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Research Article

# Enhanced production of 2,3-butanediol in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* through optimizing ratio of glucose/galactose

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Galactose and glucose are two of the most abundant monomeric sugars in hydrolysates of marine biomasses. While *Saccharomyces cerevisiae* can ferment galactose, its uptake is tightly controlled in the presence of glucose by catabolite repression. It is desirable to construct engineered strains capable of simultaneous utilization of glucose and galactose for producing biofuels and chemicals from marine biomass. The *MTH1* gene coding for a transcription factor in glucose signaling was mutated in a pyruvate decarboxylase (Pdc)-deficient *S. cerevisiae* expressing heterologous 2,3-butanediol (2,3-BD) biosynthetic genes. The engineered *S. cerevisiae* strain consumed glucose and galactose simultaneously and produced 2,3-BD as a major product. Total sugar consumption rates increased with a low ratio of glucose/galactose, though, occurrence of glucose depletion in a fed-batch fermentation decreased 2,3-BD production substantially. Through optimizing the profiles of sugar concentrations in a fed-batch cultivation with the engineered strain, 99.1 ± 1.7 g/L 2,3-BD was produced in 143 h with a yield of 0.353 ± 0.022 g 2,3-BD/g sugars. This result suggests that simultaneous and efficient utilization of glucose and galactose by the engineered yeast might be applicable to the economical production of not only 2,3-BD, but also other biofuels and chemicals from marine biomass.

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## 1 Introduction

With growing public awareness of global climate change and support for sustainable energy, efforts for the production of biofuels and chemicals from renewable biomass have attracted attention [1]. Seaweeds are regarded as a viable alternative renewable feedstock to starch-based and lignocellulosic biomass because, in contrast to lignocellulosic biomass, seaweed biomass can be easily hydrolyzed into monomeric sugars. In addition, marine biomass can be produced without land limitation whereas terrestrial biomass production competes for arable land with food crops. Among the seaweeds, red seaweeds contain a high content of glucose and galactose [2]. Therefore, efficient utilization of glucose and galactose is important for economically-viable microbial production of biofuels and chemicals from red seaweed hydrolysates.

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**Abbreviations:** **alsD**, acetolactate decarboxylase; **alsS**, acetolactate synthase; **2,3-BD**, 2,3-butanediol; **BDH1**, 2,3-butanediol dehydrogenase; **DCW**, dry cell weight; **HPLC**, high performance liquid chromatography; **LB**, Lysogeny Broth; **OD**, optical density; **Pdc**, pyruvate decarboxylase; **SNP**, single nucleotide polymorphism; **YNB**, yeast nitrogen base; **YSC**, Yeast synthetic complete

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Because of the phenomenon known as catabolite repression, wild type *Saccharomyces cerevisiae* strains consume galactose only after glucose is depleted [3, 4]. The sequential consumption of mixed sugars and diauxic growth results in the reduction of overall productivity [5]. Therefore, simultaneous consumption of glucose and galactose is necessary for the improvement of microbial fermentation processes.

2,3-butanediol (2,3-BD) is a promising chemical because of its wide applications in the chemical industry. 1,3-Butadiene, the dehydration product of 2,3-BD, is one of the primary building blocks in the renewable chemical industry [6], and is a main substance used for producing synthetic rubber [7]. More compounds can be synthesized from 2,3-BD, such as methylethyl ketone (MEK) used in liquid fuel additives and polyurethane used in drugs and cosmetic products [7, 8].

Microbial production of 2,3-BD has mainly focused on using bacterial strains such as *Klebsiella pneumoniae*, *K. oxytoca* and *Bacillus* species [9–11]. Since *S. cerevisiae* has many advantages over bacteria for industrial uses, significant research efforts have focused on the production of 2,3-BD by engineered yeast [12–14]. To block the production of ethanol, the main fermentation product of *S. cerevisiae*, pyruvate decarboxylase (Pdc) was first deleted. Because deletion of Pdc results in NADH imbalance and requirement for supplemented $C_2$-compounds, the Pdc-deleted strain was then evolved to overcome these metabolic limitations. As a result, a high titer of 2,3-BD (96.2 g/L) was produced from fed-batch cultivation with the evolved Pdc-deficient *S. cerevisiae* strain expressing the 2,3-BD synthetic enzymes [12].

In this study, we report that the evolved 2,3-BD producing *S. cerevisiae* strain is able to consume glucose and galactose simultaneously and to produce 2,3-BD with high productivity. To identify the genetic perturbations responsible for simultaneous sugar utilization phenotype, we transferred the single point mutation on the *MTH1* gene into the Pdc-deficient *S. cerevisiae* strain. The engineered strain, harboring the 2,3-BD biosynthetic pathway consisting of *B. subtilis* acetolactate synthase (*alsS*), acetolactate decarboxylase (*alsD*) and *S. cerevisiae* 2,3-butanediol dehydrogenase (*BDH1*), could ferment glucose and galactose simultaneously like the evolved strain. Moreover, the ratio of extracellular glucose and galactose concentrations largely influenced sugar consumption rates in the engineered strain. The results of this study provide fermentation strategies for producing chemicals from marine biomass hydrolysates including glucose and galactose by *S. cerevisiae*.

