Enhanced 2'-fucosyllactose production by engineered *Saccharomyces cerevisiae* using xylose as a co-substrate

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ABSTRACT

2'-fucosyllactose (2'-FL), a human milk oligosaccharide with confirmed benefits for infant health, is a promising infant formula ingredient. Although *Escherichia coli*, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum*, and *Bacillus subtilis* have been engineered to produce 2'-FL, their titers and productivities need be improved for economic production. Glucose along with lactose have been used as substrates for producing 2'-FL, but accumulation of by-products due to overflow metabolism of glucose hampered efficient production of 2'-FL regardless of a host strain. To circumvent this problem, we used xylose, which is the second most abundant sugar in plant cell wall hydrolysates and is metabolized through oxidative metabolism, for the production of 2'-FL by engineered yeast. Specifically, we modified an engineered *S. cerevisiae* strain capable of assimilating xylose to produce 2'-FL from a mixture of xylose and lactose. First, a lactose transporter (Lac12) from *Kluyveromyces lactis* was introduced. Second, a heterologous 2'-FL biosynthetic pathway consisting of enzymes Gmd, WcaG, and WbgL from *Escherichia coli* was introduced. Third, we adjusted expression levels of the heterologous genes to maximize 2'-FL production. The resulting engineered yeast produced 25.5 g/L of 2'-FL with a volumetric productivity of 0.35 g/L·h in a fed-batch fermentation with lactose and xylose feeding to mitigate the glucose repression. Interestingly, the major location of produced 2'-FL by the engineered yeast can be changed using different culture media. While 72 % of the produced 2'-FL was secreted when a complex medium was used, 82 % of the produced 2'-FL remained inside the cells when a minimal medium was used. As yeast extract is already used as food and animal feed ingredients, 2'-FL enriched yeast extract can be produced cost-effectively using the 2'-FL accumulating yeast cells.

Keywords: 2'-Fucosyllactose*; Saccharomyces cerevisiae*, Xylose, Lactose, GDP-L-fucose

1. Introduction

A fucosylated oligosaccharide in human milk, 2'-fucosyllactose (2'-FL), accounts for ~30 % of total human milk oligosaccharide (HMO) (Chaturvedi et al., 2001). 2'-FL exhibits various health benefits, such as enhancing the host immune and nerve system development (Eiwegger et al., 2004; Lucas et al., 1990), inhibiting pathogenic infections (Newburg et al., 2005), and stimulating the growth of beneficial gut microorganisms (GYÖRGY, 1953). Supplementing infant formulas with 2'-FL can be beneficial to babies who are not breast-fed (Goehring et al., 2016; Marriage et al., 2015; Morrow et al., 2001). 2'-FL can act as a decoy for norovirus by inhibiting its ability to bind to the surface of epithelial cells (Derya et al., 2020), suggesting a possibility to be used for prevention and treating norovirus infection.

In order to expand the range of 2'-FL applications as food ingredients, economical and scalable production of 2'-FL is necessary. 2'-FL can be obtained via extraction from human milk (Anderson & Donald, 1981), chemical synthesis (Fernandez-Mayoralas & Martin-Lomas, 1986), or enzymatic synthesis (Albermann et al., 2001). However, microbial biosynthesis of 2'-FL is more cost-effective and amenable for a large-scale production than chemical or enzymatic synthesis (Han et al., 2012).

Microbial biosynthesis of 2'-FL was first demonstrated in engineered *Escherichia coli* strains (Baumgärtner et al., 2013; Chin et al., 2015; Huang et al., 2017; Lee et al., 2012). Nonetheless, 2'-FL production by engineered *E. coli* has critical drawbacks, such as the risk of bacteriophage infection during a large-scale fermentation (Wünsche, 1989), possible endotoxin contamination, and unfavorable consumer perception (Rietschel et al., 1996). Therefore, *Saccharomyces cerevisiae,* which is generally recognized as safe (GRAS), has been proposed as an alternative host to produce 2'-FL.

Moreover, *S. cerevisiae* has a richer intracellular pool of GDP-D-mannose (Mattila et al., 2000) which can be converted into GDP-L-fucose, a key precursor of 2'-FL biosynthesis. In order to enable 2'-FL synthesis in engineered *S. cerevisiae*, installation of three heterologous metabolic components is necessary: a lactose transporter for internalization of lactose, a GDP-L-fucose synthetic pathway, and α-1,2-fucosyltransferase (Fig. 1). GDP-L-fucose can be produced from GDP-D-mannose (*de novo* pathway) (Mattila et al., 2000), or L-fucose (*salvage* pathway) (Coyne et al., 2005). As *S. cerevisiae* cannot utilize lactose, it requires that another carbon source is supplied to produce GDP-L-fucose as well as to support cell growth. Recently, 2'-FL production in engineered *S. cerevisiae* via the *de novo*, or *salvage* pathway has been reported (Hollands et al., 2019; Liu et al., 2018; Yu et al., 2018). These studies used glucose as a main carbon source, and lactose as a backbone for the production of 2'-FL.

The titers of 2'-FL by engineered yeast were not comparable to those by engineered *E. coli* (Baumgärtner et al., 2013; Chin et al., 2015; Chin et al., 2016; Huang et al., 2017). We reasoned that the predominantly fermentative nature of glucose metabolism by *S. cerevisiae*, and unstable and unbalanced expression of the heterologous genes might limit productivities by the engineered yeast strains (Liu et al., 2018; Yu et al., 2018). When glucose is provided, *S. cerevisiae* exhibits the Crabtree effect which is characterized by repressed respiration and facilitated ethanol production. Therefore, even after introduction of 2'-FL biosynthetic pathways, ethanol remained as a major product by engineered yeast. In addition, previous studies used episomal plasmids to overexpress the enzymes for GDP-L-fucose and 2'-FL biosynthesis. However, episomal plasmids tend to exhibit structural and segregational instability (Friehs, 2004) and gene expression from episomal plasmids can be noisy (Ryan, 2014). The instability and inconsistent expression may negatively impact 2'-FL production by engineered yeast.

To resolve these problems, we constructed an engineered yeast strain to produce 2'-FL using xylose, the second most abundant sugar in plant cell wall. The resulting yeast was able to utilize xylose as a primary carbon source instead of glucose for the enhanced production of 2'-FL. Moreover, heterologous genes encoding the 2'-FL biosynthetic enzymes were integrated into the engineered yeast chromosome for stable gene expression. To further increase 2'-FL productivity, the copy numbers of the integrated heterologous genes were optimized in the engineered yeast. As a result, our engineered yeast drastically improved 2'-FL production. With recent advances of cellulosic biofuel technologies, economic and sustainable production of xylose from non-edible cellulosic biomass is expected. Therefore, it will be feasible to produce 2'-FL by engineered yeast using a mixture of xylose and lactose.

