



# Article Mutants with Enhanced Cellobiose-Fermenting Ability from Thermotolerant *Kluyveromyces marxianus* DMKU 3-1042, Which Are Beneficial for Fermentation with Cellulosic Biomass

Masayuki Murata<sup>1</sup>, Sornsiri Pattanakittivorakul<sup>2</sup>, Toshiro Manabe<sup>3</sup>, Savitree Limtong<sup>4</sup> and Mamoru Yamada<sup>1,2,5,\*</sup>

- <sup>1</sup> Research Center for Thermotolerant Microbial Resources, Yamaguchi University, Yamaguchi 753-8515, Japan; muratam@yamaguchi-u.ac.jp
- <sup>2</sup> Graduate School of Science and Technology for Innovation, Yamaguchi University, Yamaguchi 753-8515, Japan; sornkiri@gmail.com
- <sup>3</sup> J. COMM Co., Ltd., Himi Hei 477, Saijo 793-0073, Japan; t-manabe@hokushinkai.or.jp
- <sup>4</sup> Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand; fscistl@ku.ac.th
- <sup>5</sup> Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan
- Correspondence: m-yamada@yamaguchi-u.ac.jp

Abstract: Several cellulose-hydrolysis enzymes are required for eco-friendly utilization of cellulose as renewable biomass, and it would therefore be beneficial if fermenting microbes can provide such enzymes without genetic engineering. Thermotolerant and multisugar-fermenting *Kluyveromyces marxianus* is one of the promising yeasts for high-temperature fermentation and has genes for putative oligosaccharide-degradation enzymes. Mutants obtained after multiple mutagenesis showed significantly higher activity than that of the parental strain for cellobiose fermentation. The efficient strains were found to have amino acid substitutions and frame-shift mutations in 26–28 genes including 3 genes for glucose transporters. These strains grown in a cellobiose medium showed higher  $\beta$ -glucosidase than that of the parental strain and greatly reduced glucose utilization. The introduction of *KTH2* for a glucose transporter into one of the efficient mutants reduced the cellobiose fermentation activity of the mutant. The results suggest that release from glucose repression significantly promotes the uptake of cellobiose. Co-culture of one efficient strain and the parental strain allowed good fermentation of both glucose and cellobiose, suggesting that the efficient strains are useful for conversion of cellulosic biomass to ethanol.

Keywords: Kluyveromyces marxianus DMKU3-1042; cellobiose-fermentation; KHT2; LAC12; HGT1

# 1. Introduction

Cellulose, which is the most abundant renewable biopolymer on earth, can be used for various biorefining processes including production of biofuels after being decomposed into monosaccharides such as glucose by enzymes [1,2]. Enzymes that decompose cellulose are collectively called cellulases. Cellulases include cellobiohydrolase (CBH: EC 3.2.1.91), which attacks from the end of crystalline cellulose to release cellobiose; endoglucanase (EG: EC 3.2.1.4), which randomly cleaves regions where the cellulose molecular chain is relatively disturbed; and  $\beta$ -glucosidase (BGL: EC 3.2.1.21), which produces glucose from the ends of short chains.

Currently, the most promising methods for preparation of cellulases in terms of cost are provided by filamentous fungi, mainly *Trichoderma reesei*, which secrete a large amount of highly active cellulase; technological developments have significantly improved their saccharification capacity [3–5]. The genus *Trichoderma* has relatively weak BGL activity, and



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**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). the addition of BGL prepared from other microorganisms greatly improves its monosaccharide production capacity [6,7]. BGL is found in several glycosylhydrolase (GH) families, but BGL belonging to the GH3 family derived from filamentous fungi that are highly active against cellobiose is generally used for cellulose degradation. BGL from the genus *Aspergillus* has a higher cellobiose-degrading activity than that of BGL from the genus *Trichoderma* [8–10].