## 2 Materials and methods

### 2.1 Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and manipulation. *S. cerevisiae* SOS2 with deletions of the *PDC1* and *PDC5* genes [12] was used as a host strain for the construction of the engineered strains. The deletion of both *PDC1* and *PDC5* resulted in complete loss of Pdc activity [15, 16]. For the construction of the *MTH1* mutant strain (SOS2_Mth1), a single point mutation was created using the PCR-based allele replacement method as previously reported [17] with primers listed in Table 1. The point-mutated *MTH1* gene was amplified from the evolved SOS4 strain [12] with

**Table 1.** Strains, plasmids, and primers used in this study

| Strains | Description | Reference |
|---|---|---|
| SOS2 | *S. cerevisiae* D452-2 *MATα leu2 his3 ura3 can pdc1Δ pdc5Δ* | [12] |
| SOS4 | SOS2, evolved in excess glucose | |
| SOS2_Mth1 | SOS2, $MTH1^{G241C}$ | This study |
| BD2_Con | SOS2, p423_alsSalsD, p425_BDH1 | This study |
| BD2_Mth1 | SOS2_Mth1, p423_alsSalsD, p425_BDH1 | This study |
| **Plasmids** | **Description** | **Reference** |
| pWJ1077 | Source for *K. lactis URA3* gene | [18] |
| p423_alsSalsD | *HIS3* 2μm origin $TDH3_{prom}$-*alsS*-$CYC1_{term}$ $TDH3_{prom}$-*alsD*-$CYC1_{term}$ | [41] |
| p425_BDH1 | *LEU2* 2μm origin $TDH3_{prom}$-*BDH1*-$CYC1_{term}$ | [12] |
| **Primers** | **Sequence** | |
| F_MTH1 | TCCAGCTGACCACCATGATGTTTGTTTCACCACC | |
| R_MTH1 | GATCCCCGGGAATTGCCTCAGGATACTGAATCCG | |
| F_KlURA3 | GGCAATTCCCGGGGATCGTGATTCTGGGTAGAAG | |
| R_KlURA3 | CATGGTGGTCAGCTGGACGATGATGTAGTTTCTG | |
| F_Klint5 | CTTGACGTTCGTTCGACTGATGAGC | |
| R_Klint3 | GAGCAATGAACCCAATAACGAAATC | |

primer F_MTH1 and R_MTH1. The *Kluyveromyces lactis URA3* gene was amplified from the pWJ1077 plasmid [18]. Two fused DNA fragments containing the mutant *MTH1* gene and *K. lactis URA3* gene were transformed into the SOS2 strain and plated on YNB medium without uracil. Finally, the transformed cells were selected on 5-fluoroorotic acid (5-FOA) medium to rescue the *URA3* gene. The 241st nucleotide of the *MTH1* gene on the chromosomal DNA of the SOS2_Mth1 strain was mutated from G to C. The resulting amino acid sequence was changed from alanine to proline at the 81st codon of the Mth1 protein. For introduction of the 2,3-BD biosynthetic pathway in Pdc-deficient *S. cerevisiae*, acetolactate synthase (*alsS*) and acetolactate decarboxylase (*alsD*) from *B. subtilis* and endogenous 2,3-butanediol dehydrogenase (*BDH1*) were expressed under the control of the *S. cerevisiae TDH3* promoter and *CYC1* terminator.

## 2.2 Culture conditions

*E. coli* TOP10 (Invitrogen) was grown in Lysogeny Broth (LB) medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) with 50 μg/mL of ampicillin. All yeast cells were grown at 30°C. Yeast synthetic complete (YSC) medium containing 6.7 g/L yeast nitrogen base (YNB), 20 g/L glucose, and appropriate nucleotides and amino acids was used for pre-culture of yeast strains. Mid-exponential phase cells aerobically grown in YSC medium containing 20 g/L glucose and 0.1% ethanol were used as an inoculum for the main culture. The inoculum cells were washed with double distilled water ($ddH_2O$) and transferred into the main cultures at the initial $OD_{600}$ of 1.0. The main flask cultures were performed in YP medium (10 g/L yeast extract, 20 g/L bacto-peptone) containing 80 g/L glucose, 80 g/L galactose, or a mixture of 80 g/L glucose and 80 g/L galactose. For evaluating carbon consumption rates with different glucose:galactose ratios, the engineered strain was cultivated in minimal media with 0, 25, 50, 75, and 100 g/L glucose combined with 50 g/L galactose, or 0, 25, 50, 75, and 100 g/L galactose combined with 50 g/L glucose. A solution of 0.2% ethanol was supplemented as a $C_2$-compound to support growth of Pdc-deficient *S. cerevisiae* [14]. Main cultures were carried out in 250 mL flasks with a working volume of 50 mL at 80 rpm for oxygen-limited conditions. Yeast extract, tryptone and bacto-peptone were purchased from Becton Dickinson (Sparks, MD, USA) and the others were purchased from Sigma (St. Louis, MO, USA).