2. Materials and Methods

2.1. Strains and media

E. coli Top10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] was used for the manipulation of a plasmid. The *E. coli* strain was grown in Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37 °C with ampicillin (100 μg/mL) if necessary. A xylose-fermenting *S. cerevisiae* CT2 strain—*S. cerevisiae* D452-2 derived strain with integration of two copies of expression cassettes containing *XYL1*, *XYL2*, and *XYL3* in the background of *PHO13* and *ALD6* deletion (Tsai et al., 2015)—was used as a host strain for introducing genetic modifications to produce 2'-FL. The CT2 strain, and its derived yeast strains were cultivated at 30 °C in YP medium (10 g/L yeast extract, and 20 g/L peptone) with 20 g/L glucose. For CRISPR-Cas9 based genome editing experiments,

120 μg/mL of nourseothricin, 300 μg/mL of geneticin, and 300 μg/mL of hygromycin B were added as necessary for selecting transformants.

2.2. Plasmids and strains construction

The strains used in this study are listed in Table 1. The plasmids, primers, and guide RNA (gRNA) target sequences used in this study are listed in Table S1, S2 and S3, respectively. Details of plasmid and strain construction procedures are described in the **Supplementary materials**. Recombinant DNA techniques were performed according to standard procedures. A lithium acetate transformation method with single strand carrier DNA and polyethylene glycol (Gietz & Schiestl, 2007) was used to introduce Cas9-NAT, gRNA expression vectors, and donor DNA fragment into yeast strains. Putative transformants on selection plates were confirmed by colony PCR.

2.3. Fermentation experiments

To produce 2'-FL, we precultured engineered yeast strains overnight in 5 mL of YPD20 (YP medium with 20 g/L of glucose) at 30 °C and 250 rpm. The pre-cultured yeast cells were then transferred into 40 mL of YPD20 and incubated under the same conditions. Grown cells were harvested at the mid-exponential phase and inoculated into 20 mL either YPD30L2 (YP medium with 30 g/L of glucose and 2 g/L of lactose) or YPX30L2 (YP medium with 30 g/L of xylose and 2 g/L of lactose) in a 250-mL flask with an initial cell density of OD_{600} = 10. All flasks were incubated at 30 °C and 250 rpm.

Xylose fed-batch fermentations were conducted in the BioFlo & CelliGen 115 fermenter (New Brunswick Scientific-Eppendorf, CT, USA) using either a complex medium (YP), or a defined

medium (Verduyn), respectively (van Hoek et al., 2000). For a fermentation using the complex medium, the *S. cerevisiae* CTLD2F2 strain was pre-cultured in 200 mL of YPD40 (YP medium with 40 g/L of glucose), then inoculated into 1L of YPX30 (YP medium with 30 g/L xylose) medium. After the initially added xylose was depleted, additional xylose and lactose were added to the bioreactor up to 30 g/L of xylose and 2 g/L of lactose. For a fermentation with the defined medium, the *S. cerevisiae* CTLD2F2L strain was pre-cultured in 200 mL of VD40 (Verduyn medium with 40 g/L of glucose), then inoculated into 1L of VX30 (Verduyn medium with 30 g/L of xylose). Engineered strains were cultured with feeding xylose to reach a high cell density of OD_{600} = 120, equivalent to 53.6 g/L dry cell weight (DCW), and lactose was then added to initiate 2'-FL synthesis. After the initially added lactose was completely converted to 2'-FL, additional lactose was added to the bioreactor up to 2~3 g/L. The pHs of fed-batch fermentations were controlled at pH 5.6 by adding 2N NaOH.

To compare intracellular GDP-L-fucose production by engineered strains on glucose and xylose, we pre-cultured the engineered strains in 5 mL of YPD20. We inoculated pre-cultured cells into 20 mL of YPD30 or YPX30 in a 250 mL flask with an initial cell density of OD_{600} = 0.1. The fermentation was performed at 30 °C and 250 rpm.

To compare lactose assimilation by engineered strains grown on glucose and xylose, the engineered strains were pre-cultured in 5 mL of YPD20. The pre-cultured cells were then inoculated into 3 mL of YPD10L2 (YP medium with 10 g/L of glucose and 2 g/L of lactose) or YPX10L2 (YP medium with 10 g/L of xylose and 2 g/L of lactose) in a 14 mL culture tube with an initial cell density of OD_{600} = 10. The fermentation was performed at 30 °C and 250 rpm.

2.4. Analytic methods

Cell density (OD_{600}) was monitored using a spectrophotometer (BioMate 5, Thermo Fisher Scientific, MA, USA). Dried cell weights (DCW) of engineered yeast strains were calculated from a pre-determined relationship between OD_{600} and DCW. Extracellular glucose, xylose, lactose, ethanol, and 2'-FL concentrations of culture broths were analyzed with the Agilent 1200 HPLC system equipped with a refractive index detector (Agilent Technologies, Wilmington, DE, USA) and Rezex ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, CA, USA). The flow rate of the mobile phase 0.005N H_2SO_4 was 0.6 mL/min, and the column temperature was 50 °C. To measure total (intracellular and extracellular) 2'-FL, fermentation broth containing yeast cells was boiled for 10 min to release all of the intracellular 2'-FL and centrifuged at 21,130×*g* for 10 min. 2'-FL concentrations in supernatants were analyzed by high-performance liquid chromatography (HPLC) (Canelas et al., 2009). Differences between total and extracellular 2'-FL concentrations were calculated as intracellular 2'-FL concentrations.

To measure intracellular GDP-L-fucose in engineered yeast cells, we harvested 1.8 mL of cell culture by centrifugation at 21,130×*g* for 10 min, washed twice with distilled water, and re-suspended pellet with 500 μL of distilled water. To ensure that all of the GDP-L-fucose was released, the cells were then further disrupted by continuous beating with glass beads for 40 min. After centrifugation at 25,000×*g* for 20 min at 4 °C, we injected the supernatant into a HPLC system (Shimadzu, Kyoto, Japan) equipped with a CAPCELL PAK C18 MG column (Shiseido, Tokyo, Japan) at 30 °C. The flow rate of a mobile phase composed of 20 mM triethylamineacetate and 2% (v/v) acetonitrile was set to 0.6 mL/min. GDP-L-fucose was detected at 254 nm by HPLC, and the concentration of GDP-L-fucose was calculated from its peak height using a GDP-L-fucose standard solution.

To measure the intracellular lactose content in the engineered yeast, we harvested 200 μL of the cell culture by centrifugation at 21,130×*g* for 5 min, washed twice with distilled water, and re-suspended with 200 μL of distilled water. The cells were then boiled for 10 min to release the intracellular lactose. The intracellular lactose was then measured with the Agilent 1200 HPLC system equipped with a refractive index detector (Agilent Technologies, Wilmington, DE, USA) and Rezex ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, CA, USA) as described in the above protocol.