Kluyveromyces marxianus, which is a thermotolerant yeast, has various beneficial characteristics for application in industrial ethanol fermentation including efficient production of ethanol at high temperatures, high growth rate, short doubling times, weak glucose repression, and capability for assimilation of sugars present in various raw materials, including glucose, xylose and sucrose [11–15]. K. marxianus DMKU 3-1042 has been extensively studied [11,16,17]. Its complete genome sequencing revealed that there are two genes for endo-1,3(4)- $\beta$ -glucanase 1 and endo-1,3(4)- $\beta$ -glucanase 2 in the genome [18], which catalyze endohydrolysis of (1->3)- or (1->4)-linkages in  $\beta$ -D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3. Partially purified  $\beta$ -glucanase in *Kluyveromyces lactis* has been shown to have some *p*-nitropheny- $\beta$ -D-glucopyranoside (pNPG) hydrolysis activity [19], indicating the presence of BGL. It has been reported that LAC12 can transport cellobiose, which is derived from cellulose, as well as lactose in K. lactis [20,21] and that a LAC12-CEL2 cluster may uptake and hydrolyze cellobiose in K. marxianus [22]. However, there has been no study in which these intrinsic genes in *K. marxianus* were used for ethanol fermentation with cellulosic biomass. On the other hand, high- and low-affinity glucose transporters have been studied in the yeast [22].

Despite the existence of those intrinsic genes for cellulose degradation, *K. marxianus* DMKU 3-1042 is able to utilize neither cellulose nor cellobiose as a fermentation substrate. In this study, therefore, we mutagenized the strain by UV irradiation and subjected the mutagenized strains to screening in the presence of several drugs. Larger colonies more than those of the parental strain were subsequently subjected to fermentation tests with cellobiose as a carbon source. The obtained strains that were slightly or highly efficient for cellobiose fermentation ability were compared with the parental strain by genome analysis. Fermentation experiments with cellobiose and glucose as carbon sources and introduction of specific genes into the genome of one of the efficient strains in addition to genome analysis led to an understanding of the mechanism of enhanced cellobiose fermentation. The results of these experiments and results of fermentation experiments by co-culture with one efficient strain and the parental strain suggest that the efficient strains developed in this study would contribute to reduction of the running cost in bioethanol production when used with cellulase preparations with low BGL activity.

# 2. Materials and Methods

#### 2.1. Yeast Strains and Media

The yeast strains used in this study were *Kluyveromyces marxianus* DMKU3-1042 [11] and its derivatives (Table 1). YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) was used for pre-culture at 37 °C under shaking condition at 160 rpm and a cellobiose-containing medium, YAY medium (0.17% Difco Yeast Nitrogen Base (amino acid-free) (Becton, Dickinson and Company, New jersey), 0.08% ammonium sulfate, 0.5% yeast extract), supplemented with 3% cellobiose (YAYC) was used as the medium for screening of mutants by liquid culture or culture on agar plates. YAYC and YAY media supplemented with 3% glucose (YAYD) were used for examination of fermentation ability. The inoculum size of pre-culture was 0.1% of the medium. If necessary, 1  $\mu$ M disulfiram (DSF) as an inhibitor of acetaldehyde synthesis, 0.1–0.5% (*w*/*v*) 2-deoxyglucose (2-DOG) as a glucose analogue and 4  $\mu$ g/mL Clotrimazole (CTZ) (Wako, Japan) as an inhibitor of ergosterol synthesis, were added. DSF represses acetic acid production and its resistant strains may increase ethanol production [23]. 2-DOG represses glucose utilization [24,25] and its resistant strains may enhance cellobiose uptake. CTZ increases permeability of the

cell wall and inhibits the synthesis of ergosterol and other sterols essential for cell membrane biosynthesis [26] and its resistant strains may affect glucose or cellobiose uptake.