## 2.3 Fed-batch fermentation

Fed-batch fermentations were carried out in a 1 L-benchtop fermenter (KoBiotech, Korea) according to the method of the previous study with some modifications [12]. 500 mL of YP medium containing glucose and galactose with 0.2% ethanol was used and pH was adjusted to 5.5 by the addition of 5 N HCl or 2 N NaOH intermittently. Yeast cells were inoculated into a bioreactor with an initial $OD_{600}$ of 10.0. Initial concentrations of sugars were 60 g/L glucose and 60 g/L galactose or 40 g/L glucose and 80 g/L galactose. After depletion of glucose, additional glucose and galactose were added into the medium at different glucose:galactose ratios such as 1:2, 1:1, or 1:2. Agitation speed and aeration rate were maintained at 300 rpm and 1.0 vvm throughout the cultivation.

## 2.4 Measurement of cell mass and metabolites

Cell growth was monitored by optical density (OD) at 600 nm using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ) and dry cell weight (DCW) was calculated from the pre-determined conversion factor: 0.2 $g_{DCW}/OD_{600}$. Concentrations of glucose, galactose, glycerol, acetate, acetoin and 2,3-butanediol were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series, Santa Clara, CA) equipped with a Rezex ROA-organic acid column (Phenomenex, CA). The metabolites were detected by a refractive index (RI) detector. The column was heated at 60°C and eluted with 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

## 2.5 Quantitative real-time PCR

The mid-exponential phase cells grown in YP medium containing 80 g/L glucose or a mixture of 80 g/L glucose and 80 g/L galactose were used for qRT-PCR experiments. Total RNAs were extracted with a RNeasy mini kit (QIAGEN, Valencia, CA). The cDNAs were synthesized with a PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan). The reaction mixtures were prepared by mixing SYBR premix EX Taq (Takara, Shiga, Japan). The experiments were carried out according to manufacturer's manuals. Quantitative real time PCR was conducted with a DNA Engine Opticon system (Bio-Rad, Waltham, MA) with the following conditions: after initial denaturation by 95°C for 30s and 45 repetitions by 95°C for 5s and 55°C for 30s. A melting curve from 65 to 95°C was generated at the end of the reaction. The cycle threshold values and normalized expression data were calculated using Bio-rad CFX Maneger software. The relative expression levels of the hexose transporter genes (*HXT1*, *HXT2*, *HXT3*, *HXT4*, *HXT5*, *HXT6*, *HXT7*, *GAL2*) were quantified by comparing the expression levels with the *ACT1* gene as a reference gene. The primers used in the quantitative real-time PCR are listed in Supporting information, Table S1.

## 3 Results

### 3.1 Construction of the Pdc-deficient *S. cerevisiae* strain with *MTH1* mutation

In our previous study, an evolved Pdc-deficient *S. cerevisiae* strain was generated by serial cultivation in excess glucose medium, then the evolved 2,3-BD-producing strain (BD4) was constructed by introducing genes for the 2,3-BD biosynthetic pathway [12]. Among the chromosomal mutations of the BD4 strain, a single nucleotide polymorphism (SNP) at the 241 bp position of the *MTH1* gene has been identified to alleviate the toxicity from excess glucose. Interestingly, when flask cultivations were carried out with the BD4 strain in a mixture of glucose and galactose, both sugars were simultaneously consumed as shown in Supporting information, Fig. S1. Since Mth1p is a transcription factor that regulates hexose transport in *S. cerevisiae*, the mutation on *MTH1* allowed growth in glucose media by reducing glucose uptake in the Pdc-deficient *S. cerevisiae* strain [12, 19]. From this, we hypothesized that the *MTH1* mutation might be related to the simultaneous consumption of glucose and galactose. Thus, we introduced the mutant *MTH1* allele into the Pdc-deficient *S. cerevisiae* strain (SOS2) to investigate its effects on 2,3-BD fermentation (Fig. S2). Specifically, two fused fragments harboring the point-mutated *MTH1* gene and a *URA3* gene were cotransformed into the SOS2 strain. The transformants were confirmed by PCR with primers F_KlURA3 and R_check_Mth1 (Supporting information, Fig. S3). This transformation resulted in a *URA3* gene flanked by two copies of the mutant *MTH1* gene in the *MTH1* gene locus. The *URA3* marker was then removed through pop-out recombination, yielding the SOS2_Mth1 strain. Finally, we constructed the BD2_Con and BD2_Mth1 strains by introducing the 2,3-BD biosynthetic pathway into the wild-type *MTH1* and point-mutated *MTH1* strains, respectively (Table 1).