2.5. Real-time quantitative PCR (RT-qPCR)

To determine mRNA levels of 2'-FL biosynthetic enzymes (Gmd, WcaG, and WbgL) in engineered yeast strains (CT2, CTLD1F1, and CTLD2F2), we performed RT-qPCR analysis. For RNA extraction, the strains were inoculated into 5 ml of YPX30L2 in a 14 mL culture tube and incubated at 30 °C and 250 rpm. RNA was extracted from the fresh cultures of the strains using a Qiagen RNeasy mini kit following the manufacturer's protocol. The RNA was reverse transcribed to cDNA using Superscript™ III First-Strand Synthesis System (Thermo Fisher Scientific, MA, USA). The RT-qPCR was conducted using CFX96 Real-Time system (Bio-Rad, CA, USA) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), according to the manufacture's protocol. All RT-qPCR amplicon primers are listed in Table S4. The housekeeping gene *ACT1* was used as a loading control. The relative gene expression level was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) from three biological replicates, and statistical significance was assessed by Student's t-test.

3. Results and Discussion

3.1. Comparison of glucose and xylose as a carbon source for 2'-FL production by engineered yeast

3.1.1. Intracellular GDP-L-fucose production

Ample supply of GDP-L-fucose is required to enhance 2'-FL production because GDP-L-fucose is a fucosyl donor for the fucosylation of lactose (Lee et al., 2012). The GDP-L-fucose synthesis pathway is branched from fructose-6-phosphate which is a metabolite of glycolysis (Hollands et al., 2019; Liu et al., 2018). When excess amounts of glucose are used by *S. cerevisiae*, majority of consumed glucose is converted into ethanol regardless of aerations due to the Crabtree effect (De Deken, 1966). As a result, GDP-L-fucose production in engineered *S. cerevisiae* can be extremely limited because of rapid and efficient metabolism—characterized by little or no accumulation of glycolytic intermediates—of glucose toward ethanol production. We hypothesized that use of xylose instead of glucose might increase supply of GDP-L-fucose as xylose does not induce glucose repression (Jin et al., 2004; Kwak et al., 2017). Previous studies have reported that xylose-utilizing *S. cerevisiae* strains do not exhibit glucose repression through different transcriptomic and metabolomic patterns of the central carbon metabolism (Jin et al., 2004; Matsushika et al., 2014; Feng & Zhao, 2013).

To examine the effect of xylose metabolism on GDP-L-fucose production in engineered yeast, we constructed the *S. cerevisiae* CTD strain by integrating the expression cassettes containing *gmd* and *wcaG* into the chromosome of the *S. cerevisiae* CT2 strain, which was constructed to ferment xylose previously (Tsai et al., 2015). Under glucose and xylose conditions, phenotypic changes of the CTD strain including intracellular GDP-L-fucose content were analyzed. The CTD strain

consumed 30 g/L of glucose, and xylose within 12 h and 36 h, respectively (Fig. S1). The CTD strain grew more and produced less ethanol on xylose than on glucose (Fig. S1), confirming that glucose repression is substantially alleviated when xylose is used.

The intracellular GDP-L-fucose content of the CTD cells grown on xylose was 37% more (1.02 vs. 0.74 mg/g cell) than that of the CTD cells grown on glucose. As the CTD cells grew more on xylose than glucose, the volumetric GDP-L-fucose concentration of the xylose culture was 3.0-fold higher (44.1 vs. 14.6 mg/L) than on glucose. When glucose was used, the CTD strain showed a diauxic growth pattern on ethanol after glucose depletion. However, the intracellular GDP-L-fucose content of the CTD cells grown on ethanol was much lower (0.13 mg/g cell) than those of the cells grown on glucose and xylose (Fig. 2). This result can be explained by the fact that ethanol can be converted into GDP-L-fucose via gluconeogenesis, but it is less efficient than synthesis of GDP-L-fucose through glycolysis from glucose or xylose (Foy & Bhattacharjee, 1977). In addition, biosynthesis of GDP-L-fucose requires one GTP and one ATP (Guan et al., 2018), but ethanol utilization is energetically less efficient than sugar utilization, which could limit the biosynthesis of GDP-L-fucose from ethanol (de KoK et al., 2012; Pfeiffer & Morley, 2014; Verduyn, 1991). Because of these reasons, sequential utilization of glucose and ethanol could not drive high levels of GDP-L-fucose as shown in xylose utilization in terms of both specific contents and volumetric concentrations (Fig. 2).

3.1.2. Lactose uptake

As *S. cerevisiae* cannot transport lactose (Sreekrishna & Dickson, 1985), a backbone of 2'-FL, it is necessary to introduce a heterologous lactose transporter to internalize lactose for fucosylation. To avoid the potential challenges of expressing a prokaryotic lactose transporter gene

in eukaryotic cells, we decided to express a eukaryotic lactose transporter in our engineered yeast. Therefore, a lactose transporter (Lac12) from *Kluyveromyces lactis* under the control of the *TDH3* promoter was integrated into the genome of the *S. cerevisiae* CT2 strain. Glucose is known to trigger the inactivation of transporters and enzymes needed for utilizing other sugars in *S. cerevisiae* (Lucero et al., 2002). Specifically, glucose triggers ubiquitination of maltose and galactose transporters causing endocytosis and proteolysis of the sugar transporters in the vacuole (Horak & Wolf, 1997; Lucero et al., 1993). As such, we reasoned that Lac12 might not be stable or functional when glucose is present, thereby limiting 2'-FL production by engineered yeast.

To examine the functionality of Lac12 in *S. cerevisiae* under different carbon sources, we measured both extra and intracellular lactose concentrations in the *S. cerevisiae* CTL strain—a CT2-derived strain with an integrated *LAC12* expression cassette— after incubating the CTL cells with 2 g/L lactose, and either 10 g/L of glucose or 10 g/L of xylose. Under glucose and xylose conditions, intracellular lactose concentrations of the CTL strain were increased in proportion to reduced extracellular lactose concentrations. Counterintuitively, the CTL strain was able to assimilate lactose efficiently even under the presence of glucose (Fig. S2). This result indicates that Lac12 in *S. cerevisiae* is stable and capable of transporting lactose under xylose conditions and even under glucose conditions.

