Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of 2'-fucosyllactose

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Abstract

Human milk oligosaccharides (HMOs) have been known for their abundance of nutrients for infants and its protection from pathogens and infectious diseases. 2'-fucosyllactose (2-FL) is the most abundant HMO, and it has a potential to be facilitated as nutraceutical and pharmaceutical purpose. Currently, many scientists are investigating the production of 2-FL in *E. coli*, but the research has not been optimized to produce 2-FL in yeast. Thus, the production of 2-FL in *S. cerevisiae* yeast strain D452-2 was proposed to be optimized for a similar level of 2-FL production in *E. coli*. For efficient 2-FL production, *S. cerevisiae* strain SK1, based on D452-2, was mutated for upper and lower glycolysis pathways, enhanced GDP-mannose synthetic pathway, and developed via transformation with the appropriate fucosyltransferase and transporter. The levels of GDP-mannose and GDP-fucose affect the production of 2-FL according to the previous studies *in E. coli*. Therefore, the overproductions of GPD-mannose and GDP-fucose were performed. These experiments found that engineered SK1 base strains had higher levels of GDP-mannose and GPD-fucose productions than developed D452-2 base strains. However, SK1P2L-gwf strain with widely used fucosyltransferase (FucT2) and transporter (LAC12) did not show the higher level of both intracellular and extracellular 2-FL productions than the D452L-gwf strain. The result of this experiment described a toxicity of lactose to produce 2-FL so that it was repeated with varied lactose concentrations. Repeating the experiment revealed that the low lactose concentration produced more 2-FL and the fucosytransferase and the transporter did not properly function because intracellular 2-FL was more highly accumulated than extracellular 2-FL. To be optimized the strain for efficient 2-FL production, SK1P2-w(b)gwh and D452-w(b)gwh were constructed with WbgL and mutant

HXT2.4, as a fucosyltransferase and a lactose transporter, respectively, in the lower initial lactose concentration. It determined SK1P2-w(b)gwh had the highest productivity of 2-FL.

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Introduction

Human milk oligosaccharides (HMOs) are found in human breast milk and are necessary for infants to develop as they have abundant nutrients.[1] Previous researchers identified approximatly 140 different types of HMOs. They were found to prevent pathogens (e.g., *Salmonella* and *Helicobacter pylori*), reduce the risks of bacterial and viral infections, and improve immune response.[1–3] Among different HMOs, the most abundant is 2'-fucosyllactose (2-FL) that over 30% of HMOs.[3,4] Due to its abundance and significant role in protection against pathogens and infectious diseases, 2-FL has potential effects as a nutraceutical and pharmaceutical substance. Production of 2-FL requires fucosyltransferases such as α-1,2-fucosyltransferase from *H. pylori* (FucT2) to synthesize the final product, 2-FL, from GDP-fucose and lactose. Previous studies demonstrated that GDP-fucose could be efficiently synthesized in *Escherichia coli.* Therefore, many metabolic engineering approaches have been achieved to produce 2-FL in *E. coli* by introducing the widely used FucT2 gene .[3,5]

Even though 2-FL in the bacteria can be cultured and investigated easily in the laboratory, there is the limitation of 2-FL production in bacteria as a nutraceutical and pharmaceutical substance in the industry because people have a negative point of view about the usage bacteria for human. Also, many studies revealed yeast is more stable than bacteria, and *Saccharomyces cerevisiae* yeast strain D452-2 is commonly used as industrial *yeast* strain. [3,6] Therefore, we proposed the production of 2-FL in yeast, especially *Saccharomyces cerevisiae*, to increase the utilization and preference of 2-FL in the industry. Also, the study of 2-FL production in yeast has a novelty because the study of 2-FL production in *E. coli* was recently continued, but there are no cases associated with yeast. Over the years, *S. cerevisiae* has been the most widely used as engineered yeast species in the laboratory.[7,8] An understanding of the 2-FL

biosynthetic metabolic pathway in yeast is important to produce 2-FL efficiently via metabolic engineering. Enhanced GDP-mannose synthesis is needed for an overproduction of GDP-fucose, a precursor for 2-FL synthesis, by the overexpression of genes (e.g., *PMI40* and *PSA1*) through Cas9-based promoter substitution. Phosphomannose isomerase (*PMI40*) converts fructose-6-phosphate (Fruc-6-P) to mannose-6-phosphate (Man-6-P).[8] Phosphomannomutase encoded by *SEC53* converts Man-6-P to mannose-1-phosphate (Man-1-P). Man-1-P is converted to GDP-mannose by mannose-1-phosphate guanylyltransferase, encoded by *PSA1*, using guanosine-5-triphosphate (GTP) molecules. Fucosyltransferase will produce 2-FL from GDP-fucose, and a transporter will facilitate the transport of 2-FL out of the cell (Figure 1).

For efficient production of 2-FL, I proposed to overproduce GDP-mannose and GDP-fucose using a mutant *S. cerevisiae* D452-2 strain, called SK1, to develop the construction of the strain. SK1 is an engineered strain via *phosphofructokinase (PFK)* mutation in glycolysis for upper and lower glycolysis pathways (Figure 1 and Table 1). In our lab, both D452-2 and SK1 strains were already succeeded in CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 system, which is a strong genome-editing method in the laboratory. CRISPR-Cas9 system generates double-strand breaks of the genome of host cell at a precise location to screen correct mutant strains after removing endogenous DNA and/or to add new DNA since Cas9 nuclease complex is carried with a synthetic guide RNA (gRNA) into the cell.[9] We placed drug resistance markers NAT1 (nourseothricin resistance) to express Cas9 and gRNA in both D452-2 and SK1 strains as the industrial *S. cerevisiae* strains.[10] Moreover, an expression system of *S. cerevisiae* strains relies on the selected vector. This system contains cassettes including the strong *GPD* promoters and *CYC1* terminator. Expression cassettes were positioned in pRS series plasmids containing *HIS3, LEU2, URA3,* and *TRP1* markers (Table 2). Constructed expression

vectors are useful for the cloning and the gene expression in the genetic background of used strains.[6] I hypothesized that the engineered and developed SK1 strain would have a higher productivity of GDP-mannose, GDP-fucose, and 2-FL than developed parental strain D452-2. I performed several attempts to improve and analyze the production of 2-FL in the developed SK1 base strains compared to developed D452-2 base strains. Regardless of the *S. cerevisiae* strain developments with the changes of fucosyltransferase and transporter, the strains underwent *de novo* pathway of GDP-fucose that overexpressed GDP-D-mannose-4,6-dehydratase (*gmd*) and GDP-L-fucose synthase (*wcaG*) (Figure 1). Furthermore, a significant amount of 2-FL in the developed SK1 base strain would be expected to be produced the similar level of 2-FL production in *E. coli* using fermentation equipment.

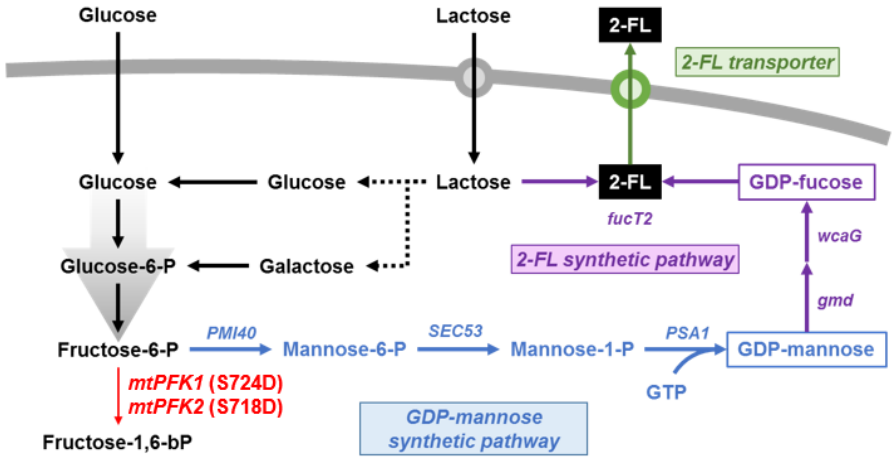


Figure 1. Metabolic pathways for GDP-mannose, GDP-fucose, and 2'-fucosyllactose (2-FL) biosynthesis in *S. cerevisiae* yeast. Unlike parental strain D452-2, only engineered *S. cerevisiae* strain SK1 was mutated kinase enzymes such as *PFK1 and PFK2* (red-colored arrow).