### 3.2 The engineered 2,3-BD producing *S. cerevisiae* strains strain with *MTH1* mutation consumed glucose and galactose simultaneously in batch cultivations

To investigate the effect of the *MTH1* mutation on 2,3-BD production by the engineered *S. cerevisiae* strains, batch cultivations of both BD2_Con and BD2_Mth1 strains were

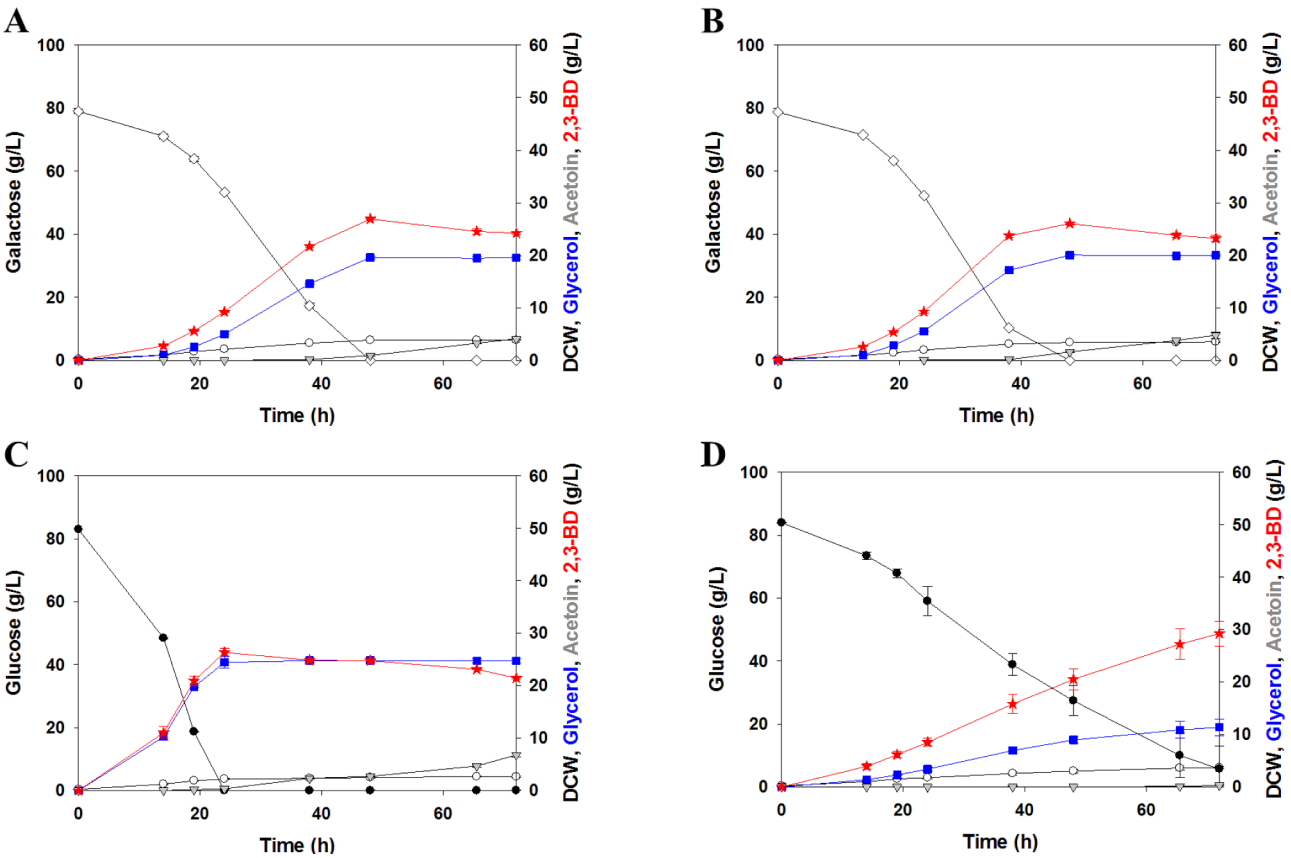


**Figure 1.** Batch fermentation profiles of the BD2_Con (**a** and **c**) and BD2_Mth1 (**b** and **d**) strains cultivated in minimal medium containing 80 g/L galactose (**a** and **b**) or 80 g/L glucose (**c** and **d**). Symbols: glucose (filled circle), galactose (diamond), dry cell weight (open circle), glycerol (square), acetoin (inverted triangle), and 2,3-BD (star). Results are the averages of duplicate experiments with error bars representing standard deviations.