3.1.3. 2'-FL production by engineered yeast

We constructed the *S. cerevisiae* CTLD by integrating the expression cassettes containing *gmd* and *wcaG* into the chromosome of the CTL strain, which allowed the CTLD strain to assimilate lactose and produce GDP-L-fucose intracellularly. A heterologous α-1,2-fucosyltransferase (FT) is also necessary to produce 2′-FL in the CTLD strain. Instead of

using *Helicobacter pylori futC,* which resists functional expression in a heterologous host strain (Chin et al., 2015; Lee et al., 2012), we decided to introduce an alternative FT gene, *wbgL,* found in *E. coli.*

To examine the advantages of using xylose over glucose for the production of 2'-FL by engineered yeast, the *S. cerevisiae* CTLD1F1—a CT2-derived strain with chromosomal integration of *LAC12*, *gmd, wcaG,* and *wbgL* expression cassettes—was cultured in the YPD30L2 and YPX30L2. In the YPD30L2, 30 g/L of glucose was depleted within 4 hours, and the CTLD1F1 cells continued to grow using ethanol produced from glucose. The 2 g/L of lactose initially added into the culture medium was not detected after 48 hours, indicating that all of the added lactose was internalized in the CTLD1F1 cells. However, 2'-FL concentration in the culture media was only 0.7 g/L, suggesting that produced 2'-FL might remain inside the cells. When the cells were lysed by boiling, the 2'-FL concentration in the boiled culture medium was 1.5 g/L. This result indicates that the CTLD1F1 strain produced 0.7 g/L of extracellular 2'-FL and 0.8 g/L of intracellular 2'-FL. The volumetric productivity of 2'-FL was 0.04 g/L·h and the yield of total 2'-FL from lactose was 0.53 mol/mol when the CTLD1F1 is cultured in the YPD30L2 (Fig. 3a).

When the YPX30L2 was used, the CTLD1F1 strain consumed 30 g/L of xylose in 30 h and produced less ethanol (4.7 vs. 13.1 g/L) and grew to higher cell densities (OD_{600} 40.9 vs. 29.4) than when the YPD30L2 was used. The extracellular concentration of 2'-FL was 0.9 g/L and the total concentration of extracellular and intracellular concentrations of 2'-FL from the boiled cell culture was 2.3 g/L. Therefore, intracellular concentration of 2'-FL was calculated to be 1.4 g/L. The 2'-FL productivity was 0.11 g/L·h, and the yield of 2'-FL from lactose was 0.81 mol/mol when the CTLD1F1 is cultured in the YPX30L2 (Fig. 3b).

While the CTLD1F1 strain produced 2'-FL, WcaG uses NADPH as a cofactor, so we hypothesized that improved NADPH production might enhance 2'-FL production as (Fig. 1). To increase intracellular concentrations of NADPH in the CTLD1F1 strain, we overexpressed genes reported to increase NADPH levels in *S. cerevisiae* (Oh et al., 2013). Specifically, we expressed either *SsZWF1* (*Scheffersomyces stipitis* glucose-6-phosphate dehydrogenase) or *IDP2* (*S. cerevisiae* isocitrate dehydrogenase). However, the effects of the overexpression of *SsZWF1* or *IDP2* on 2'-FL production were marginal (Fig. S3). We suspect that endogenous *ZWF1* and *IDP2* might be already upregulated under xylose conditions so that their overexpression did not impact on 2'-FL production. The RNA-seq data of a xylose-fermenting engineered *S. cerevisiae* (SR7*pho13*Δ) that has a similar genetic background with the CT2 strain (Kim et al., 2015) supported this idea. According to the RNA-seq data, *ZWF1* and *IDP2* of the SR7*pho13*Δ strain were 2.35- and 22.3-fold upregulated under xylose conditions as compared to when they were under glucose conditions.

We speculate that the improved production of 2'-FL from xylose as compared to glucose might be caused by better energetics of xylose utilization than glucose fermentation. Both GDP-L-fucose synthesis and lactose transportation require cellular energy (GDP-L-fucose: ATP and GTP, Lactose transport: ATP) (Guan et al., 2018; Guimarães et al., 2008). As such, the xylose-utilizing *S. cerevisiae*, which can produce more ATP due to alleviation of glucose repression on oxidative phosphorylation (Jin et al., 2004; Kwak et al., 2017), can produce more 2'-FL than the glucose/ethanol-utilizing *S. cerevisiae*. This hypothesis is supported by the observation that lactose uptake rates were slowed when ethanol was consumed after glucose depletion more than rates when xylose was consumed (Fig. S2, Fig. 3a). This is consistent with findings in a previous study

(Liu et al., 2018) where about 40 % of lactose initially added was not transported into a cell during ethanol consumption after glucose depletion.

Lac12 is a proton symporter which co-transports a proton with each lactose. Consequently, transmembrane electrochemical gradients of protons (ΔP) is a driving force of lactose transport. In *S. cerevisiae*, ΔP is generated largely by plasma membrane ATPase (Pma1p). It has been reported that at least 10% of cellular ATP is consumed by Pma1p for transporting maltose by a proton symporter when the yeast is growing on maltose (Guimarães et al., 2008). As such, substantial amounts of ATP will be necessary for lactose transport. Ethanol assimilation is energetically inefficient (Pfeiffer & Morley, 2014; Verduyn, 1991) so that sequential utilization of glucose and ethanol might not be suited to providing for sufficient intracellular lactose, hampering the efficient production of 2'-FL. Therefore, higher energetic efficiency of xylose metabolism than glucose metabolism in *S. cerevisiae* could be a factor that improved 2'-FL titer and productivity under xylose conditions.

3.2. The effects of copy numbers of *gmd*, *wcaG*, and *wbgL* on 2'-FL production

The yield of 2'-FL from lactose (0.81 mol/mol) by the CTLD1F1 strain in the YPX30L2 (Fig. 3) was still lower than the theoretical maximum (1.0 mol/mol). This lower yield also suggests that lactose might be degraded or modified slowly into other cellular metabolites via promiscuous activities of endogenous enzymes in yeast. In order to compete with such reactions draining lactose, *in vivo* activities of GDP-L-fucose synthesizing enzymes (Gmd and WcaG) and fucosyltransferase (WbgL) might need to be optimized. We constructed three engineered *S. cerevisiae* strains (CTLD2F1, CTLD1F2, and CTLD2F2) with different integrated copies of the expression cassettes with *gmd, wcaG*, and *wbgL* in the genomes via Cas9-based genome editing.

The CTLD2F1 strain contained two copies of the *gmd* and *wcaG* expression cassette and one copy of the *wbgL* expression cassette in the genome. The CTLD1F2 strain contained one copy of the *gmd* and *wcaG* expression cassette and two copies of the *wbgL* expression cassette in the genome. Lastly, the CTLD2F2 strain contained two copies of the *gmd* and *wcaG* expression cassette and two copies of the *wbgL* expression cassette in the genome. We examined the 2'-FL production capacities of these engineered strains along with their parental strain (CTLD1F1) in the YPX30L2.

