Glucose/Xylose Co-Fermenting *Saccharomyces cerevisiae* Increases the Production of Acetyl-CoA Derived n-Butanol From Lignocellulosic Biomass

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Efficient xylose catabolism in engineered *Saccharomyces cerevisiae* enables more economical lignocellulosic biorefinery with improved production yields per unit of biomass. Yet, the product profile of glucose/xylose co-fermenting *S. cerevisiae* is mainly limited to bioethanol and a few other chemicals. Here, we introduced an n-butanol-biosynthesis pathway into a glucose/xylose co-fermenting *S. cerevisiae* strain (XUSEA) to evaluate its potential on the production of acetyl-CoA derived products. Higher n-butanol production of glucose/xylose co-fermenting strain was explained by the transcriptomic landscape, which revealed strongly increased acetyl-CoA and NADPH pools when compared to a glucose fermenting wild-type strain. The acetate supplementation expected to support acetyl-CoA pool further increased n-butanol production, which was also validated during the fermentation of lignocellulosic hydrolysates containing acetate. Our findings imply the feasibility of lignocellulosic biorefinery for producing fuels and chemicals derived from a key intermediate of acetyl-CoA through glucose/xylose co-fermentation.

**Keywords:** *Saccharomyces cerevisiae*, glucose/xylose co-fermentation, n-butanol, acetyl-CoA, acetate, lignocellulosic biomass

**INTRODUCTION**

Lignocellulosic biomass offers a sustainable and environmentally friendly source of raw materials for producing fuels and chemicals (Ko and Lee 2018). Commercial bioethanol production has been achieved using the yeast *Saccharomyces cerevisiae* and sugar- or starch-based biomass. To improve the economic feasibility of lignocellulosic biorefinery, *S. cerevisiae* strains have been engineered to co-ferment glucose and xylose, the most abundant hexose and pentose sugars in lignocellulosic hydrolysates, respectively, resulting in significantly improved lignocellulosic bioethanol yields, titers, and productivity (Hoang Nguyen Tran et al., 2020). In addition, increasing efforts have also been devoted to expanding the product profile of *S. cerevisiae* to include advanced fuels and chemicals demonstrating the great potential of microbial cell factories for biorefinery (Ekas et al., 2019). Yet, the production of non-native products using both glucose and xylose derived from...
This study shows the potential of a glucose-CoA-fermenting strain in the production of acetyl-CoA-derived n-butanol from lignocellulosic biomass.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**
All strains used in this study are shown in Table 1. The yeast strains used in this study were isogenic with respect to *S. cerevisiae* S288C BY4741. The yeast strains were routinely cultivated at 30 °C in yeast synthetic complete (YSC) medium composed of xylose (and/or glucose), 6.7 g/L of yeast nitrogen base (Difco, Detroit, MI, United States), and CSM-HIS-LEU-URA (MP Biomedicals, Solon, Ohio, United States). *Escherichia coli* DH10β cells were employed for DNA manipulation and were cultured at 37°C in LB medium supplemented with 100 μg/ml ampicillin.

**Plasmid Construction**
All plasmids used in this study are shown in Table 2. The homologous genes used in this study were ERG10 and ETR1, and the heterologous genes were EutE from *E. coli*, *Kr* and *Htd* from *Yarrowia lipolytica*, and *Hbd*, *Crt*, *AdhE2*, and *BdhB* from *Clostridium acetobutylicum*. The start codon of *BdhB* was changed from GTG to ATG, and the mitochondria-targeting sequences of the *Kr*, *Htd*, and *Etr1* proteins were predicted and excluded using TargetP software version 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and the MITOPROT online tools (https://ihg.gsf.de/ihg/mitoprot.html) according to the reference by Lian and Zhao (2015). Each gene cassette was cloned into an expression vector using the Gibson Assembly method and introduced in *S. cerevisiae* using the Frozen-EZ Yeast Transformation II Kit (Zymo Research), according to the manufacturer’s instructions.

**Predicting Mitochondria-Targeting Sequences**
The online MITOPROT tool was used to predict and exclude mitochondria-targeting signal peptide sequences, which were further validated using TargetP, version 1.1 (Lian and Zhao 2015). The nucleotide sequences encoding the excluded signal peptides are as follows: TTCCGACTCACCCTGCCCAATTGCTTCTGTGGGCACACCTCGCCACGTGTCC (for *Kr*), CGAAACCTATATATACCGGT CCGGT CTTCCTCCACCCCTCTTAGCCAGGAA (for *Htd*), and CTTCCTCCCATTCAAGGTATCAT (for *Etr1*). Additionally, an intron sequence of *Htd* was excluded along with mitochondria-targeting sequence.