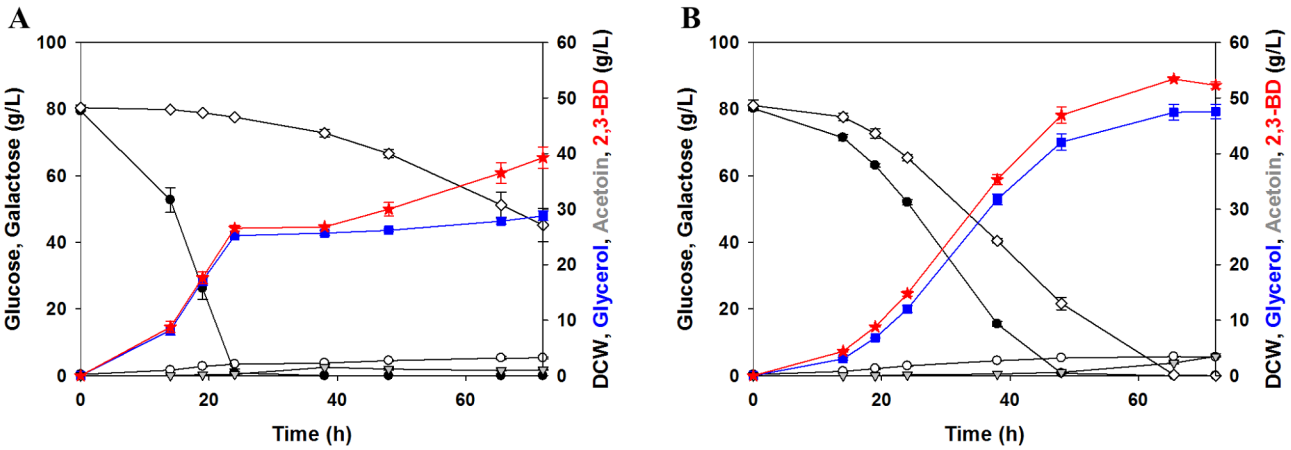


**Figure 2.** Batch fermentation profiles of the (**a**) BD2_Con and (**b**) BD2_Mth1 strains cultivated in minimal medium containing mixture of 80 g/L glucose and 80 g/L galactose. Symbols: glucose (filled circle), galactose (diamond), dry cell weight (open circle), glycerol (square), acetoin (inverted triangle), and 2,3-BD (star). Results are the averages of duplicate experiments with error bars representing standard deviations.

carried out in minimal medium with 80 g/L glucose or galactose as a sole carbon source (Fig. 1). When galactose was used as a sole carbon source (Fig. 1A and 1B), the 2,3-BD productivities of the BD2_Con strain (0.56 ± 0.00 g/L/h) and the BD2_Mth1 strain (0.55 ± 0.00 g/L/h) were similar despite of a slight increase of galactose consumption rate in the BD2_Mth1 strain (1.80 ± 0.01 $g_{Galactose}$/L/h) compared with the BD2_Con strain (1.62 ± 0.01 $g_{Galactose}$/L/h). Additionally, the fermentation patterns did not change when cells were pre-grown in galactose medium (Supporting information, Fig. S4). On the other hand, with glucose as a sole carbon source (Fig. 1C and 1D), the glucose consumption rates were substantially reduced in the BD2_Mth1 strain (1.09 ± 0.10 $g_{glucose}$/L/h) compared with the BD2_Con strain (3.39 ± 0.12 $g_{glucose}$/L/h). Glycerol, which accounts for the main by-product in 2,3-BD production, substantially decreased in the BD2_Mth1 strain (0.146 ± 0.007 $g_{glycerol}/g_{glucose}$) as compared to the BD2_Con strain (0.295 ± 0.015 $g_{glycerol}/g_{glucose}$). The 2,3-BD yield of the BD2_Mth1 strain (0.376 ± 0.005 $g_{2,3\text{-}BD}/g_{glucose}$) was higher than that of the BD2_Con strain (0.319 ± 0.010 $g_{2,3\text{-}BD}/g_{glucose}$).

In order to evaluate if the *MTH1* mutation affects the fermentation performances in a mixture of glucose and galactose, flask batch cultivations were conducted in minimal medium containing a mixture of 80 g/L glucose and 80 g/L galactose (Fig. 2). While the BD2_Con strain utilized glucose (3.08 ± 0.23 $g_{glucose}$/L/h) and galactose (0.49 ± 0.08 $g_{glucose}$/L/h) sequentially with a prolonged lag phase like the wild type *S. cerevisiae* [4], the BD2_Mth1 strain was able to co-ferment both sugars with consumption rates of glucose (1.66 ± 0.00 $g_{glucose}$/L/h) and galactose (1.25 ± 0.05 $g_{galactose}$/L/h). Additionally, the glucose consumption rate of the BD2_Mth1 strain in the mixed sugar fermentation was 52% higher than that in pure glucose medium. Simultaneous consumption of glucose and galactose in the BD2_Mth1 strain increased the overall substrate consumption rate (2.46 ± 0.03 $g_{sugars}$/L/h) compared with the BD2_Con strain (1.59 ± 0.08 $g_{sugars}$/L/h). The 2,3-BD productivity of the BD2_Mth1 strain (0.82 ± 0.00 g/L/h) was substantially higher than that of the BD2_Con strain (0.55 ± 0.03 g/L/h), but 2,3-BD yields were similar between the BD2_Con (0.344 ± 0.000 $g_{2,3\text{-}BD}/g_{substrate}$) and the BD2_Mth1 (0.333 ± 0.003 $g_{2,3\text{-}BD}/g_{substrate}$) strains.