The extracellular, total 2'-FL, and volumetric 2'-FL productivities by the four engineered yeast strains (CTLD1F1, CTLD2F1, CTLD1F2, and CTLD2F2) (Table 1) were measured (Fig. 4). Overall, the changes in the total 2'-FL titers were not significant in the four engineered strains but extracellular 2'-FL titer and 2'-FL productivity levels differed in the engineered yeast strains with altered copies of the expression cassettes. When we doubled the expression cassette of *gmd* and *wcaG*, the CTLD2F1 strain increased 2'-FL productivity (0.144 $\pm$ 0.003 vs. 0.109 $\pm$ 0.002 g/L·h) but not the extracellular 2'-FL concentration as compared to the CTLD1F1 strain. In contrast, when we doubled the *wbgL* expression cassette, the CTLD1F2 strain showed improvements in both the extracellular 2'-FL titer (1.10 $\pm$ 0.00 vs. 0.87 $\pm$ 0.01 g/L) and the 2'-FL productivity (0.142 $\pm$ 0.008 vs. 0.109 $\pm$ 0.002 g/L·h) as compared to the CTLD1F1 strain. When the expression cassettes of *gmd*, *wcaG*, and *wbgL* were doubled, the CTLD2F2 strain produced 1.6 g/L of extracellular 2'-FL, which was 1.7-fold higher than that of the CTLD1F1 strain (0.9 g/L), under the YPX30L2 conditions. Moreover, the CTLD2F2 strain exhibited 1.7-fold higher 2'-FL productivity over the CTLD1F1 strain (0.187 $\pm$ 0.001 and 0.109 $\pm$ 0.002 g/L·h), indicating that the rate of 2'-FL biosynthesis might be an influencing factor of 2'-FL secretion in engineered yeast. To compare 2'-FL biosynthetic enzyme mRNA levels (Gmd, WcaG, and WbgL) in the CTLD1F2 and the CTLD2F2 strains, we performed RT-qPCR (Fig. S4). The mRNA levels

of Gmd, WcaG, and WbgL in the CTLD2F2 strain were higher than levels in the CTLD1F1 strain, suggesting that the increased copy numbers of *gmd*, *wcaG*, and *wbgL* elevated enzyme mRNA levels in the CTLD2F2 strain. Overall, the increased mRNA levels of 2'-FL biosynthetic enzymes resulted in enhanced 2'-FL productivity and secretion in the CTLD2F2 strain.

In the presence of lactose, engineered yeast strains that carry a lactose transporter with no β-galactosidase activity will accumulate lactose in the cytosol. This unmetabolized lactose can restrict the uptake of other carbon sources (Lodi et al., 2005; Liu et al., 2018). Therefore, the highest 2'-FL productivity by the CTLD2F2 strain with additional copies of the 2'-FL biosynthetic genes might have been caused by less lactose accumulation (Fig. S5). As the 2'-FL biosynthesis pathway converts intracellular lactose into 2'-FL, efficient 2'-FL biosynthesis might alleviate the toxicity derived from lactose accumulation. Indeed, the CTLD2F2 strain exhibited faster xylose and lactose consumption rates and a lower intracellular lactose level than the CTLD1F1 strain (Fig. S5).

3.3. 2'-FL production by engineered yeast in a bioreactor

To increase the titer of 2'-FL and to investigate the feasibility of large scale production of 2'-FL, we examined the performance of the CTLD2F2 strain—the best strain identified from shake flask fermentations—in a fed-batch bioreactor with feeding xylose and lactose to maximize cell growth and 2'-FL production. We conducted two fed-batch fermentations using complex (YP) and defined (Verdyun) media. When the fed-batch fermentation included YP media, 185.3 g/L of xylose and 14.7 g/L of lactose was consumed and produced a total of 17.2 g/L of 2'-FL (12.3 g/L of extracellular and 4.9 g/L of intracellular) with a volumetric productivity of 0.17 g/L·h (Fig. 5a). The final yield of 2'-FL from lactose was 0.82 mol/mol. A majority of the produced 2'-FL (71.5%)

was secreted into the medium at the end of the fed-batch fermentation, which was consistent with the flask fermentation results (Fig. 4, Fig. 5a). While the YP media offered rapid growth of the engineered yeast, there are limiting nutrients in the YP media so that the final cell density was OD_{600}= 62.2 (28.0 g/L DCW) which was not enough to provide a high volumetric productivity. As the CTLD2F2 is a leucine auxotroph strain, we constructed a prototrophic strain (CTLD2F2L) by introducing a functional *LEU2* into the CTLD2F2 strain. When we examined the results of the fed-batch fermentations (Fig. S6), the CTLD2F2L strain final cell density was higher than that of the CTLD2F2 (OD_{600}: 80.0 vs. 62.2) strain. However, the CTLD2F2L strain productivity and total 2'-FL titers were similar to those produced by the CTLD2F2 strain.

We also conducted a fed-batch fermentation using a defined (Verdyun) media for achieving a higher volumetric productivity with a higher cell density (van Hoek et al., 2000). Researchers reported previously that unmetabolized lactose restricts the uptake of carbon sources in engineered yeast strains carrying a lactose transporter with no β-galactosidase activity (Lodi et al., 2005; Liu et al., 2018). We also observed the lactose inhibition on xylose utilization of our engineered yeast (Fig. S7). Therefore, we designed a two-phase fermentation to minimize lactose toxicity in the fed-batch fermentation. In the first phase, we grew cells up to a cell density of OD_{600}=120. In the second phase, we added low levels of lactose into the medium to initiate 2'-FL synthesis (Fig. 5b). The CTLD2F2L strain produced a total of 25.5 g/L of 2'-FL (4.5 g/L of extracellular and 21.0 g/L of intracellular) with a volumetric productivity of 0.35 g/L·h which are substantially higher than those (15.0 g/L of 2'-FL and 0.22 g/L·h) reported in a glucose-limited fed-batch fermentation previously (Hollands et al., 2019). Using xylose instead of glucose as a substrate, we achieved a high 2'-FL production titer without any complicated feeding algorithms or devices that are necessary for a glucose-limited fed-batch fermentation. Moreover, the cell-specific 2'-FL

412 productivity of the CTLD2F2L strain was about 2.9 times higher than that of the previous report
413 (0.43 vs. 0.15 g 2'-FL/g cell) (Hollands et al., 2019). These results suggest that although a glucose-
414 limited feeding strategy can alleviate the glucose repression, xylose consumption might be better
415 suited to providing necessary GDP-L-fucose than glucose. In addition, excess xylose feeding
416 might be better than limited feeding of glucose for the production of 2'-FL.

417 Although we obtained promising results for the production of 2'-FL using xylose as a
418 substrate, further improvements to the CTLD2F2L strain can be considered. During the high cell
419 density fermentation using the Verduyn media (Fig. 5b), 82.4 % of the produced 2'-FL was not
420 secreted into the medium and remained inside the cell, which is consistent with a previous report
421 (Hollands et al., 2019). Depending on a process design to produce 2'-FL, extracellular secretion
422 of 2'-FL into a culture medium can be favored. According to our study results, there might be
423 endogenous transporters capable of exporting 2'-FL in yeast and activities of the endogenous
424 transporters might be different in the YP and Verdyun media. While the synthesized 2'-FL by the
425 engineered yeast was efficiently secreted out of the cell when the YP medium was used, the
426 majority of the produced 2'-FL was inside of the cells when the Verdyun medium was used (Fig.
427 5). We speculated that components in the YP media might induce expression of the endogenous
428 transporters or increase activities of the transporters. Nonetheless, the endogenous transporters
429 might not have enough activity to secrete 2'-FL entirely. Therefore, identification of highly
430 efficient 2'-FL transporters will improve 2'-FL biosynthesis and secretion.