Materials and Methods

Strains and media

Laboratory *S. cerevisiae* yeast strain D452-2 and engineered *S. cerevisiae* strain SK1 were used in this study (Table 1). The yeast strains were cultured in three different types of medium depending on experiments as follows: yeast extract peptone (YP; 10 g/L yeast extract and 20 g/L peptone) supplemented with glucose (YPD) alone or together with lactose. Synthetic complete (SC; 6.7g/L yeast nitrogen base with ammonium sulfate and 0.65 g/L CSM-HIS-URA-LEU or 0.79 g/L CSM, MP Biomedicals) supplemented with glucose (SCD) and 50 mM pH 5.5 potassium hydrogen phthalate (KHP) buffer. SC medium without histidine, uracil, and leucine (SCD-H-U-L) were used for the confirmation of auxotrophic phenotype. Verduyn (15 g/L $(NH_4)_2SO_4$, 8 g/L KH_2PO_4, 3 g/L $MgSO_4$, 10 mL/L trace element, and 12 mL/mL vitamin solution) supplemented with glucose (VD), lactose, and 50 mM pH 5.5 KHP buffer.

Plasmid preparation

All plasmids used in this study are described in Table 1. *E. coli* Top 10 was used for plasmids propagation. *E. coli* was grown in 5 mL of LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0) containing 100 µg/mL of ampicillin at 37°C shaker for 12 hours. Propagated *E. coli* was centrifuged at maximum speed for 10 minutes. Using Qiagen miniprep kit, 30 µL of plasmid samples were obtained and their concentrations were measured by Nanodrop.

Polymerase chain reaction (PCR) for donor DNA cloning

Primers and its sequences that listed in Table 1 are used in this study before yeast strain transformation. 10 µL of gRNA expression vector down/up (primers), 100 µL of 2X Phusion High-Fidelity Master Mix, 4 µL of DNA template (plasmid vector), and 76 µL of distilled water

were mixed thoroughly. After PCR amplifications via PCR program, products were purified through ethanol/acetate precipitation.

Yeast strain construction

For CRISPR-Cas9 based genome editing, yeast strains harboring Cas9-NAT were regenerated in 5 mL of YPD medium with 120 µg/mL of clonNAT antibiotic at 30℃ for 24 hours. Cells were subsequently transferred into 10 mL YPD medium containing clonNAT. Initial optical density at 600 nm (OD) was 0.5 and cultures were incubated at 30℃ until OD approached to 2. A lithium acetate transformation method[11] was used. Transformants were screened by spreading on YPD plates containing corresponding antibiotics and gRNA plasmids, and incubated at 30℃ until transformants were ready to be picked. The transformed yeast strain on a plate was confirmed by colony PCR and gel electrophoresis. Colony PCR mixtures composed of 5X Q5 reaction buffer, 10 mM dNTPs, 10 µM forward primer, 10 µM reverse primer, yeast cell paste from a single colony of the plate, Q5 DNA polymerase, and nuclease-free water. Colony PCR products were loaded on 1% agarose gel with 1kb DNA ladder. Transformation of auxotrophic 2-micron plasmids expressing heterogenous genes were performed by same lithium acetate method[11] and correct transformants were screened on SCD-H-U-L plate.

Cultivation of engineered strains in medium and analytical methods

Depending on the type of plasmids in engineered strains, pre-cultured yeast cells were inoculated and grown on 10 mL of YPD with corresponding antibiotics, SCD, SCD-H-U-L, or VD to maintain episomal plasmids. An initial OD was adjusted to 0.1 for confirmations of GDP-mannose and GDP-fucose and OD = 0.5 or 1 for the production of 2-FL in 50 mL of fermentation medium containing glucose alone or with lactose. All the flasks used for fermentation were sealed with aluminum foil and incubated at 30℃ and a 250 rpm in a

MaxQ4000 orbital shaker (Thermo Fisher Scientific Inc., USA). Samples of yeast cultures were obtained to monitor cell growth and extracellular and intracellular metabolites. Cell growth was monitored by OD of samples using BioMate 5 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA) after dilution. To quantify extracellular and intracellular metabolites, samples were centrifuged at 16,400 rpm for 10 minutes. The supernatants were analyzed using a 1200 series high-performance liquid chromatography (HPLC, Agilent Technologies Inc., USA) to measure extracellular metabolites such as glucose, lactose, glycerol, acetate, ethanol, and 2-FL in culture broth. A Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex, USA) was used with the mobile phase 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50°C. To measure intracellular metabolites, harvested cells were washed, and vortexed with proper amounts of glass beads and distilled water to break the cell. The supernatant was separated from cell debris and glass beads by centrifugation at 4°C maximum speed for 10 min. GDP-mannose and GDP-fucose were identified and quantified at 254 nm by HPLC equipped with UV detector (Shimadzu Scientific Instruments, Japan) and Capcell PAK C18 column (Shimadzu, Japan). 15mM ammonium acetate 5% of acetonitrile were flowed with 95:5 ratio at 0.4 mL/min as a mobile phase. Intracellular lactose and 2-fucosyllactose were measured by same HPLC system used for analyzing extracellular metabolites.

Table 1. Description of strains that used in this study.

Name	Description	Reference
S. cerevisiae Strain		
D452-2	D452-2(*MATα*, *leu2*, *his3*, *ura3*, and *can1)*	12
D452P	D452-2 in which *GPD* promoter substituted *PMI40* promoter	This study
D452P2	D452P in which *GPD* promoter substituted *PSA1* promoter	This study
D452L	D452-2 in which the P_{GPD}-*LAC12*-T_{CYC1} was integrated on Chr VII	This study
D452-gw	D452-2, pRS423GPD-gmd, pRS425GPD-wcaG, pRS426GPD	This study
D452L-gwf	D452L, pRS423GPD-gmd, pRS425GPD-wcaG, pRS426GPD-FucT2	This study
D452-w(b)gwh	D452-2, pRS423GPD-WbgL, pRS425GPD-gmd-wcaG, pRS426GPD-mtHXT2.4	This study
SK1	D452-2 harboring *mtPFK1* and *mtPFK2*	Kwak, S

SK1P	SK1 in which *GPD* promoter substituted *PMI40* promoter	This study
SK1P2	SK1P in which *GPD* promoter substituted *PSA1* promoter	This study
SK1P2L	SK1P2 in which the P_{GPD}-*LAC12*-T_{CYC1} was integrated on Chr VII	This study
SK1P2-gw	SK1P2, pRS423GPD-gmd, pRS425GPD-wcaG, pRS426GPD	This study
SK1P2L-gwf	SK1P2L, pRS423GPD-gmd, pRS425GPD-wcaG, pRS426GPD-FucT2	This study
SK1P2-w(b)gwh	SK1P2, pRS423GPD-WbgL, pRS425GPD-gmd-wcaG, pRS426GPD-mtHXT2.4	This study

Table 2. Description of plasmids that used in this study.