**Fermentation**
For seed cultures, yeast cells from a glycerol stock were inoculated in YSC medium containing 2% glucose. The yeast cells were then transferred to fresh YSC medium containing 2% glucose, 2% xylose, or 2% glucose plus 2% xylose with an inoculum size of 5%, and grown aerobically.
TABLE 1 | Strains used in this study.

| Strain          | Description                                                                 | References                |
|-----------------|------------------------------------------------------------------------------|---------------------------|
| XUSEA           | BY4741 gre3Δykl4Δ3 TAL1 ΔKSS1 RPE1 aggre3Δapho13 Δasc1 evolved on xylose    | Ko et al. (2019)          |
| XUSEAS7         | BY4741 ykl4Δ3 TAL1 ΔKSS1 RPE1 aggre3Δapho13 evolved on xylose and acetic acid stress | This study                |
| XUSEA-B         | XUSEAS7 p425-Hbd-Crt, p425-Erg10-cps1-Etr1, and p426-BdhB-EutE               | Hoang Nguyen Tran et al. (2020) |
| WT-B            | XUSEA-B ace6Δ415F from Salmonella enterica and asch from E. coli            |                           |
| XUSEA-B (XA-4)  | XUSEA BY4741 p425-Hbd-Crt, p425-Erg10-cps1-Etr1, and p426-BdhB-EutE        |                           |
| XA-1            | XUSEA p423-Kr-cps1-Htd, p425-Erg10-cps1-Etr1, and p426-AdhE2-EutE          |                           |
| XA-2            | XUSEA p423-Kr-cps1-Htd, p425-Erg10-cps1-Etr1, and p426-AdhE2-EutE          |                           |
| XA-3            | XUSEA p423-Hbd-Crt, p425-Erg10-cps1-Etr1, and p426-AdhE2-EutE              |                           |
| XA-5            | XUSEA p423-Hbd-Crt, p425-Erg10-cps1-Etr1, and p426-AdhE2                   |                           |
| XA-6            | XUSEA p423-Hbd-Crt, p425-Erg10-cys1-Etr1, and p426-AdhE2                   |                           |

TABLE 2 | Plasmids used in this study.

| Plasmid     | Characteristics                                                                 |
|-------------|---------------------------------------------------------------------------------|
| p423-Kr-cps1-Htd | GPDP-cps1-Kr-PRM9t and TEFp-cps1-Htd-CPS1t                                         |
| p423-Hbd-Crt   | GPDP-Hbd-PRM9t and TEFp-Crt-CPS1t                                                   |
| p423-Hbd-Crt-AdhE2 | GPDP-Hbd-PRM9t, TEFp-Crt-CPS1t, and HXT7p-Ter-TP1t                                   |
| p423-Hbd-Crt-TEFp-cps1-Etr1 | GPDP-Hbd-PRM9t, TEFp-Crt-CPS1t, and HXT7p-cps1-Etr1-TP1t                                |
| p425-Erg10-cps1-Etr1 | TEFp-Erg10-CYCl1 and HXT7p-cps1-Etr1-TP1t                                           |
| p426-AdhE2     | CYClp-AdhE2-SPGSt                                                                    |
| p426-BdhB      | CYClp-BdhB-SPGSt                                                                    |
| p426-BdhB-EutE | CYClp-BdhB-SPGSt and PGKlp-EutE-CYC1t                                               |
| p426-BdhB-EutE-Erg10 | CYClp-BdhB-SPGSt and PGKlp-EutE-CYC1t and TEFp-Erg10-CYC1t                     |

Using a Hi-Plex H column (Agilent Technologies, Palo Alto, CA, United States). The system was operated using 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.6 mL/min, and the column temperature was maintained at 65 °C. Ethanol, n-butanol, and acetal concentrations were analyzed using a gas chromatograph instrument (Agilent Technologies, CA, United States) equipped with a flame ionization detector, using an HP-INNOWax polyethylene glycol column (30 m x 0.32 µm x 0.25 µm).