431 Intracellular accumulation of 2'-FL by engineered yeast also can be advantageous for
432 implementing a simplified downstream process if pure 2'-FL is not necessary. For instance, 2'-FL
433 containing cells can be easily harvested from the culture medium and used directly for applications.
434 To test this idea, we harvested and lysed the 2'-FL accumulated cells. The soluble fraction of the

435 cell lysate was then dried to make a yeast extract in a powder form. The resulting yeast extract
436 contained 12% (wt/wt) 2'-FL. We envision that 2'-FL enriched yeast extract can be used as food
437 and animal feed ingredients.
438
439 **4. Conclusions**
440 In this study, we highlight potential advantages of using xylose as a carbon source for the
441 production of 2'-fucosyllactose. Use of xylose instead of glucose can provide better cell growth
442 and energetics, and robust supply of GDP-L-fucose for the production of 2'-FL by engineered
443 yeast. We also report that the increased enzyme levels in the 2'-FL biosynthetic pathway can lead
444 to enhanced 2'-FL production by engineered yeast. The best engineered yeast strain with double
445 copies of *gmd*, *wcaG*, and *wbgL* produced 25.5 g/L of 2'-FL with a productivity of 0.35 g/L·h from
446 a fed batch fermentation using xylose. The capacity of the engineered yeast to secrete 2'-FL was
447 quite different depending on the culture media. While 71.5 % of the produced 2'-FL was secreted
448 to the YP media, 82.4 % of the produced 2'-FL remained inside the cells in the Verduyn media.
449 Through our results, the proof-of-concept level production of 2'-FL by yeast was promoted to the
450 industrial level production using xylose as a carbon source.
451
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Figure legends

Fig. 1. A schematic diagram for 2'-FL production by engineered *S. cerevisiae*. Three metabolic elements are necessary to enable 2'-FL production in yeast: (1) lactose internalization by expression of a heterologous transporter (Lac12: lactose permease) (2) *de novo* production GDP-L-fucose production by expression of heterologous metabolic enzymes (Gmd: GDP-mannose 4,6-dehydratase; WcaG: GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase), and (3) expression of heterologous fucosyltransferase (WbgL: α-1,2-fucosyltransferase).

Fig. 2. GDP-L-fucose production during glucose (YPD30) and xylose (YPX30) utilization by the *S. cerevisiae* CTD strain (a CT2-derived strain with the integrated Gmd and WcaG expression cassettes). 12 h and 36 h indicate the time points of glucose and xylose depletion, respectively. The gray section indicates an ethanol consumption phase. Patterns: glucose (plain) and xylose (coarse)., N.D: not detected., Gmd, WcaG (-) means CT2 strain. Results are the mean of duplicated experiments. Error bars represent standard deviations and are not visible when smaller than the symbol size.

Fig. 3. Batch fermentation profiles of the engineered yeast strain (CTLD1F1) on (a) glucose and (b) xylose conditions. Symbols: OD_{600} (closed circle), glucose (hexagon), xylose (triangle down), ethanol (open circle), lactose (triangle up), extracellular 2'-FL (diamond), and total 2'-FL (square). Results are the mean of duplicated experiments. Error bars represent standard deviations and are not visible when smaller than the symbol size.

Fig. 4. Comparison of engineered yeast strains (CTLD1F1, CTLD2F1, CTLD1F2, and CTLD2F2) for (a) 2'-FL production and (b) volumetric total 2'-FL productivity (g/L·h) on xylose (YPX30L2). Results are the mean of duplicated experiments. Error bars represent standard deviations and are not visible when smaller than the symbol size.

Fig. 5. Fed-batch fermentation profiles of the CTLD2F2 strain using the YP medium (a) the CTLD2F2L strain using the Verduyn medium (b). Symbols: OD_{600} (closed circle), xylose (triangle down), ethanol (open circle), lactose (triangle up), extracellular 2'-FL (diamond), and total 2'-FL (square).

Table 1. Strains used or constructed in this study

Strain	Description	Source
CT2	D452-2 *pho13*Δ::XYL123 *ald6*Δ::XYL123 *his3, leu2, ura3*	Tsai, C.-S (2015)
CTD	CT2 in which the linearized pRS403 (P_{TDH3}-*gmd*-T_{CYC1}, P_{PGK1}-*wcaG*-T_{PGK1}) has been integrated *HIS3* locus	This study
CTL	CT2 in which the P_{TDH3}-*Lac12*-T_{CYC1} cassette has been integrated on chr XVI	This study
CTLD	CTL in which the linearized pRS403 (P_{TDH3}-*gmd*-T_{CYC1}, P_{PGK1}-*wcaG*-T_{PGK1}) has been integrated on *HIS3* locus	This study
CTLD1F1	CTLD in which the P_{TDH3}-*wbgL*-T_{CYC1} cassette has been integrated on chr VII	This study
CTLD2F1	CTLD1F1 in which the linearized pRS406 (P_{TDH3}-*gmd*-T_{CYC1}, P_{PGK1}-*wcaG*-T_{PGK1}) has been integrated on *URA3* locus	This study
CTLD1F2	CTLD1F1 in which the P_{TDH3}-*wbgL*-T_{CYC1} cassette has been integrated on chr VIII	This study
CTLD2F2	CTLD2F1 in which the P_{TDH3}-*wbgL*-T_{CYC1} cassette has been integrated on chr VIII	This study
CTLD2F2L	CTLD2F2 in which the linearized pRS405 has been integrated on *Leu2* locus	This study