Name	Description	Reference
Plasmid		
Cas9-NAT	Cas9 expression plasmid, NAT1 marker	10
pRS423GPD-gmd	*HIS3* marker in yeast episomal vector, P_{GPD}-*gmd*- T_{CYC1}	Liu, J. J
pRS425GPD-wcaG	*LEU2* marker in yeast episomal vector, P_{GPD}-*wcaG*- T_{CYC1}	Liu, J. J
pRS426GPD	*URA3* marker in yeast episomal vector, P_{GPD}	6
pRS424GPD-LAC12	*TRP1* marker in yeast episomal vector, P_{GPD}-*LAC12*- T_{CYC1}	Liu, J. J
pRS423GPD-FucT2	*HIS3* marker in yeast episomal vector, P_{GPD}-*FucT2*- T_{CYC1}	Liu, J. J
pRS423GPD-WbgL	*HIS3* marker in yeast episomal vector, P_{GPD}-*WbgL*- T_{CYC1}	Jin lab
pRS423GPD-WcfB	*HIS3* marker in yeast episomal vector, P_{GPD}-*WcfB*- T_{CYC1}	Jin lab
pRS425GPD-gmd-wcaG	*LEU2* marker in yeast episomal vector, P_{GPD}-*gmd-wcaG*-T_{CYC1}	Kwak, S
pRS426GPD-mtHXT2.4	*URA3* marker in yeast episomal vector, P_{GPD}-*H*- T_{CYC1}	Jin lab
pRS42K-CS6	G418(antibiotic) in yeast episomal vector,	Kwak, S

Table 3. Description of primers that used in this study.

Name	Description	Reference
Primer		
Jin4963	*GPD* promoter forward for *PMI40* promoter substitution (tggttattgtagcaatttttgtcgcgtccgtgtccttttcg CACGCTTTTTCAGTTCG)	Jin lab
Jin4964	*GPD* promoter reverse for *PMI40* promoter substitution (gcacatacctgcatctaacctgaacagcttgttggacat TTTGTTTGTTTATGTGTGTTT)	Jin lab
Jin5034	*PMI40* promoter sequence forward (gcataaacctgtagcgc)	Jin lab
Jin5035	*PMI40* promoter sequence reverse (ctgaacagcttgttggac)	Jin lab
Jin4967	*GPD* promoter forward for *PSA1* promoter substitution (aagtgtgcaaactactttacattcgctaactctttttttctgt CACGCTTTTTCAGTTCG)	Jin lab
Jin4968	*GPD* promoter reverse for *PSA1* promoter substitution (tctggtaccgtaaccaccgactaaaattaaacctttcat TTTGTTTGTTTATGTGTGTTT)	Jin lab
Jin5038	*PSA1* promoter sequence forward (cagctagaaatgcctcgg)	Jin lab
Jin5039	*PSA1* promoter sequence reverse (ggtctcaatctggtaccg)	Jin lab
Soo587	CS6 Int dDNA forward (AACCTCGAGGAGAAGTTTTTTTACCCCTCTCCACAGATC caggaaacagctatgaccatg)	Jin lab
Soo588	CS6 Int dDNA reverse (TAATTAGGTAGACCGGGTAGATTTTTCCGTAACCTTGGTGTC tgtaaaacgacggccagt)	Jin lab
Jin2382	Before GPD Sequence/Confirm (aaatcagagagagcagaagg)	Jin lab
Soo595	-700 of NGG of CS6 (GTCTGCCGAAATTCTGTG)	Jin lab

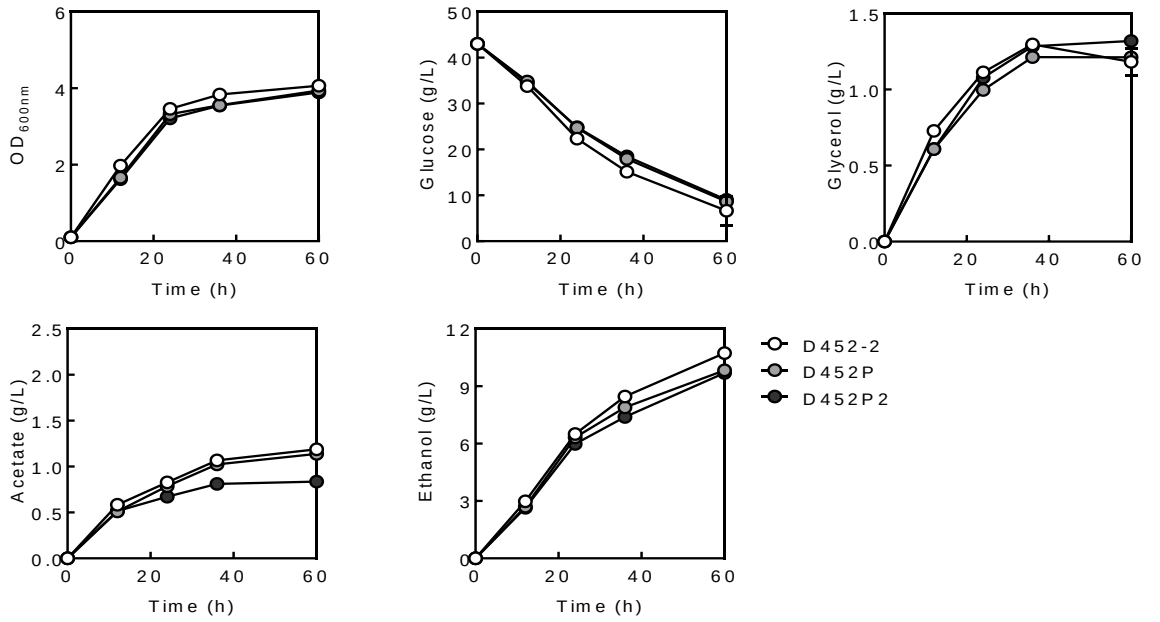
Results and Discussion

Comparison of GDP-mannose overproduction among varied strains based on parental strain D452-2 and engineered strain SK1 via aerobic cultivation

As followed the hypothesis of this study, aerobic cultures for comparing GDP-mannose levels were conducted with yeast strains developed from a wild-type strain D452-2 and an PFK mutant strain SK1 illustrated in Table 1. Since *PFK1* and *PFK2* of SK1 were mutated by site-directed mutagenesis on a Serine residue, specifically Ser724Asp of *PFK1* and Ser718Asp of *PFK2*, upper glycolysis of SK1 cannot be activated by the allosteric up-regulator, fructose-2,6-bisphosphate (Fructose-1,6-bP) (Figure 1). A strong constitutive GPD promoter was amplified from D452-2 genomic DNA with Jin4963 and Jin4964 primers and introduced in front of *PMI40* coding sequences of D452-2 and SK1. The promoter substitution was confirmed with Jin5034 and Jin5035 primers, and resulting strains were named as D452P and SK1P. *PSA1* promoters of D452P and SK1P were further substituted in the same manner using Jin4967 and Jin4968 primers. The resulting strains confirmed with Jin5038 and Jin5039 primers were named as D452P2 or SK1P2 (Table 1, 2, and 3). To compare the productivity of GDP-mannose among representative colonies of parental and engineered strains, aerobic cultures were conducted with SCD containing full amino acid and 40 g/L glucose for 60 hours. The cell growth in 600 nm of SK1 base stains (SK1, SK1P, and SK1P2) showed higher cell mass titer as compared to D452 parental base strains (D452-2, D452P, and D452P2). Most metabolites showed similar trends excepting glycerol (Figure 2A and 2B). I concluded based on this that upper and lower glycolysis pathways in SK1 were balanced. Therefore, SK1 did not need to use the glycerol synthetic pathway in order to re-generate phosphate from sugar phosphates.[13] At 24 hours, intracellular GDP-mannose levels of each strain were measured. The production of GDP-mannose was

approximately 5.5 times greater in SK1P2 than in parental strain D452-2 (Figure 3 and Table 4). The result indicated that slow upper glycolysis is beneficial in terms of GDP-mannose synthesis since mutated Pfk1 and Pfk2 are unable to catalyze fructose-6-P phosphorylation to fructose-1,6-bP. Therefore, more fructose-6-P could pass through GDP-mannose synthetic pathway to produce more GDP-mannose rather than fructose-1,6-bP.

A.



B.