**Transcriptomic Analysis**

For transcriptomic analysis, cells were cultured and harvested at exponential phase during glucose fermentation and glucose/xylose co-fermentation. Cell pellets were collected by centrifugation at 1,000 g for 5 min. Total RNA extraction was performed using Trizol reagent (Invitrogen, CA, United States) according to the manufacturer’s protocol provided by Ebiogen (Seoul, Republic of Korea). Each of the total RNA samples was evaluated for RNA quality control based on the 28S/18S ratio and RIN measured on the 2,100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany). The cDNA library was constructed using the Clontech SMARTer Stranded RNA-Seq kit (Clontech, Mountain View, CA, United States). High-throughput sequencing was performed on an Illumina HiSeq 2,500 system (Illumina, Inc, San Diego, CA, United States).

**RESULTS**

**Introduction of a n-Butanol Production Pathway in a Glucose/Xylose Co-fermenting Strain**

To develop an S. cerevisiae strain capable of n-butanol production, genes that were previously reported to support butanol biosynthesis were introduced in an efficient glucose/xylose co-fermenting strain, XUSEA (Hoang Nguyen Tran et al., 2020), with various combinations. Specifically, Hbd and Crt originated from *C. acetobutylicum* (a representative n-butanol-producing bacterium), and the orthologous genes (Kr and Htd, respectively) originated from *Y. lipolytica*, a yeast

Analytical Methods

Cell growth was analyzed by measuring the OD at 600 nm with a Cary 60 Bio UV–Vis spectrometer (Agilent Technologies, United States). Glucose and xylose concentrations were analyzed using a high-performance liquid chromatography system (HPLC 1260 Infinity, Agilent Technologies, CA, United States) equipped with a refractive index detector, in flasks for 1.5–2 days. Subsequently, the yeast cells were harvested and finally transferred to fresh YSC medium. The pH of the fermentation medium was maintained by adding 70 mM phthalate buffer (pH 5.0) or 80 mM phosphate buffer (pH 6.5). Micro-aerobic fermentation was carried out in 125 mL serum bottles with a final working volume of 40 mL at a low cell density (initial optical density [OD] of 0.2) and a high cell density (initial OD of 15). The serum bottles were capped with rubber stoppers, with a needle for carbon dioxide release during fermentation. Lignocellulosic-biomass hydrolysates (*Miscanthus sacchariflorus* Goedae-Uksae 1), pretreated with dilute acid, were purchased from Sugaren Co., Ltd (Korea). The hydrolysates contained 30.6 g/L of glucose, 15.1 g/L of xylose, 1.3 g/L of arabinose, 0.15 g/L of formic acid, 0.05 g/L of acetic acid, 0.08 g/L of levulinic acid, 0.06 g/L of 5-HMF, and 0.07 g/L of furfural.

**Determining the Expression of the Butanol Biosynthesis Pathway**

The expression of the butanol biosynthesis pathway was determined using high-throughput sequencing. The cDNA library was constructed using the Clontech SMARTer Stranded RNA-Seq kit (Clontech, Mountain View, CA, United States). High-throughput sequencing was performed on an Illumina HiSeq 2,500 system (Illumina, Inc, San Diego, CA, United States).
Heterologous expression of these C. acetobutylicum comprised of gene combinations, the XA-3 strain harboring the set comprised of n-butanol synthesis. Of the strains expressing heterologous expression of the indicated increased butanol production more than heterologous EutE BdhB, did not significantly improve performance, when expressed in a heterologous host. The phylogenetic tree does not seem to guarantee better cells (such as eukaryotic cells will be expressed at higher levels in eukaryotic often expected that genes originating from yeasts or other E. coli biosynthesis. The genes were sourced from S. cerevisiae (Sc), Y. lipolytica (Yl), E. coli (Ec), and C. acetobutylicum (Ca). Low-cell density fermentations (initial CO of 0.2) were conducted with 20 g/L xylose as the sole carbon source. (A) n-butanol pathway showing the genes used in this study. (B) n-butanol titer of the strains expressing six combinations of genes used to construct various n-butanol-production pathways. The error bars represent the standard deviations obtained using biological triplicates. *: below the detection limit of 2.5 mg/L.

with an efficient β-oxidation pathway. These genes were heterologously expressed along with the homologous genes, ERG10 and cytETR1, to construct the reverse-β-oxidation pathway, thereby enabling the conversion of acetyl-CoA into butyryl-CoA (Dellomonaco et al., 2011; Lian and Zhao 2015). To generate a complete butanol-production pathway, Adhe2 or BdhB from C. acetobutylicum were heterologously expressed with or without EutE from E. coli (Figure 1A). Heterologous expression of these C. acetobutylicum genes increased butanol production more than heterologous expression of the indicated Y. lipolytica genes, even without codon optimization. When expressing heterologous genes, it is often expected that genes originating from yeasts or other eukaryotic cells will be expressed at higher levels in eukaryotic cells (such as S. cerevisiae). However, the distance in a phylogenetic tree does not seem to guarantee better performance, when expressed in a heterologous host.