663 **Graphic abstract**

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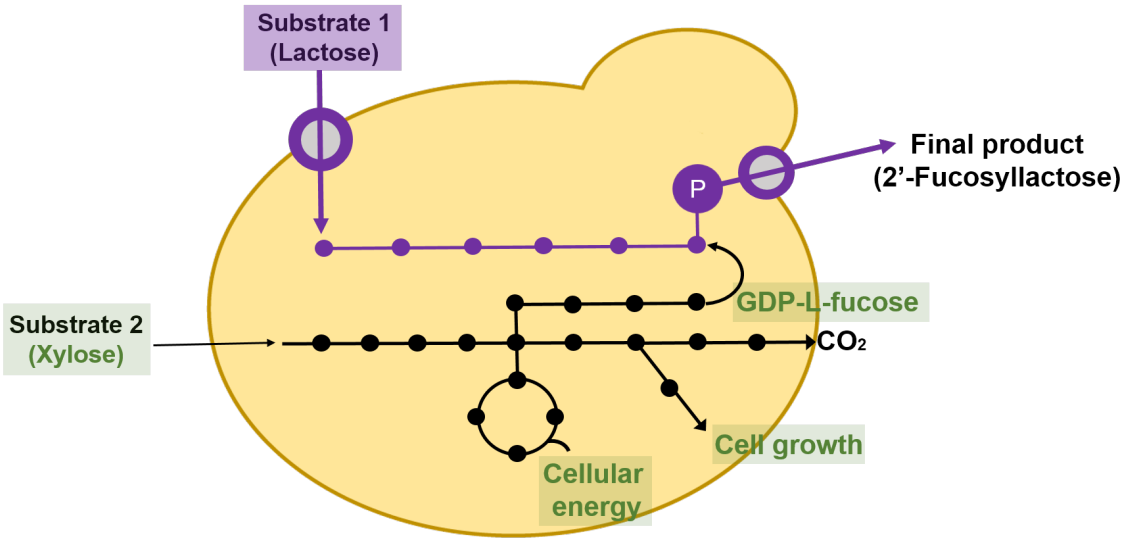

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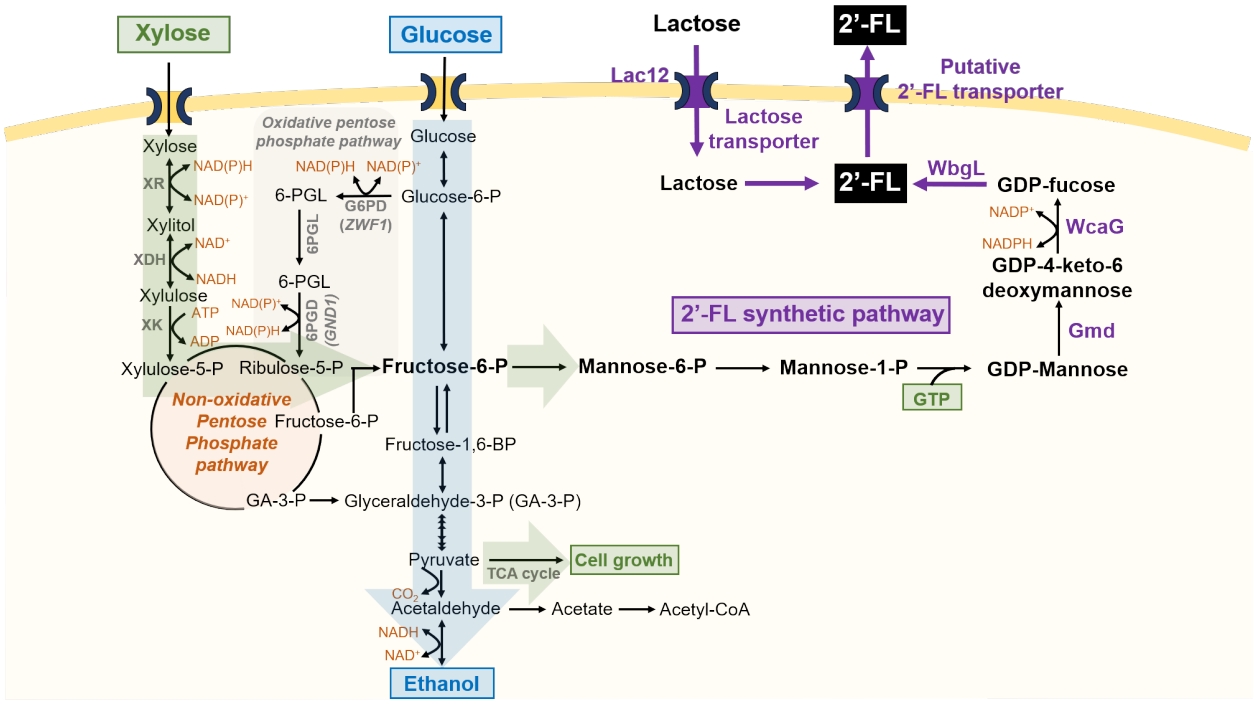

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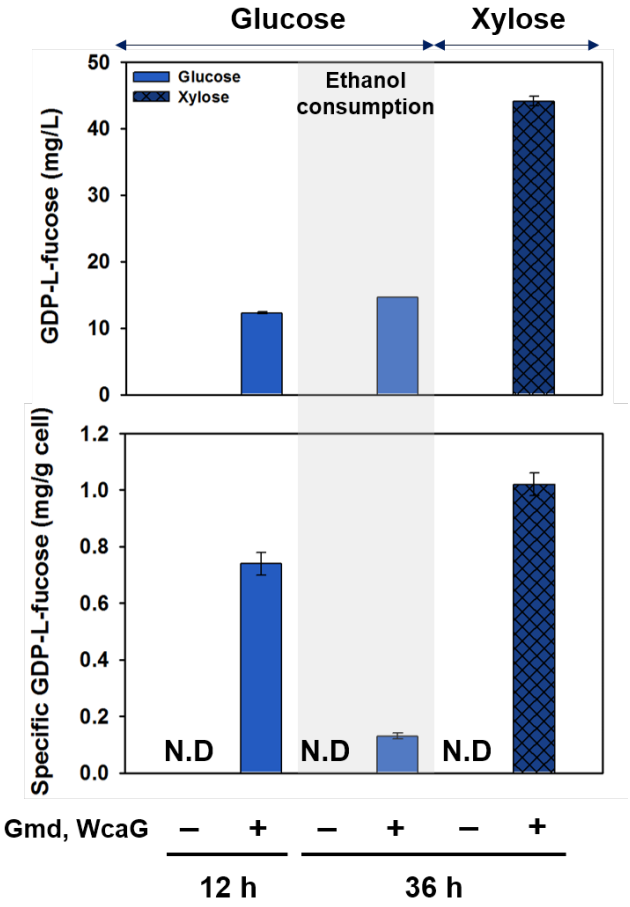