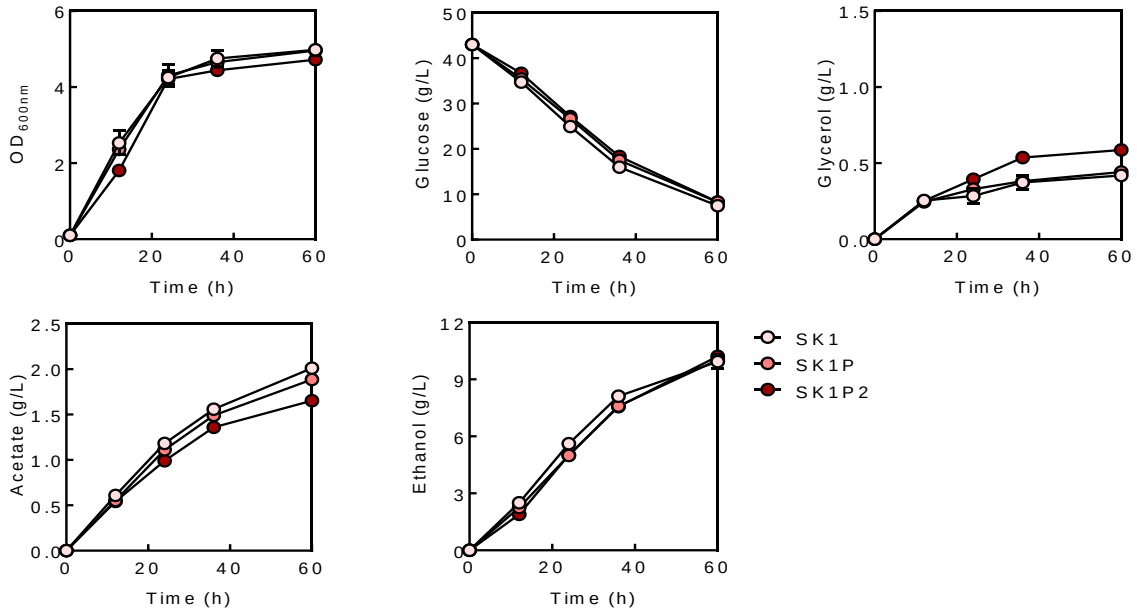


Figure 2. Aerobic culture profiles of *S. cerevisiae* strain D452-2 (A) and the engineered *S. cerevisiae* stain SK1 (B) that were cultured in SCD medium. Mean values of duplicate experiments are obtained, and error bars indicate standard deviations.

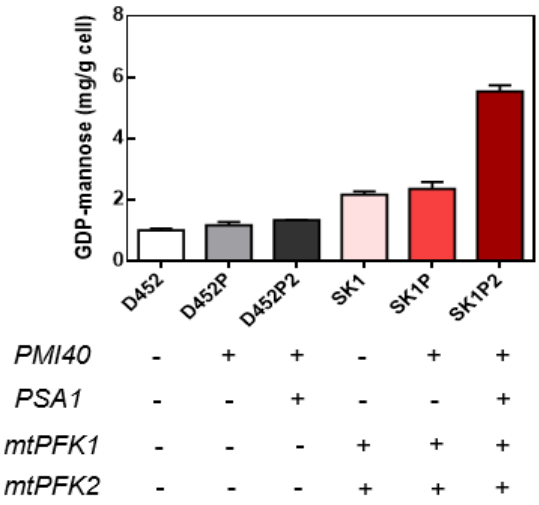


Figure 3. GDP-mannose levels at 24 hours. Mean values of duplicate experiments are obtained, and error bars indicate standard deviations. (+) symbol indicates a presence of each expression while (-) symbol indicates an absence of the expression.

Comparison of GDP-fucose production between engineered strains D452-gw and SK1P2-gw via aerobic cultivation

It was demonstrated that SK1P2 has better capabilities to synthesize GDP-mannose. I extended this experimental concept, the combination of slow upper glycolysis and enhanced GDP-mannose pathway, to the production of GDP-fucose. To test the production of GDP-fucose in yeast and *de novo* pathway of GDP-fucose, yeast episomal vectors for expression of GDP-D-mannose-4,6-dehydratase (*gmd*) and GDP-L-fucose synthase (*wcaG*) were introduced into D452-2 and SK1P2. GDP-mannose is converted into GDP-4-keto-6-dexoymannose by the Gmd enzyme, and GDP-fucose is produced by WcaG enzyme from GDP-4-keto-6-dexoymannose.[14–16] GDP-fucose producing strains were constructed by introduction of pRS423GPD-gmd, pRS425GPD-wcaG, and pRS426GPD into D452-2 and SK1P2, and they were named D452-gw and SK1P2-gw (Table 1). The aerobic fermentation was performed in SCD-H-U-L medium

containing 40 g/L of glucose and 50 mM pH 5.5 KHP buffer. At 36 hours, GDP-mannose of 0.649 mg and 2.514 mg of GDP-mannose were measured per g cell mass in D452-gw and SK1P2-gw, respectively. GDP-fucose levels in D452-gw and SK1P2-gw were 0.314 mg/g cell and 0.414 mg/g cell, respectively (Figure 5). I expected GDP-fucose synthesis in both strains to follow the previous trend of GDP-mannose, but the increase of GDP-fucose level was not significant (25%). GDP-fucose was biosynthesized from its substrate GDP-4-keto-6-deoxymannose that led to in the expression of gmd vector.[3,14,16] GDP-4-keto-6-deoxymannose could be accumulated before it synthesized GDP-fucose in the expression of wcaG, and it could lead to more GDP-fucose.

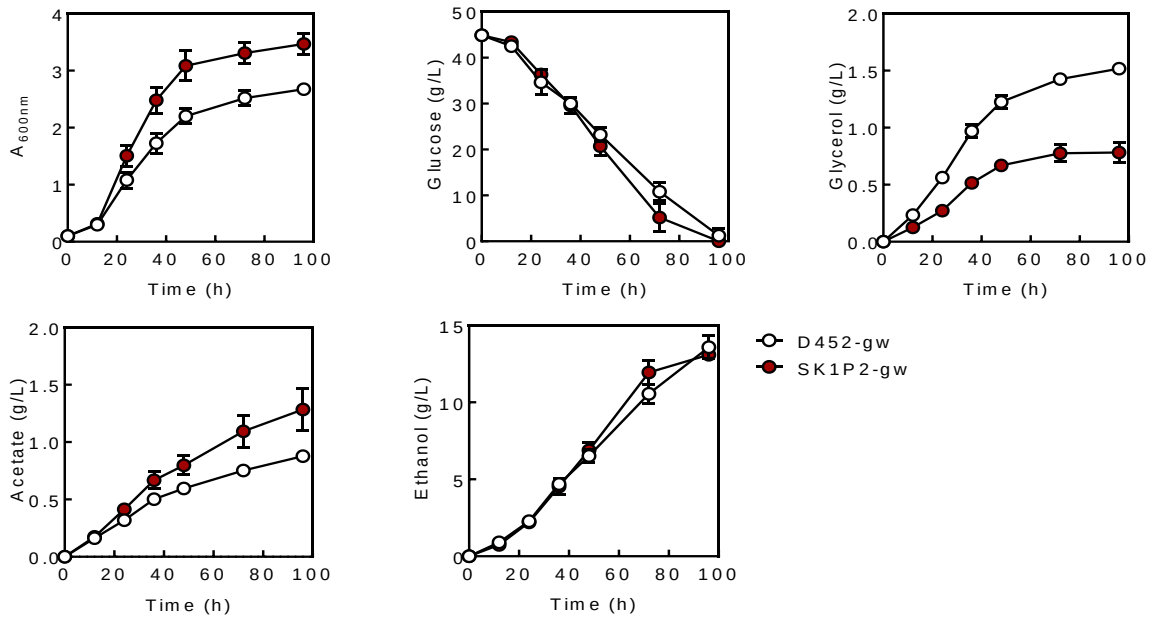


Figure 4. Aerobic culture profiles of D452-gw (white) and SK1P2-gw (red). These were cultured in SCD medium. Mean values of triplicate experiments are obtained, and error bars indicate standard deviations.