The choice of the alcohol dehydrogenase used, either Adhe2 or BdhB, did not significantly affect the butanol titer. Although Adhe2 is a bi-functional enzyme that converts butyryl-CoA into butyraldehyde and then to n-butanol, it cannot fully support butanol production without EutE expression, suggesting that sufficient enzyme levels are required for each step in n-butanol synthesis. Of the strains expressing heterologous gene combinations, the XA-3 strain harboring the set comprised of Hdb, Crt, EutE, and AdhE2 resulted in the highest butanol titer of 7.2 mg/L, followed by the XA-4 strain harboring the set comprised of Hdb, Crt, EutE, and BdhB (6.8 mg/L) (Figure 1B). Based on the similar butanol titer with a half size of BdhB compared to Adhe2, the butanol production pathway genes in the XA-4 strain was selected as the combination of choice for further experiments throughout this study.

The n-butanol production pathway with the selected genes were introduced into both a xylose utilizing strain of XUSEA and a wild-type strain of S. cerevisiae BY4741, generating XUSEA-B and WT-B, respectively. Then, the n-butanol production performance of XUSEA-B and WT-B was compared during glucose fermentation, xylose fermentation, and glucose/xylose co-fermentation (Figure 2). During glucose fermentation, the XUSEA-B strain showed 2.1-fold higher n-butanol production than the WT-B strain (14.2 mg/L and 6.6 mg/L, respectively; Figure 2A). The XUSEA-B strain produced the same amount of n-butanol during xylose fermentation, whereas the WT-B strain produced no n-butanol because it could not utilize xylose. During glucose/xylose co-fermentation, XUSEA-B produced 26.3 mg/L of n-butanol within 72 h, which was 3.9-fold higher than that produced by WT-B (6.7 mg/L; Figures 2C,D), suggesting that the xylose-utilizing strain could serve as a promising host for producing n-butanol, an acetyl-CoA-derived product.

Transcriptomic Landscape Revealed the Redirected Carbon Flux for Improved n-Butanol Production in a Xylose-Utilizing Strain

To understand the mechanism underlying efficient n-butanol production by the xylose-utilizing strain, we analyzed the global transcript profiles of XUSEA-B and WT-B during glucose fermentation and glucose/xylose co-fermentation. Compared with WT-B, the XUSEA-B strain showed marked differences in the gene-expression landscape (170 upregulated and 84 downregulated genes with glucose fermentation versus 172 upregulated and 82 downregulated genes with mixed-sugar fermentation) (Figure 3). Transcriptional changes in genes associated with core carbon metabolism and linked with the butanol-production pathway were similar between both fermentation conditions, despite the use of different carbon sources. The XUSEA-B strain appeared to show elevated central carbon flux through the n-butanol-production pathway, resulting from pathway upregulation due to increased cofactor generation and reduced use of the acetyl-CoA precursor. The ZWF1, SOL3, and GND1 genes (involved in the oxidative pentose phosphate pathway, from which the factor NADPH is generated), were upregulated in XUSEA-B compared to WT-B by 2.81-, 4.11-, and 7.82-fold during glucose fermentation and by 3.74-, 5.54-, and 9.93-fold during glucose/xylose co-fermentation, respectively (Figure 3, Table 3).

It should be noted that genes involved in the non-oxidative pentose phosphate pathway (such as RPE1 and TAL1) and XKSI were highly upregulated in the XUSEA-B strain, compared to WT-B (Figure 3, Table 3). These differences were mainly due to the genetic background XUSEA (the parental strain of XUSEA-B), in which the above-mentioned genes were overexpressed (Tran Nguyen Hoang et al., 2018; Hoang Nguyen Tran et al., 2020). However, it is noteworthy that RPE1 mRNA expression was upregulated by approximately 100-fold in the XUSEA-B strain. This finding suggests that carbon flux was enhanced
through glycerate-3-phosphate, an interconnection point between the oxidative and non-oxidative pentose phosphate pathways. The transcription of PDC6, which is involved in converting pyruvate to acetaldehyde, was also strongly upregulated suggesting that the acetyl-CoA was increased in XUSEA-B. In contrast, the FBA1 and TDH2 genes (involved in glycolysis), were down-regulated more than 2-fold in XUSEA-B, leading to a low carbon flux through central carbon metabolism via glycolysis.