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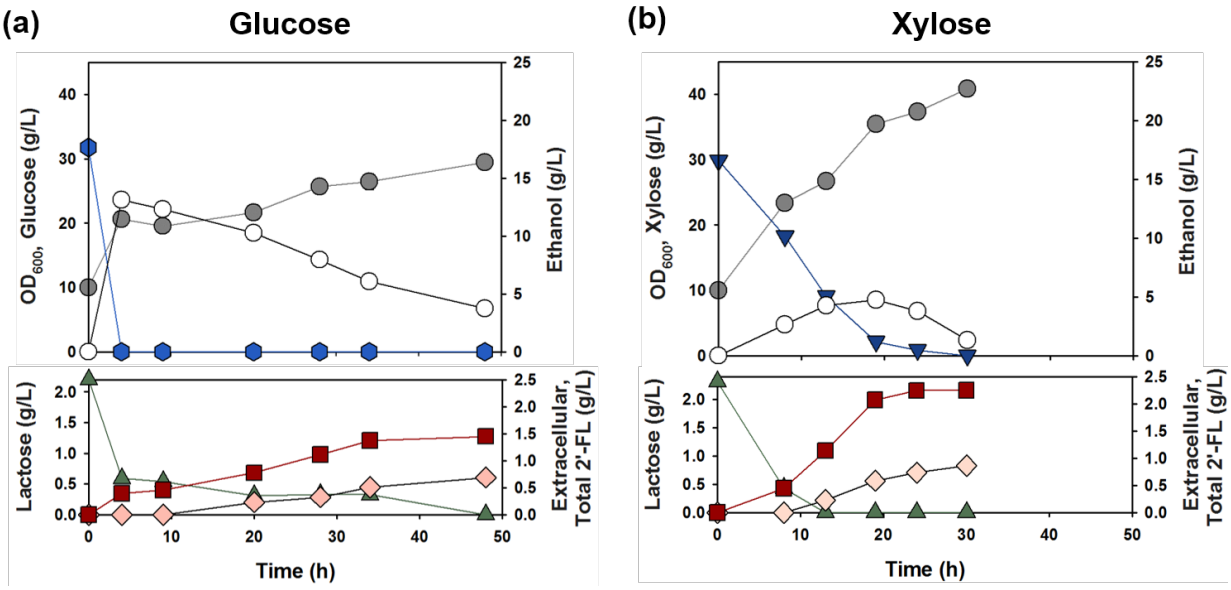

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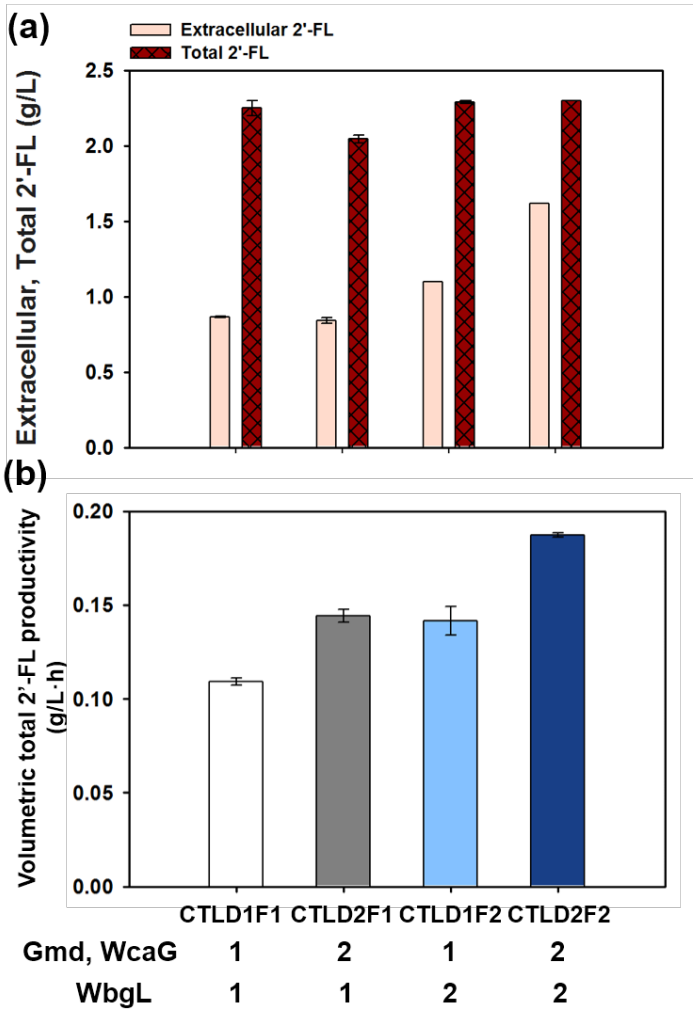

Fig. 4. Comparison of engineered yeast strains (CTLD1F1, CTLD2F1, CTLD1F2, and CTLD2F2) for (a) 2'-FL production and (b) volumetric total 2'-FL productivity (g/L·h) on xylose condition (YPX30L2).

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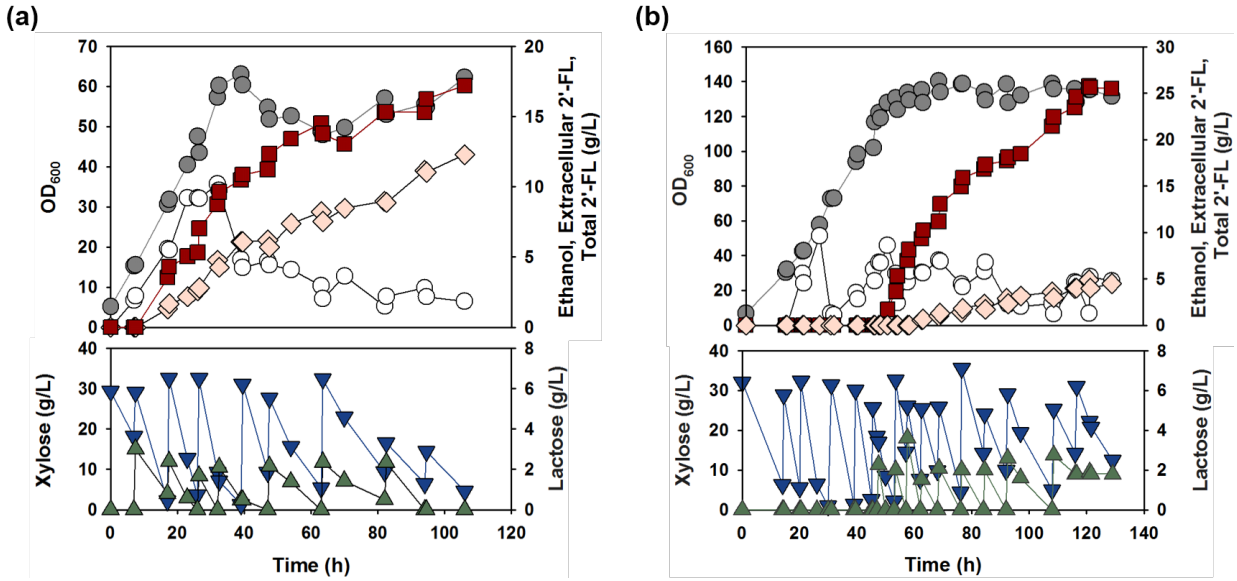

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708 **Fig. 5.** Fed-batch fermentation profiles of (a) the CTLD2F2 strain under YP medium (b) the

709 CTLD2F2L strain under Verduyn medium. Symbols: OD_{600} (closed circle), xylose (triangle down),

710 ethanol (open circle), lactose (triangle up), extracellular 2'-FL (diamond), and total 2'-FL (square).

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