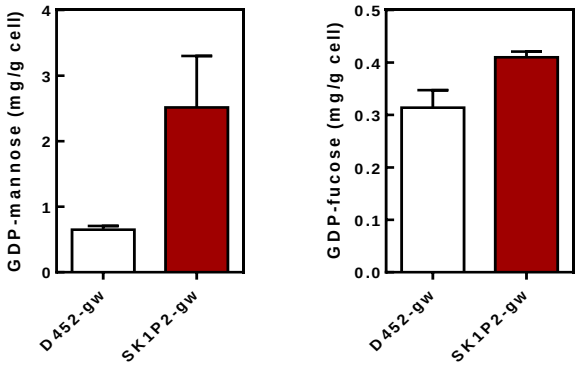


Figure 5. GDP-mannose and GDP-fucose levels of D452-gw and SK1P2-gw strains at 36 hours. Mean values of triplicate experiments are obtained, and error bars indicate standard deviations. Bars: D452-gw (white) and SK1P2-gw (red).

Comparison of 2-FL production between engineered strains D452L-gwf and SK1P2L-gwf

I tested the production of GDP-fucose in de novo pathway and confirmed the GDP-fucose productivity in D452-gw and SK1P2-gw. To test a relationship between GDP-fucose synthesis and 2-FL production, I integrated lactose transporter (Lac12) and overexpressed fucosyltransferase (FucT2). The pRS plasmids transformation was also performed to overexpress FucT2. I named these strains as D452L-gwf and SK1P2L-gwf (Table 1). “L” indicates LAC12 expression cassette integration on CS6 intergenic site on Chr VII using pRS424GPD-LAC12 and pRS42K-CS6, as a template of the expression cassette and gRNA plasmid for CRISPR-Cas9-based genome editing, respectively. “gwf” indicates episomal vectors pRS423GPD-gmd, pRS425GPD-wcaG, and pRS426GPD-FucT2 (Table 2). Representative colonies of the transformation were selected from the SCD-H-U-L plate and performed the fermentation in YPD containing 40 g/L of glucose and 4 g/L of lactose. SCD medium was used for preculture to maintain episomal plasmids. Unlike the results of previous experiments, SK1 base strain (SK1P2L-gwf) was not grown well and had a lower glucose consumption than D452-2 parental base strain (D452L-gwf). After 48 hours, an analysis using HPLC shown the amount of lactose

was increased in both D452L-gwf and SK1P2L-gwf medium (Figure 6) and quantified 2-FL in D452L-gwf that 0.0173 g/g cell inside of cells and 0.00147 g/g cell outside of cells. In SK1P2L-gwf strain, 2-FL was produced that 0.0133 g/g cell inside of cells and 0.00223 g/g cell outside of cells. It showed that D452L-gwf strain was produced more 2-FL than SK1P2L-gwf (Figure 7 and Table 5). I did not expect that lower productivity of SK1P2L-gwf, therefore, I analyzed intracellular and extracellular 2-FL and lactose productions with samples at 12 hours and 48 hours. As times went by, the intracellular levels of both lactose and 2-FL were increased (Figure 7). SK1P2L-gwf contained more lactose but less 2-FL inside and outside of cells than D452L-gwf. Strains could consume lactose, but lactose could not be efficiently utilized to produce 2-FL due to inefficient activities of FucT2. A significant amount of lactose was intracellularly accumulated and disrupted the production of 2-FL.

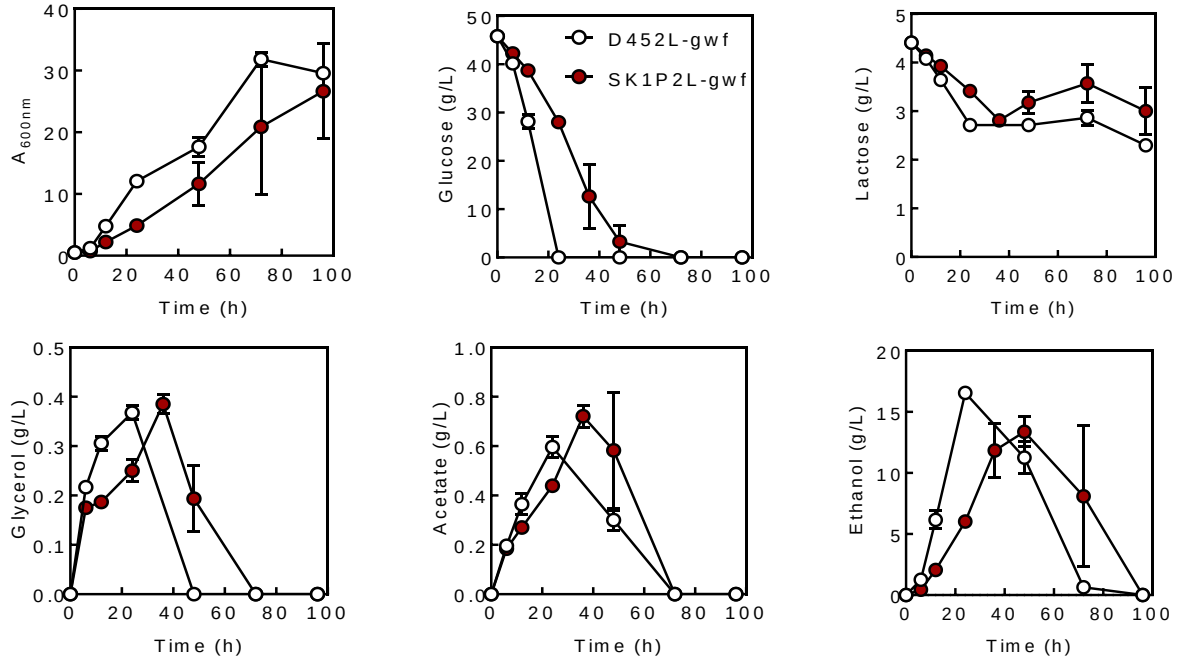


Figure 6. Culture profiles of D452L-gwf and SK1P2L-gwf. D452L-gwf (white) and SK1P2L-gwf (red) were cultured in YP medium containing 40 g/L glucose and 4 g/L lactose. Mean values of triplicate experiments are obtained, and error bars indicate standard deviations.

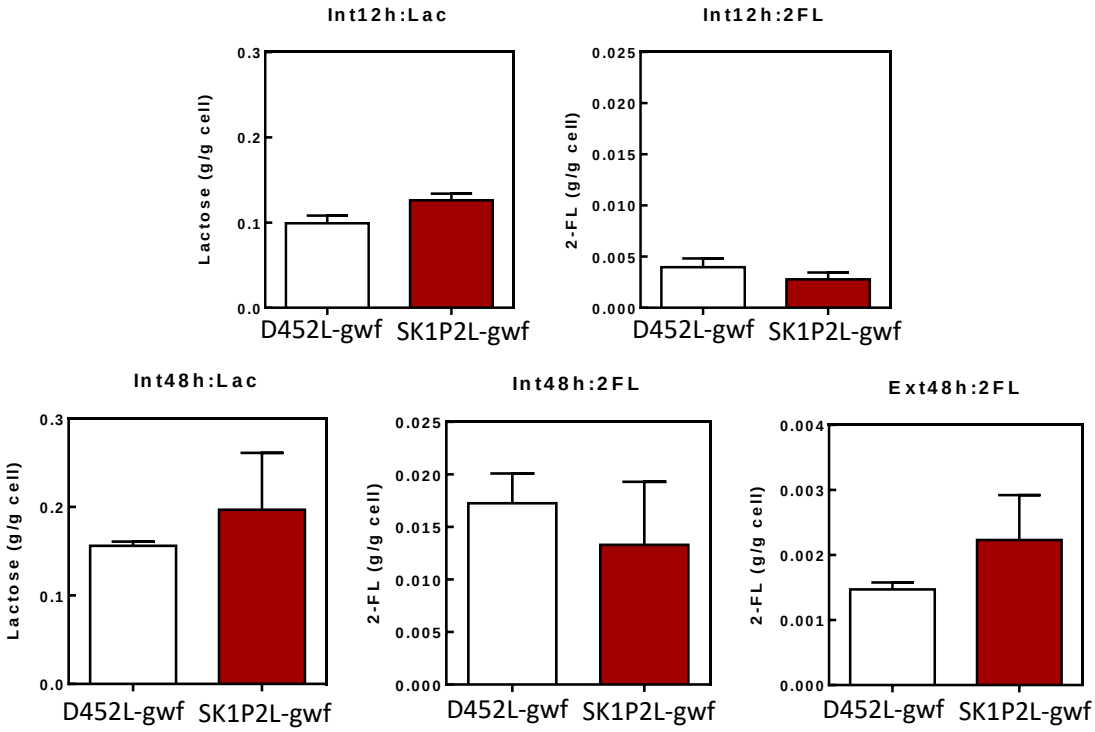


Figure 7. Intracellular lactose and 2-FL concentrations at 12 hours and 48 hours. Mean values of triplicate experiments are obtained, and error bars indicate standard deviations. Bars: D452L-gwf (white) and SK1P2L-gwf (red).