**Supplementation With Acetate Improved n-Butanol Production by S. cerevisiae**

After confirming the positive effect of increased acetyl-CoA availability in XUSEA-B, based on transcriptomic analysis, the effect of acetate supplementation on n-butanol production was investigated as a strategy for supplying additional acetyl-CoA. To this end, n-butanol production by XUSEA-B was evaluated during glucose and/or xylose fermentation supplemented with various concentrations of acetate (0 g/L, 1 g/L, or 2 g/L). During glucose fermentation, acetate supplementation increased n-butanol production by XUSEA-B (Figure 4). XUSEA-B strains produced 40% or 28% more n-butanol in the presence of one or 2 g/L acetate, respectively. In contrast, acetate supplementation did not result in increased n-butanol production during xylose fermentation. The production of n-butanol decreased by 56% in the presence of 1 g/L acetate during 48 h of xylose fermentation. No n-butanol was produced in the presence of 2 g/L acetate in the medium because the cell growth was significantly inhibited (data not shown).

Interestingly, the negative effect of acetate supplementation on n-butanol production during xylose fermentation was compensated in the presence of glucose. During glucose/xylose co-fermentation, n-butanol production was reduced by 23% in the presence of 1 g/L acetate (Figure 4B).

Given the both positive and negative effects of acetate supplementation on n-butanol production, we introduced the n-butanol-production pathway into an acetate-tolerant glucose/xylose co-fermenting strain of XUSAE-57 (Ko et al., 2019). To promote the conversion of acetate into acetyl-CoA, a heterologous acetate-utilization pathway was additionally integrated by overexpressing a mutant version of ACS<sup>L641P</sup> from *Salmonella enterica* and AADH from *E. coli*, thereby generating XUSAE-A-B. The XUSAE-A-B, an acetate tolerant and utilizing strain, showed...
dramatically improved n-butanol production resulting in an n-butanol titer of 46.5 mg/L with acetate supplementation (1 g/L) during glucose/xylose fermentation at pH 6.5, under which condition the inhibitory effect of acetate has been shown to decrease (Figure 5). Encouraged by the increased n-butanol production during glucose/xylose co-fermentation in the presence of acetate, we evaluated the n-butanol-production performance of XUSAEA-B from lignocellulosic hydrolysates of Miscanthus sacchariflorus Goedae-Uksae, which were prepared through a H₂SO₄-catalyzed hydrothermal process (Figure 6). During a 21 h fermentation, XUSAEA-B completely utilized glucose and xylose, and produced 60.1 mg/L of n-butanol (Figures 6A,C). Interestingly, n-butanol production from lignocellulosic hydrolysates was 14% higher than that from YSC medium which was supplemented with same glucose and xylose concentrations as Micanthus hydrolysate (60.1 mg/L and 52.8 mg/L for lignocellulosic hydrolysates and YSC medium, respectively) (Figure 6C).

TABLE 3 | Fold-changes in the expression levels of genes involved in metabolic pathways in XUSEA-B strain, versus the WT-B strain. G: glucose, GX: glucose and xylose.