### 3.3 Influence of glucose and galactose ratio on 2,3-BD fermentation by the BD2_Mth1 strain

As reported recently, the expression of the genes responsible for galactose metabolism are regulated by the intracellular ratio of glucose and galactose [20]. Thus, we speculated that the extracellular ratio of glucose and galactose in the medium would affect the rates of sugar consumption and 2,3-BD production. To explore the influence of sugar ratios on carbon consumption rates in 2,3-BD-producing Pdc-deficient *S. cerevisiae*, batch cultivations were carried out with the BD2_Mth1 strain with different ratios of glucose and galactose (Fig. 3 and Supporting information, Fig. S5). The substrate inhibition became significant when sugar concentration was above 150 g/L [21–23]. Thus, to exclude a possible effect of total sugar concentrations on sugar uptake rate, the sum of sugar concentrations was set between 50 and 150 g/L. The BD2_Mth1 strain consumed both sugars simultaneously under all tested conditions, however, the galactose consumption rate decreased as glucose concentrations increased. The galactose consumption rate (1.28 g/L/h) when cultivated with 50 g/L galactose only was substantially higher than the glucose consumption rate (0.51 g/L/h) when cultivated with 100 g/L glucose. On the other hand, glucose consumption rates decreased by only 19.1% with

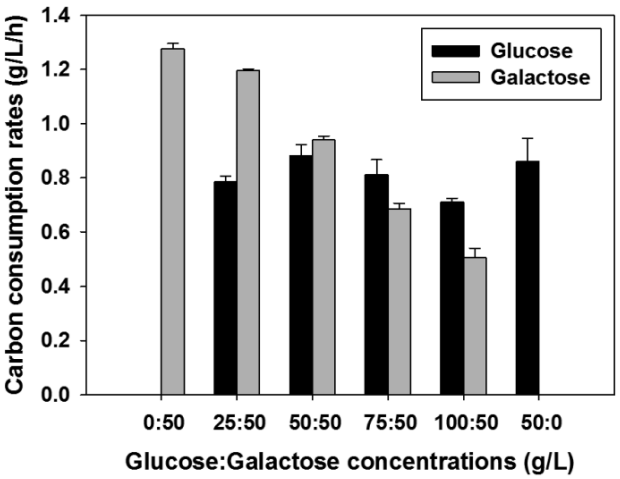


**Figure 3.** Glucose and galactose consumption rates of the BD2_Mth1 strain according to different ratios of glucose and galactose in minimal medium. Symbols: glucose consumption rate (grey bar), and galactose consumption rate (black bar). Results are the averages of duplicate experiments representing standard deviations.

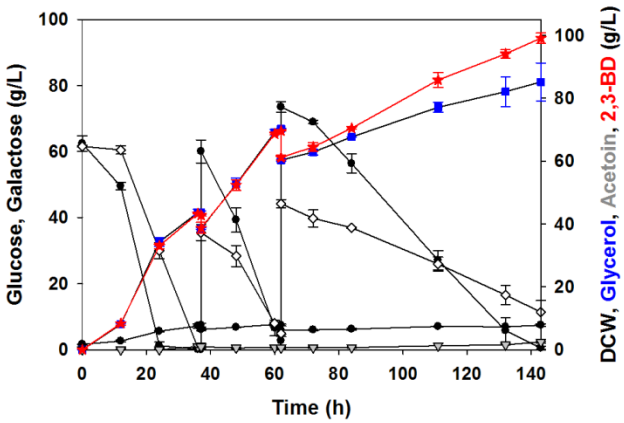


**Figure 4.** Fed-batch fermentation profile of the BD2_Mth1 strain cultivated in a bioreactor. Symbols: glucose (filled circle), galactose (diamond), dry cell weight (open circle), glycerol (square), acetoin (inverted triangle), and 2,3-BD (star). Results are the averages of duplicate experiments with error bars representing standard deviations.

the addition of 100 g/L galactose (from 1.36 to 1.10 g/L/h). A sum of sugar consumption rates in 1:2 of glucose:galactose ratio (1.98 g/L/h ) was the best among the tested conditions (Fig. 3). This condition was selected for subsequent fed-batch fermentations.

### 3.4 Optimization of fed-batch fermentation by the BD2_Mth1 strain in mixed sugars

In order to evaluate the fermentation behavior of the BD2_Mth1 strain in a mixture of glucose and galactose for high production of 2,3-BD, fed-batch fermentations were carried out with intermittent feeding of sugars (Fig. 4 and Supporting information, Fig. S6). In Fig. S6A, with the optimized condition from batch cultivation (1:2 ratio of glucose and galactose), both of sugars were simultaneously utilized by the BD2_Mth1 strain. However, the galactose uptake rate was sharply reduced compared with the glucose uptake rate in the late phase of fermentation. In addition, when galactose was solely consumed after glucose depleted, a large amount of acetoin was produced as a by-product (Fig. S6A). Such fermentation behavior was also observed for a 1:1 ratio of glucose and galactose used (Fig. S6B). 80.0 g/L 2,3-BD was produced in 180 h of fermentation with 1:1 sugar ratio. Therefore, the ratio of glucose and galactose concentrations was re-optimized for fed-batch fermentation with the BD2_Mth1 strain (Fig. 4). To balance the consumption rates of both sugars and to avoid a glucose-depleted period, the initial concentrations of sugars were set at 60 g/L glucose + 60 g/L galactose and the feeding ratio of glucose and galactose was changed to 2:1 from the results of Fig. S6. As a result, the BD2_Mth1 strain produced 99.1 ± 1.7 g/L 2,3-BD in 143 h of cultivation with a yield of 0.353 ± 0.022 $g_{2,3\text{-BD}}/g_{sugars}$. It is interesting to note that 85.1 ± 6.1 g/L glycerol was produced as a main byproduct while acetoin accumulated to less than 2.5 g/L.