Strain	Mean value of 2-FL product (g/g cell)		
	Intracellular Lactose	Intracellular 2-FL	Extracellular 2-FL
D452L-gwf	0.156	0.0173	0.00147
SK1P2L-gwf	0.197	0.0133	0.00223

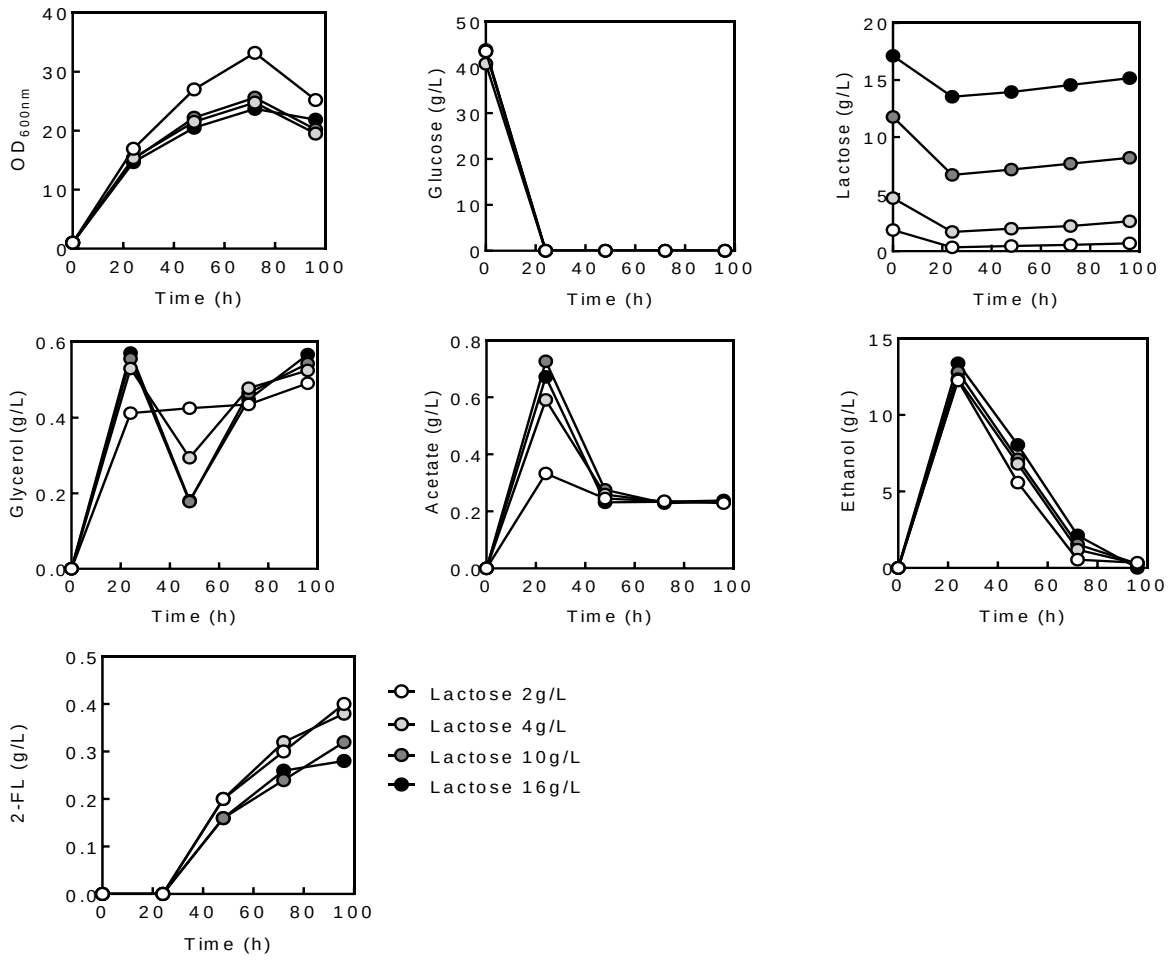
Table 4. Numerical values of culture profiles of lactose and 2-FL levels for different strains at 48 hours. Mean values of productions are illustrated in Figure 7.

Comparison of 2-FL production in accordance with varied lactose concentrations between engineered strains D452L-gwf and SK1P2L-gwf

To determine the effect of initial lactose concentrations on 2-FL production of both D452L-gwf and SK1P2L-gwf, previous culture experiments were repeated with varied lactose concentrations (e.g., 2g/L, 4g/L, 10g/L, and 16g/L) and the initial optical cell density was OD = 1 at 600nm instead of OD = 0.5. After glucose depletion, acetate and ethanol were *assimilated*. Interestingly, extracellular lactose was accumulated during the acetate and ethanol assimilation

phase. (Figure 8). After the fermentation was done at 96 hours, extracellular 2-FL was quantified using HPLC (Figure 9 and Table 6); lower lactose concentrations produced more 2-FL in both D452L-gwf and SK1P2L-gwf, even though the differences among varied lactose concentrations were minor. I concluded that the high lactose concentration negatively affected cell growth and 2-FL titer.

A.



B.