| Gene symbol | Expression fold-change | Function |
|-------------|------------------------|----------|
| Glycolysis  |                        |          |
| TDH2        | 0.42                   | 0.40     | Glyceraldehyde-3-phosphate dehydrogenase |
| FBA1        | 0.49                   | 0.50     | Fructose 1,6-bisphosphate aldolase |
| EN D 2      | 0.43                   | 0.48     | Phosphopyruvate hydratase |
| Pentose phosphate pathway |       |          |
| ZWF1        | 2.81                   | 3.74     | Glucose-6-phosphate dehydrogenase |
| SOL3        | 4.11                   | 5.54     | 6-phosphogluconolactonase |
| GND1        | 7.82                   | 9.93     | 6-phosphogluconate dehydrogenase |
| Non-oxidative branch |       |          |
| RPE1        | 100                    | 95.1     | D-ribulose-5-phosphate 3-epimerase |
| XKS1        | 9.76                   | 29.5     | Xylulokinase |
| TAL1        | 3.15                   | 3.96     | Transaldolase |
| Electron transport change |       |          |
| CYC1        | 0.36                   | 0.48     | Cytochrome c, isofrom 1 |
| AAC3        | 2.24                   | 2.2      | Mitochondrial inner membrane ADP/ATP translocator |
| TCA cycle and glyoxylate cycle |       |          |
| CIT2        | 0.32                   | 0.50     | Citrate synthase |
| ACO1        | 0.27                   | 0.49     | Aconitase |
| PyC1        | 0.29                   | 0.42     | Cytoplasmic pyruvate carboxylase; decarboxylates pyruvate to oxaloacetate |
| PyC2        | 0.44                   | 0.49     | Cytoplasmic pyruvate carboxylase; decarboxylates pyruvate to oxaloacetate |
| Ethanol fermentation |       |          |
| ADH4        | 0.1                    | 0.09     | Alcohol dehydrogenase isoenzyme IV |
| ADH5        | 0.39                   | 0.43     | Alcohol dehydrogenase isoenzyme V |
| PDC6        | 14.4                   | 6.07     | Pyruvate decarboxylase; decarboxylates pyruvate to acetalddehyde |
| ALD6        | 3.36                   | 0.77     | Cytosolic aldehyde dehydrogenase |
| Glycerol biosynthesis and degradation |       |          |
| GPP1        | 0.34                   | 0.27     | Glycerol-1-phosphatase |
| GCY1        | 3.98                   | 14       | Glycerol dehydrogenase |
| Amino acid biosynthesis |       |          |
| ILV3        | 0.28                   | 0.39     | Dihydroxy-acid dehydratase |
| EEB1        | 0.14                   | 0.28     | Medium-chain fatty acid ethyl ester synthase/esterase |
| LEU1        | 0.26                   | 0.43     | Isopropylmalate isomerase |
| LEU2        | 0.29                   | 0.30     | Beta-isopropylmalate dehydrogenase |
| LEU9        | 0.36                   | 0.26     | Alpha-isopropylmalate synthase I |
| TRP2        | 0.46                   | 0.39     | Anthranilate synthase |
| Other functions |       |          |
| OYE3        | 7.16                   | 12.21    | NADPH oxidoreductase containing a flavin mononucleotide |
| SER33       | 3.88                   | 3.44     | 3-phosphoglycerate dehydrogenase |
| YEF1        | 3.69                   | 7.64     | ATP-NAD kinase; phosphorylates both NAD and NADH |
| ERG10       | 0.32                   | 0.27     | Cytosolic acetyl-CoA C-acetyltransferase |

Cellulosic n-Butanol Production Using Glucose/Xylose Co-fermenting S. cerevisiae

Encouraged by the increased n-butanol production during glucose/xylose co-fermentation in the presence of acetate, we
our knowledge, this study demonstrates n-butanol production from lignocellulosic hydrolysate by using a glucose/xylose co-fermenting *S. cerevisiae* for the first time whereas other previous studies mainly showed n-butanol production from synthetic glucose media (Lian et al., 2014; Sakuragi et al., 2015; Swidah et al., 2015; Schadeweg and Boles, 2016a; Schadeweg and Boles, 2016b).

**FIGURE 4** | Effect of acetate supplementation on n-butanol production by XUSEA-B strains during glucose and/or xylose fermentation. Fermentation was conducted with an initial OD of 0.2 and various concentrations of acetate (0, 1, or 2 g/L). *(A)* n-butanol production during glucose (pattern, blue) or xylose (solid, yellow) fermentation. *(B)* n-butanol production during glucose/xylose co-fermentation without (white) and with (yellow) acetate supplementation (0.2 g/L, 0.5 g/L, or 1 g/L). The error bars represent standard deviations obtained using biological triplicates.

**FIGURE 5** | Comparison of n-butanol production by the XUSEA-B strain and the acetate-tolerant strain of XUSAEA-B during glucose/xylose co-fermentation. Fermentation was conducted using an initial OD of 0.2 with 0 g/L (solid, white) and 1 g/L (pattern, blue) of acetate supplementation at pH 5.0. n-butanol production at pH 6.5 was also evaluated with 1 g/L of acetate supplementation (solid, yellow). The error bars represent standard deviations obtained using biological triplicates.