Strain/Plasmid	Relevant Properties	Strain/Plasmid Source/Reference
Kluyveromyces		
marxianus	Parental strain	[27]
DMKU3-1042		
SY5-1-17	Cellobiose-fermenting strain	This work
SY5-1-17-40	Cellobiose-fermenting strain	This work
SY8	Cellobiose-fermenting strain	This work
SY10	Cellobiose-fermenting strain	This work
SY12	Cellobiose-fermenting strain	This work
SY13	Cellobiose-fermenting strain, 1 µM DSF <sup>R</sup>	This work
SY14	Cellobiose-fermenting strain, 1 µM DSF <sup>R</sup> , 0.1% 2 DOG <sup>R</sup>	This work
SY15-1	Cellobiose-fermenting strain, 1 µM DSF <sup>R</sup> , 0.1% 2 DOG <sup>R</sup> , 4 µg/mL CTZ <sup>R</sup>	This work
SY15-2	Cellobiose-fermenting strain, 10 µM DSF <sup>R</sup> 0.1% 2 DOG <sup>R</sup>	This work
SY15-3	Cellobiose-fermenting strain, 1 µM DSF <sup>R</sup> 0.5% 2 DOG <sup>R</sup>	This work
pUC19	amp <sup>R</sup>	[28]
pUC-KHT2-bleR	pUC19 with the $KHT2$ and <i>bleR</i> genes	This work
pUC-LAC12-	pUC19 with the <i>LAC12</i> and <i>bleR</i> genes	This work

Table 1. Strains and plasmids used in study.

#### 2.2. Mutagenesis and Screening

UV irradiation was performed under the condition of 1% of the cells being able to survive [29]. DMKU3-1042 cells were grown in YPD medium at 37 °C under a shaking condition at 160 rpm. The culture was diluted with  $H_2O$  to adjust  $OD_{660}$  to 1.0 and poured into a glass petri dish with an outer diameter of 10 cm, which was put under a sterilization lamp (GL-15, manufactured by Firefly Co., Ltd., wavelength = 250 nm) in a clean bench. The sample was irradiated with ultraviolet rays from a distance of 20 cm for 5 min, and then the UV-treated cells were mixed with 10 mL of YAYC medium and cultured under a shaking condition at 40 °C and 160 rpm for 24 h. The cells were then spread on YAYC plates and incubated at 37 °C for 48 to 72 h. Among colonies on the plates, larger colonies were subjected to cellobiose fermentation tests. One strain selected at each mutagenesis step was used for the next mutagenesis.

# 2.3. Fermentation Tests and Analysis of Fermentation Parameters

Fermentation tests were carried out to examine abilities for cellobiose assimilation and ethanol production. Cells were pre-cultivated in YPD medium at 37 °C under a shaking condition at 160 rpm for 16 h. The cell was inoculated into a 100 mL flask containing 30 mL of YAYC at OD<sub>660</sub> of 0.1, followed by incubation at 40 °C under a shaking condition at 160 rpm for an appropriate time. Cell density was determined by measurement on a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Fermentation parameters were analyzed by using a high-performance liquid chromatography (HPLC) system (Hitachi, Japan) consisting of a Hitachi Model D-2000 Elite HPLC system Manager, L-2130 column oven, L-2130 pump, L-2200 auto-sampler and L-2490 RI detector equipped with a GL-C610H-S gel pack column at 60 °C with 0.5 mL/min eluent of H<sub>2</sub>O.