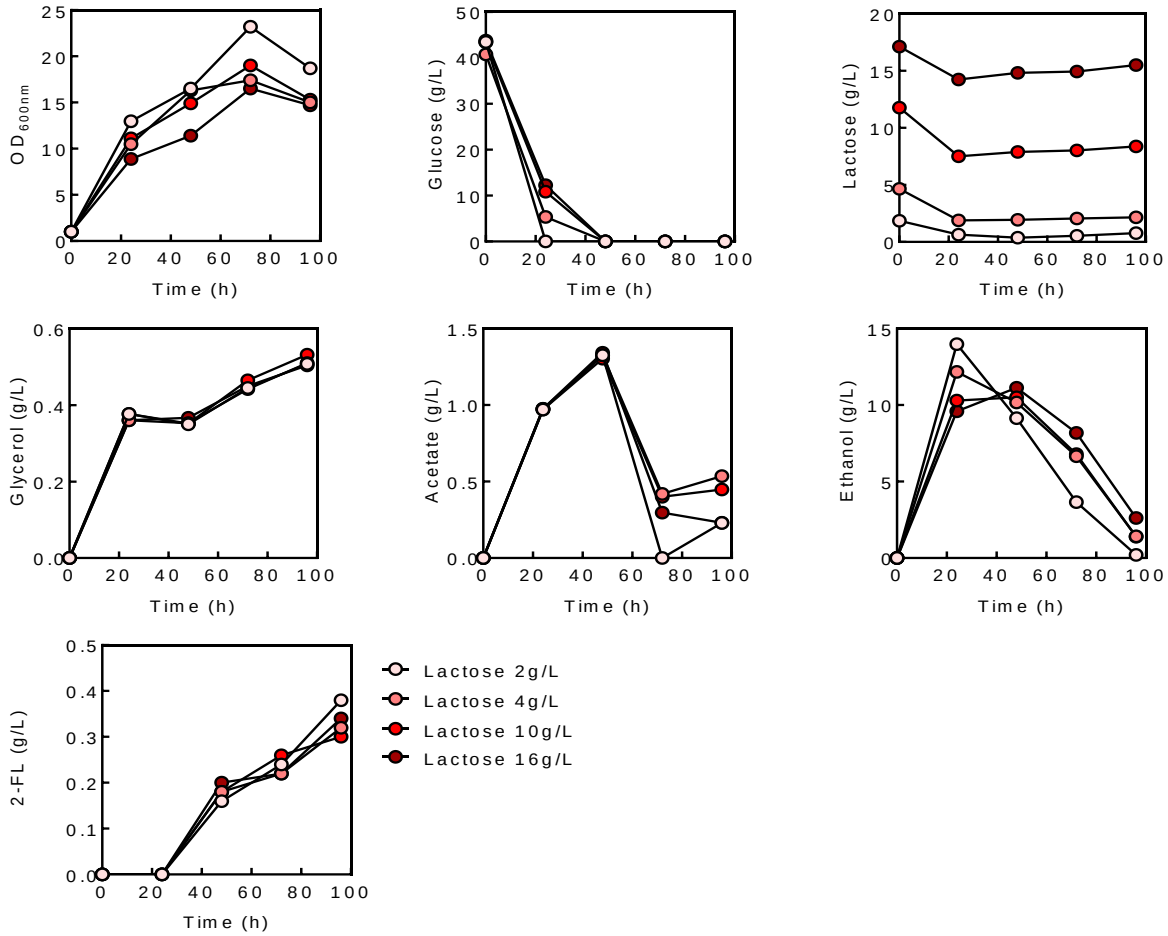


Figure 8. Effect of varied lactose concentrations on production of 2-FL for D452L-gwf (A) and SK1P2L-gwf (B) that were cultured in YPD medium.

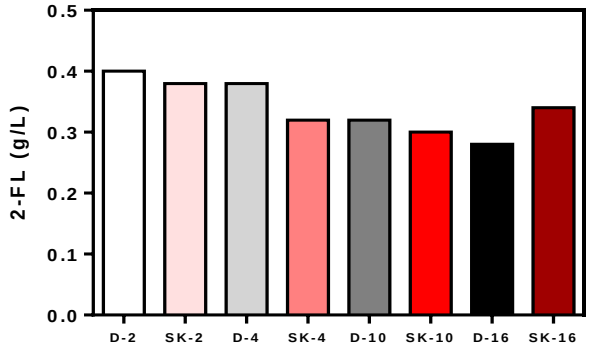


Figure 9. Comparison of 2-FL production at 96 hours (illustrated in Figure 7) for different strains with varied lactose concentrations. Bars: D-2(D452L-gwf; lactose 2g/L), SK-2(SK1P2L-gwf; lactose 2g/L), D-4(D452L-gwf; lactose 4g/L), SK-4(SK1P2L-gwf; lactose 4g/L), D-10(D452L-gwf; lactose 10g/L), SK-10(SK1P2L-gwf; lactose 10g/L), D-16(D452L-gwf; lactose 16g/L), and SK-16(SK1P2L-gwf; lactose 16g/L).

Strain	The production of 2-FL (g/L)			
	Lactose 2g/L	Lactose 4g/L	Lactose 10g/L	Lactose 16g/L
D452L-gwf	0.40	0.38	0.32	0.28
SK1P2L-gwf	0.38	0.32	0.30	0.34

Table 5. Numerical values of culture profiles of 2-FL for different strains with varied lactose concentrations at 96 hours that illustrated in Figure 9.

Comparison of 2-FL production in newly developed strains, D452-w(b)gwh and SK1P2-w(b)gwh, based on D452-2 and SK1P2 strains

In the previous experiment described in Figure 7, I found a significant amount of 2-FL was accumulated inside of the cell. Since transporter did not function properly, it could not take 2-FL out of the cell. Also, since the fucosyltransferase FucT2 has a low level of activity in yeast strains, it causes the low productivity of 2-FL.[5,17] New α1,2-fucosyltransferase from *E. coli* O126 (WbgL), which exhibited higher capacity for synthesizing 2-FL, was recently identified.[17] Also mutant HXT2.4 has proven to be a better 2-FL production with a different type of GDP-fucose synthesis pathway by another researcher in our lab. Therefore, new fucosyltransferase WbgLand mutant Hxt2.4 substituted FucT2 and Lac12 of previous engineered yeasts, D452L-gwf and SK1P2L-gwf, and resulting strains were named D452-w(b)gwh and SK1P2-w(b)gwh, respectively (Table 1). Both strains were cultured in Verduyn medium containing 30g/L glucose and 2g/L lactose due to the negative effect of initial lactose concentration on the production of 2-FL (Figure 9). When ethanol was entirely consumed at 60 hours and 80 hours, I added 5 g/L and 17 g/L of ethanol each time to grow the cells. 2-FL was produced with noticeable levels after 60 hours (Figure 10). At 120 hours, the intracellular and the extracellular 2-FL were quantified using HPLC. Specific intracellular 2-FL productions of D452-w(b)gwh and SK1P2-w(b)gwh were 0.00454 g/g and 0.00397 g/g cell, respectively. Specific extracellular 2-FL productions of D452-w(b)gwh and SK1P2-w(b)gwh were 0.0133 g/g cell and 0.0173 g/g, respectively (Figure

11). The results described that both the new transporter and fucosyltransferase effectively functioned according to the increased total production of 2-FL. SK1P2-w(b)gwh produced more 2-FL than SK1P2L-gwf strain and D452 base strains (e.g., D452L-gwf and D452-w(b)gwh) (Figure 7 and 11).

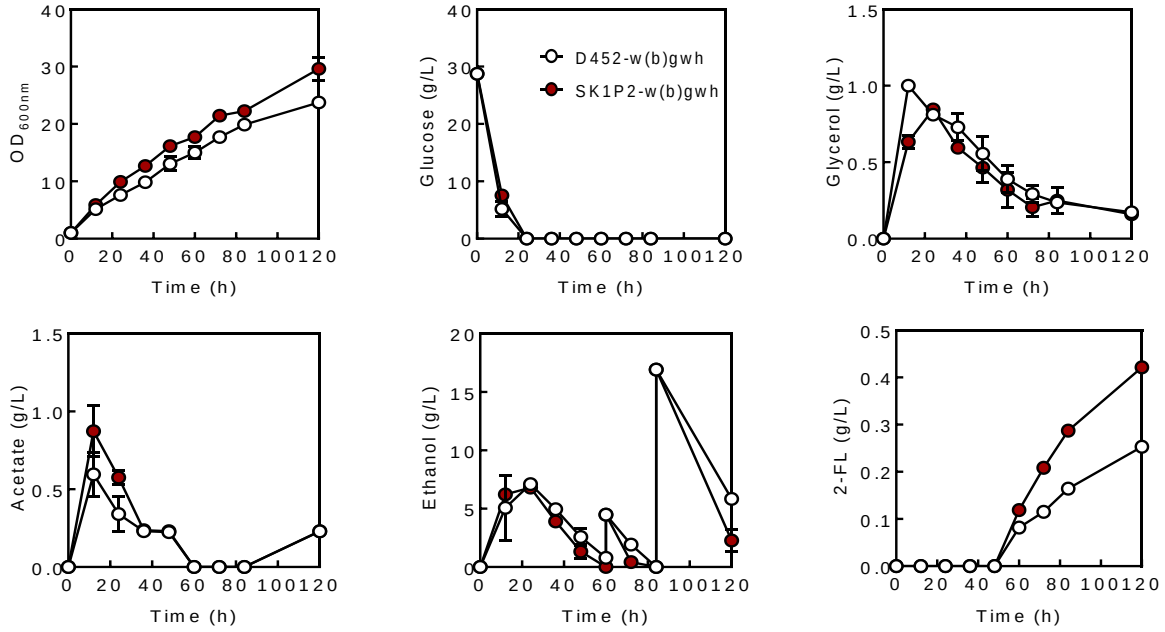


Figure 10. Aerobic culture profiles of 2-FL production for newly engineered strains based on D452-2 (white) and SK1P2 (red). Mean values of duplicate experiments are obtained, and error bars indicate standard deviations.

