENTS
This work was supported by the Intelligent Synthetic Biology Center of the Global Frontier Project (grant no. NRF-2014M3A6A8066443) and by the Advanced Biomass R&D Center (ABC) of Global Frontier Project (grant no. ABC-2015M3A6A2074238) funded by the Ministry of Science and ICT (MSIT).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

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**How to cite this article:** Cha JW, Jang SH, Kim YJ, Chang YK, Jeong KJ. Engineering of Klebsiella oxytoca for production of 2,3-butanediol using mixed sugars derived from lignocellulosic hydrolysates. *GCB Bioenergy*. 2020;12:275–286. https://doi.org/10.1111/gcbb.12674