**DISCUSSION**

Yeast engineering for lignocellulosic biorefinery has primarily focused on either the expansion of substrate ranges or product profiles through individual approaches. However, the product profiles of *S. cerevisiae* capable of xylose utilization are limited to only a few compounds, including isobutanol, carotene, and 1-hexadecanol (Brat and Boles, 2013; Guo et al., 2016; Sun et al., 2019). Recent reports highlighting the prospect of engineering a xylose-utilizing strain as a promising host for the production of acetyl-coA derived products (Kwak and Jin, 2017) offer easier diversion of product profiles from ethanol to other products, for a glucose/xylose co-utilizing *S. cerevisiae*.

In this study, we demonstrated the expansion of product profiles of a glucose/xylose co-fermenting *S. cerevisiae* to include the acetyl-coA derived product, n-butanol, which is a short chain alcohol similar to ethanol, however, has only been produced by *S. cerevisiae* from glucose with limited success. Despite the numerous attempts to develop n-butanol producing *S. cerevisiae*, the production titer remained suboptimal even with extensive metabolic engineering. There are several benefits associated with using a glucose/xylose co-fermenting strain for n-butanol production. For instance, it has a higher metabolic flux through acetyl-CoA compared to strains capable of only utilizing glucose (Kwak and Jin, 2017; Li et al., 2019). Additionally, it has an associated higher conversion yield per unit of biomass. These benefits were demonstrated in the current study. Specifically, the glucose/xylose co-fermenting *S. cerevisiae* strain, XUSEA-B, showed 3.9-fold higher n-butanol titer compared to the wild-type strain capable of only utilizing glucose during glucose/xylose co-fermentation. Given that the amount of xylose present was nearly half that of glucose, the additional n-butanol produced by XUSEA-B demonstrates a synergistic effect with an additional carbon source (xylose) in
metabolic transcriptomic landscape in XUSEA-B, in which the increased glucose/xylose co-fermenting strain through analysis of the hydrolysates and an increased acetyl-CoA availability, supported by xylose catabolism in XUSEA-B.

In the study, we pointed out the increased acetyl-CoA pool of a glucose/xylose co-fermenting strain through analysis of the transcriptomic landscape in XUSEA-B, in which the increased metabolic flux was expected toward pyruvate, acetalddehyde, acetate, and cytosolic acetyl-CoA by re-directing the carbon flux through competing pathways such as the tricarboxylic acid (TCA) cycle, glyoxylate cycle, amino acid biosynthesis, and ethanol production (Figure 3, Table 1). The metabolic flux through pyruvate, specifically, could be directed toward acetalddehyde based on the highly upregulated expression of PDC6 accompanied by downregulation of genes associated with pyruvate carboxylation, and those involved in the TCA cycle, glyoxylate cycle and amino acid biosynthesis employing pyruvate as a precursor. Moreover, the fate of acetalddehyde through the ethanol-fermentation pathway also appeared to be redirected toward acetate formation, based on the decreased expression of alcohol dehydrogenases encoded by ADH4 and ADH5 and increased expression of aldehyde dehydrogenase encoded by ALD6 (Figure 3, Table 3) in XUSEA-B. Meanwhile, the high availability of the cytosolic acetyl-CoA contributed substantially to the improved n-butanol production in XUSEA-B during glucose/xylose fermentation, thus demonstrating the advantage of employing xylose-utilizing S. cerevisiae for the production of acetyl-CoA-derived biofuels and biochemicals from lignocellulosic biomass.

In XUSEA-B, the oxidative pentose phosphate pathway, a major route for NADPH production (Stincone et al., 2015), appeared to be actively regulated via upregulation of ZWF1, SOL3, and GND1, possibly supporting the notion that improved NADPH availability improves metabolic flux through the NADPH-dependent rate-limiting step in the n-butanol production pathway. Indeed, the n-butanol pathway in the glucose/xylose co-fermenting strain, containing an NADPH-prefering cytoETR1 protein, was better supported by the sufficient NADPH cofactor present in the XUSEA-B strain.