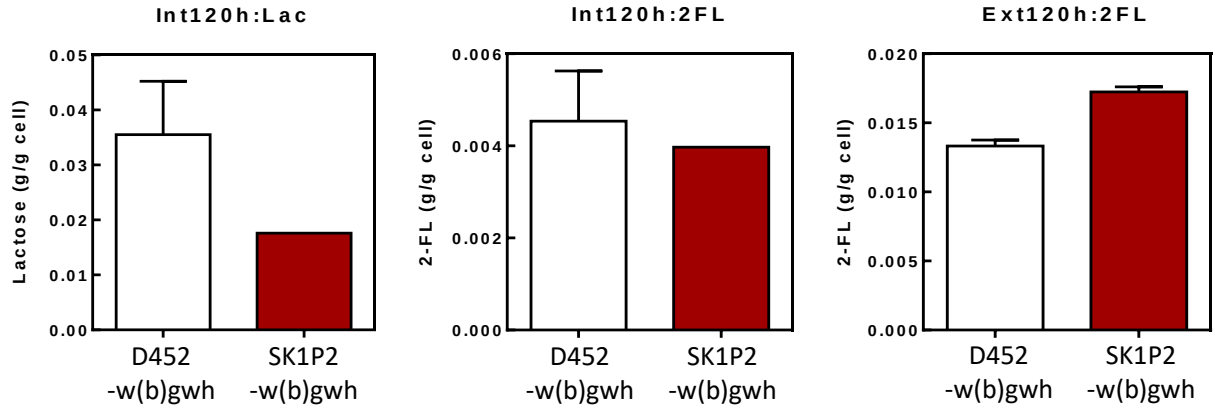


Figure 11. Intracellular lactose and 2-FL concentrations at 120 hours. Mean values of duplicate experiments are obtained, and error bars indicate standard deviations. Bars: D452-w(b)gwh (white) and SK1P2-w(b)gwh (red).

Conclusions and Future studies

The aim of this study was to construct a new developed *S. cerevisiae* strains for efficient 2-FL production by increasing metabolic fluxed toward GDP-fucose synthesis. SK1 was engineered to down-regulate upper glycolysis. First, GDP-mannose synthetic pathway was enhanced to overproduce GDP-mannose in both SK1 base strains (SK1P and SK1P2) and D452-2 base strains (D452P and D452P2), and SK1P2 strain exhibited the highest GDP-mannose level. The synthesis of GDP-mannose in SK1P2 was 5.5 times greater than wild-type strain D452-2. From this end, GDP-fucose production was determined in de novo pathway of GDP-fucose. D452-gw and SK1P2-gw strains were cultivated and quantified. SK1 base strains SK1P2-gw had a high GDP-mannose and GDP-fucose level compared to D452-gw. The result of GDP-fucose synthesis allowed the strain to continue 2-FL production. D452L-gwf and SK1P2L-gwf strains were constructed by introducing fucosyltransferase FucT2 and lactose transporter Lac12. Unexpectedly, SK1P2L-gwf exhibited a lower production of 2-FL than D452L-gwf strain. The result indicated lactose toxicity. To test the effect of lactose concentration on the production of 2-FL, the engineered yeast strains were cultured with different lactose concentrations. Both D452L-gwf and SK1P2L-gwf strains produced the highest 2-FL production levels with the lowest lactose concentration, 2 g/L. It demonstrateed that the lactose concentration negatively affected on the 2-FL production. D452-2 and SK1P2 strains were further engineered with mutant HXT2.4WbgL with *de novo* pathway of GDP-fucose. SK1P2-w(b)gwh had the highest 2-FL production among engineered yeasts in this study. Moreover, the amounts of 2-FL production between intracellular and extracellular showed that mutant Hxt2.4 allowed yeast strains to more efficiently transfer 2-FL from the inside of the cell to the outside of the cell, concluding that mutant Hxt2.4 is more appropriate transporter regarding yeast production of 2-FL as compared to

Lac12. Especially, SK1P2-based strains exhibited the highest 2-FL production among engineered yeast strains. I finally optimized the efficient 2-FL production strain based on SK1 with WbgL and Lac12 (Figure 12).

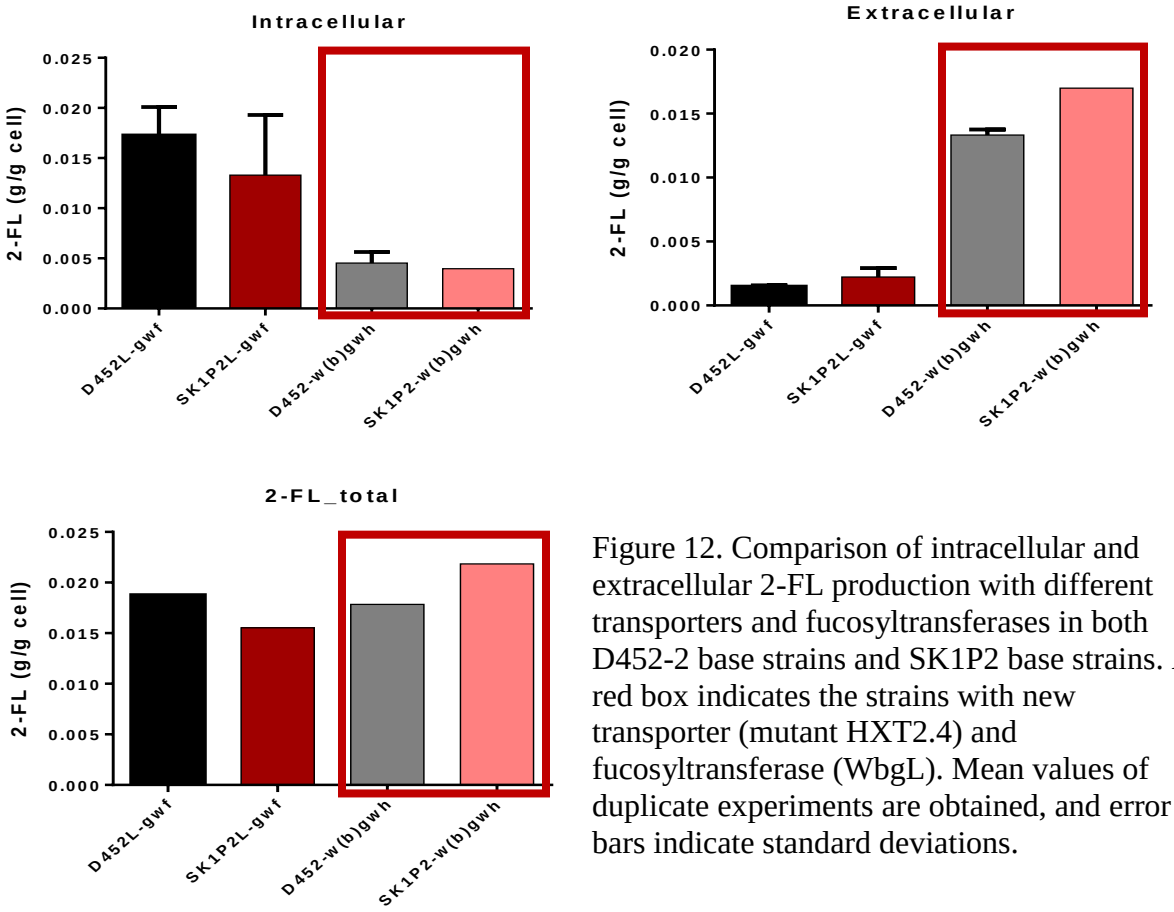


Figure 12. Comparison of intracellular and extracellular 2-FL production with different transporters and fucosyltransferases in both D452-2 base strains and SK1P2 base strains. A red box indicates the strains with new transporter (mutant HXT2.4) and fucosyltransferase (WbgL). Mean values of duplicate experiments are obtained, and error bars indicate standard deviations.

Due to better health condition of cell, pH is important for this study. At the end of the last experiment, I measured pH of each strain. Surprisingly, pH values of all cultures were approximately 3.5. In the future, calcium carbonate or a higher concentration of buffer solution would be considered to maintain pH. When pH is well maintained during 2-FL fermentation, the comparison of engineered yeast strains regarding 2-FL production would be more trustworthy and comparable to 2-FL production in *E. coli*.

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