## 4 Discussion

Glucose plays a regulatory role in wild type *S. cerevisiae* in a phenomenon known as catabolite repression, where genes that are required for respiratory metabolism and utilization of other carbon sources are repressed [24]. In this system, the expression of the genes for galactose metabolism is induced up to a 1000-fold by galactose, but is tightly regulated and repressed by glucose [25]. Recently, it has been shown that the induction of galactose metabolic genes occurs at a constant external ratio of glucose and galactose across a wide range of sugar concentrations, rather than glucose levels below a threshold [20]. Thus, the ratio of glucose and galactose is an important factor influencing 2,3-BD productivity by engineered yeast. Our research demonstrated that a single amino acid change on Mth1 enables simultaneous glucose and galactose consumption in 2,3-BD producing strains despite of the presence of high external glucose concentrations. In agreement with previous reports that a yeast responds to a ratio of glucose and galactose [16], galactose consumption rates in our engineered strains were still impacted by high glucose concentrations. Interestingly, we consistently observed the highest total volumetric sugar consumption rates when our engineered strain was cultivated in a mixture of glucose and galactose (Fig. 3 and Supporting information, Fig. S5). While simultaneous sugar utilization is often desired in the implementation of continuous fermentation processes, our result indicates that simultaneous glucose and galactose

utilization for 2,3-BD production is an effective strategy to enhance overall biotransformation rates and fermentation capacity.

Furthermore, a similar trend was observed in flask fermentations with equivalent amounts of glucose and galactose. The glucose uptake rate of the BD2_Mth1 strain was much lower than that of the control strain when glucose was used as a sole carbon source (Fig. 1C and 1D). However, the glucose uptake rate of the BD2_Mth1 strain was substantially increased when both sugars were presented in the medium (Fig. 2B). As a result, the overall carbon uptake rate and 2,3-BD productivity were enhanced compared to those of the control strain because the BD2_Mth1 strain consumes glucose and galactose simultaneously in contrast to the control strain which consumed galactose after glucose depleted with a prolonged lag period (Fig. 2). The specific carbon consumption rate of the BD2_Mth1 strain in mixed sugars ($0.72 \pm 0.02$ $g_{sugars}/g_{cells}/h$) was 2.4-fold higher than in glucose as a sole carbon source ($0.30 \pm 0.01$ $g_{sugars}/g_{cells}/h$) (Fig. 1D and Fig. 2B). *GAL2* is one of the 18 putative hexose transporter genes in *S. cerevisiae* [26], and Gal2 is classified as a high affinity glucose transporter with apparent $K_m$ values of 1.5 mM [27]. Expression of *GAL2* could be induced by 2% galactose [27]. As expected, the expression levels of the *GAL2* gene in mixed sugar medium substantially increased compared with that in the glucose medium (Supporting information, Fig. S7A). The increased carbon uptake rate for mixed sugars may be due to increased expression of hexose transporters by *GAL2* expression. Therefore, a single amino acid change in the *MTH1* gene results in not only simultaneous consumption of glucose and galactose, but also an enhancement of 2,3-BD productivity in mixed sugar fermentation.

Extracellular glucose generates an intracellular signal through the transmembrane glucose sensors Snf3 and Rgt2, which then activate the membrane-bound casein kinase I (Yck1/Yck2). The activated casein kinase I then promotes degradation of Mth1 by serial phosphorylation and ubiquitination [28]. Our observation of simultaneous glucose and galactose consumption in the BD2_Mth1 strain suggests that the point mutation of Mth1 (A81P) might result in Mth1 gaining resistance to proteolytic degradation [12]. Thus, we conclude that the mutation on *MTH1* kept Mth1 active in spite of the extracellular glucose signal. By inhibiting the phosphorylation of the transcription factor Rgt1, active Mth1 decreases *HXT* gene expression regardless of excess glucose in the medium [29] and blocks the signal from Rgt2 and Snf3 [30, 31]. The mutant Mth1 of the BD2_Mth1 strain might lead to a large protein structure change compared with the other alleles of Mth1 (*HTR1-23*, *DGT1-1*, *BPD1-1*) [31, 32] because of the exceptional conformational rigidity of the proline residue. Thus, The efficiecy of phosphorylation for protein degradation might be different according to the various Mth1 alleles, but additional studies were needed for evaluating the levels of relieving catabolite repression between the mutant Mth1 alleles. As seen in Fig. 1, the glucose uptake rate of the *MTH1* mutant strain was substantially reduced compared to the control strain, as reported previously [12, 19, 33]. It has been reported that glucose repression is dependent on the expression levels of hexose transporters as a whole rather than the expression of specific transporters individually [34, 35]. Likewise, analysis of expression levels of hexose transporter genes (*HXT1*, *HXT2*, *HXT3*, *HXT4*, *HXT5*, *HXT6*, and *HXT7*) by quantitative real-time PCR revealed that the expression of transporter genes was reduced by the mutant *MTH1* gene (Supporting information, Fig. S7B). Especially, expression of low affinity transporters (Hxt1 and Hxt3) which are induced in cells growing on high glucose medium [26] were substantially reduced. The signal for glucose repression is determined by the intracellular glucose concentration in *S. cerevisiae* [36]. Thus, it is likely that reduced glucose transport results in catabolite de-repression of the BD2_Mth1 strain regardless of excess glucose in medium.