In terms of glycerol-metabolism, expression of the glycerol-1-phosphatase gene (GPP1), which is important for glycerol biosynthesis, decreased (Figure 3, Table 3), whereas transcription of the glycerol dehydrogenase gene (GCY1), which participates in glycerol catabolism under micro-aerobic conditions, was increased. Similarly, suppressed glycerol synthesis combined with disruption of the ethanol-fermentation pathway reportedly stimulates n-butanol production (Lian et al., 2014; Swidah et al., 2015; Schadeweg and Boles, 2016b) by increasing the abundance of NADH, a driving force of the n-butanol pathway (Kim et al., 2015; Schadeweg and Boles, 2016a). Furthermore, the upregulation of genes related to cofactor regeneration, such as OYE3, SER33, and YEF1, suggest that XUSEA-B maintained an optimal balance by varying redox cofactors, not only for efficient n-butanol production. Understanding the transcriptomic characteristics of XUSEA-B strain not only help to explain the improved butanol production during glucose/xylose fermentation, but also could offer engineering strategies to further improve the butanol titer in this minimally engineered strain of XUSEA-B. These engineering targets could also be used for rewiring biosynthetic routes to produce acetyl-CoA-derived chemicals in the context of lignocellulosic bioenergy.

Previously, acetate supplementation increased n-butanol production in a native producer of Clostridium sp. by upregulating the expression levels of CoA-transferase genes, thereby increasing the availability of acetyl-CoA and butyryl-CoA, two main precursors of the n-butanol synthesis pathway (Chen and Blaschek, 1999). During lignocellulosic fermentation by S. cerevisiae, however, acetate is regarded as a major inhibitory compound impeding cell growth and sugar utilization rate, and thus limits the fermentation performance of S. cerevisiae (Helle et al., 2003; Jönsson et al., 2013), particularly during xylose fermentation (Ko et al., 2015). S. cerevisiae endogenously expresses ACS1 and ACS2, which encode acetyl-CoA synthetases to convert acetate into acetyl-CoA using ATP (van den Berg et al., 1996). Therefore, acetate supplementation could be expected to support additional carbon flux through the n-butanol biosynthetic pathway by enhancing acetyl-CoA, a crucial precursor for n-butanol production. As expected, in the current study n-butanol production increased during glucose
fermentation in the presence of acetate. This could be explained due to the increased acetyl-CoA pool through the conversion of acetate by native ACS genes. The sufficient intracellular ATP supplied from glucose could also have alleviated the inhibitory effect of acetate (Zhang et al., 2016). During xylose fermentation, however, acetate supplementation did not positively affect n-butanol production, possibly due to insufficient detoxification and limited ATP generation by xylose (Casey et al., 2010). In fact, increased acetate concentration caused a decrease in the n-butanol titer during xylose fermentation (Figure 4A). Several strategies have been proposed to overcome the toxic effects of acetic acid while maintaining fermentation performance in S. cerevisiae. Ko et al. improved acetate tolerance of a xylose fermenting S. cerevisiae through adaptive laboratory evolution (Ko et al., 2019). Zhang et al. have coupled an acetate reduction pathway with xylose catabolism during cellulosic fermentation to improve sugar-to-product conversion yield while converting acetate into less inhibitory products (Zhang et al., 2016). When an acetate-tolerant glucose/xylose co-fermenting strain harboring an acetic acid conversion pathway, XUSAEA-B, was used, the beneficial effects of acetate supplementation were recovered resulting in significantly improved n-butanol production (Figure 5). Moreover, during lignocellulosic fermentation, XUSAEA-B produced 60.1 mg/L of n-butanol, accounting for a 14% higher yield compared to that obtained during fermentation using synthetic media with the same concentrations of sugars (52.8 mg/L), possibly due to the positive effect of acetate on elevating acetyl-CoA for the n-butanol pathway in lignocellulosic hydrolysates.

CONCLUSION

In this study, we investigated the production of the acetyl-coA-derived product, n-butanol, by a glucose/xylose co-fermenting S. cerevisiae strain. The transcription profiles of S. cerevisiae with efficient xylose catabolism revealed a modified cellular network that better supported the generation of acetyl-CoA and cofactors required for n-butanol fermentation. Incorporating acetate catabolism further improved n-butanol production from lignocellulosic hydrolysates. Consequently, the results of this study show the potential of using a glucose/xylose co-fermenting strain and lignocellulosic biomass as more attractive production host and resource for biorefinery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

AUTHORS CONTRIBUTIONS

YL: investigation, writing—original draft, writing—review and editing, PH: writing—original draft, writing—review and editing. JK: supervision, writing—review and editing. GG, YU, and SH: formal analysis, writing—review and editing. S-ML: conceptualization, formal analysis, writing—review and editing.

FUNDING

This research was supported by the Korea Institute of Science and Technology (KIST) Institutional Program (grant number. 2E31853) and the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (Information and Communication Technology) (grant number. NRF-2020M1A2A2080847).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.826787/full#supplementary-material

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