Glycerol is the main byproduct in 2,3-BD fermentations by acting as a redox sink for regeneration of cytosolic $NAD^+$. Because one mole of NADH is consumed per one mole of 2,3-BD produced, glycerol is produced to maintain redox balance in Pdc-deficient *S. cerevisiae* with the 2,3-BD biosynthetic pathway. In batch cultivation on glucose with the BD2_Mth1 strain, however, glycerol production was substantially reduced compared to that of the BD2_Con strain. When the BD2_Mth1 strain was cultivated on glucose as a sole carbon source, regeneration of NADH via oxidative phosphorylation may be enhanced because of the reduced glucose uptake rate. Meanwhile, faster total sugar consumption resulted in increased glycerol production when the BD2_Mth1 strain was cultivated in a mixture of glucose and galactose.

Although the engineered strain efficiently produced 2,3-BD from a mixture of glucose and galactose, several challenges remain for enhancing 2,3-BD production. First, glycerol is produced as the main byproduct during 2,3-BD production by Pdc-deficient *S. cerevisiae* strains because excess NADH is produced in oxygen-limited conditions (Fig. 4). In our previous study, oxidation of cytosolic NADH by expression of *Lactococcus lactis* NADH oxidase substantially reduced glycerol production and increased 2,3-BD production in Pdc-deficient *S. cerevisiae* strains [14]. Additionally, deletion of *GPD1* and/or *GPD2* could be a target for reducing glycerol production in 2,3-BD fermentation. Alternatively, the redox potential may be harnessed in driving other useful reactions such as detoxification of inhibitors in hydrolysates [37]. A second challenge is highlighted by the observation that while the BD2_Mth1 strain consumed glucose and galactose simultaneously, galactose consumption was slower than glucose consumption. Further, with increasing 2,3-BD concentrations in medium, galactose consumption rates

largely decreased compared to glucose consumption rates. A possible explanation for this problem might be limited expression levels of galactose metabolic genes in the engineered strain during 2,3-BD fermentation. Finally, since hexose transporters of *S. cerevisiae* have different affinities to glucose and galactose [38], the ratio of glucose and galactose influences the uptake rates by competition for transport between the sugars [20]. Therefore, to maintain the expression levels of galactose metabolic genes, deletion of negative regulators [39], over-expression of galactose metabolic enzymes [40], or protein engineering to reduce effects of transport competition may be necessary to improve galactose consumption rates in engineered strains.

The present study demonstrates that a point mutation on the *MTH1* gene allows *S. cerevisiae* to alleviate catabolite repression during fermentations of glucose and galactose mixtures. With our engineered 2,3-BD-producing *S. cerevisiae* strain, glucose and galactose were consumed simultaneously and 2,3-BD was produced with a higher productivity than the control strain. This demonstration of simultaneous consumption of glucose and galactose could facilitate economic production of 2,3-BD from marine biomass.

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## 5 References

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**Cover illustration**

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Hyung Joon Cha, Noriho Kamiya and S. Vikineswary Sabaratnam, covers the most advanced biotech research from Asian Congress of Biotechnology 2015. This issue includes articles on drug delivery, enzyme engineering, cellular therapy, biosensors, etc. The 30Kc19 protein derived from the silkworm hemolymph consists of two domains, which are 30Kc19α (blue) and 30Kc19β (red). The cover image shows that 30Kc19α has multifunctional properties, which are cell penetration, protein stabilization, and cargo delivery. The Image is provided by Jina Ryu, Hyoju Kim, Hee Ho Park, Hong Jai Lee, Ju Hyun Park, Won Jong Rhee and Tai Hyun Park authors of "Protein-stabilizing and cell-penetrating properties of α-helix domain of 30Kc19 protein" (http://dx.doi.org/10.1002/biot.201600040).

*Biotechnology Journal* – list of articles published in the November 2016 issue.

Research Article

**Enzymatically prepared redox-responsive hydrogels as potent matrices for hepatocellular carcinoma cell spheroid formation**

*Kousuke Moriyama, Shono Naito, Rie Wakabayashi, Masahiro Goto and Noriho Kamiya*

*http://dx.doi.org/10.1002/biot.201600087*

Research Article

**Theoretical calculations on the feasibility of microalgal biofuels: Utilization of marine resources could help realizing the potential of microalgae**

*Hanwool Park, Choul-Gyun Lee*

*http://dx.doi.org/10.1002/biot.201600041*