# 2.4. ß-Glucosidase Assay

Cells were inoculated into 30 mL of YAYD or YAYC medium for 24 h at 40 °C under a shaking condition at 160 rpm. After 12 h or 24 h, 300  $\mu$ L of the sample was collected by centrifugation at 14,000 rpm for 1 min to remove the supernatant. The precipitate was dissolved in 300  $\mu$ L of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA. Then, 3  $\mu$ L of 10 mg/mL Zymolase was added to the suspension and mixed gently, and the mixture was incubated at 37 °C. After 1 h incubation, the mixture was centrifugated at 5000 rpm for 10 min at 4 °C to remove the supernatant. Then, 300  $\mu$ L of 10 mM acetic acid buffer (pH 5.5) containing 38 mM  $\beta$ -mercaptoethanol was added to the precipitate and vigorously mixed. The mixture was subjected to sonic oscillation 3 times for 15 min each time and incubated on ice for 30 min followed by centrifugation at 14,000 rpm for 5 min. After that, the supernatant was used as a sample for  $\beta$ -glucosidase assay. The protein amounts in the supernatants were determined by the Lowry method [30]. In the  $\beta$ -glucosidase assay, a sample was mixed with 700  $\mu$ L of 10 mM acetic acid buffer (pH 5.5) containing 38 mM  $\beta$ -mercaptoethanol. The reaction was initiated by the addition of 160  $\mu$ L of pNPG followed by incubation at 40 °C. After 30 min incubation, the reaction was stopped by the addition of 400  $\mu$ L of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. The absorbance at 420 nm was then measured by a UV-VIS spectrophotometer (Shimadzu, Japan). Enzyme units are expressed as nmoles of *p*-nitrophenol formed per minute [19].

#### 2.5. Growth Tests of Mutants on YAY Agar Plates Containing Glucose, Lactose or Cellobiose

To compare the abilities of mutants and the parental strain for assimilation of sugars, growth tests on YAY agar plates containing glucose, lactose or cellobiose were carried out. To prepare an inoculum, cells were grown in YAY medium at 30 °C for 18 h and recovered by low-speed centrifugation. The cells were suspended with sterile distilled water, adjusted to  $OD_{660}$  of 1.0, and ten-fold sequentially diluted. Five microliters of the cell suspension were spotted onto the surface of YAY agar plates supplemented with 0.2% glucose, 2% glucose, 0.2% lactose, 2% lactose, 0.2% cellobiose or 2% cellobiose. The plates after spotting were incubated at 40 °C and photographs of the plates were taken at 12 h and 24 h. Experiments were performed in triplicate.

# 2.6. Preparation of Genomic DNA, Genomic Sequencing and Determination of Mutations

The genome DNAs of mutated strains were isolated as described previously [31] from cultured cells and further purified using a Genomic-tip 20 kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genome sequencing was carried out by a massively parallel sequencer (MiSeq; Illumina KK, Tokyo, Japan) as reported previously [32]. The sequenced reads were screened by a quality score higher than the Phred score of 30 and were trimmed 12 bases from the 5' end and 20 bases from the 3' end. Truncated reads less than 150 bases or with ambiguous nucleotides were excluded from further analysis. Accession numbers of sequence data are DRR361628, DRR361629, DRR361630 and DRR361632 for SY10, SY13, SY14 and SY15-2, respectively.

For genome mapping analysis, the reference genome sequence of *K. marxianus* DMKU 3-1042 (GenBank acc. No: AP012213–AP012221) was downloaded from NCBI ftp site, ftp://ftp.ncbi. nlm.nih.gov/ (accessed on 28 March 2020). Mutation sites were searched for by read mapping using CLC Genomics Workbench version 7.5 (Qiagen, Venlo, The Netherlands) with the following parameters: match score: 1, mismatch cost: 2, insertion/deletion cost: 3, length fraction: 0.7, similarity fraction: 0.9. The filter settings for SNP and In-del calling were the same as those used in a previous study [33]. All mutations in coding and non-coding regions in all sequenced mutants were confirmed by the Sanger method [34]. Physiological functions of mutated genes were analyzed by a BLAST search at NCBI (https://www.ncbi.nlm.nih.gov (accessed on 28 March 2020)) or with the STRING database (https://string-db.org (accessed on 28 March 2020)).

#### 2.7. Insertion of Transporter Genes into the Genome of a Mutant Strain, SY14

To examine the effects of insertion of transporter genes into the genome of the SY14 strain, transformation with fused DNA fragments of *LAC12* or *KHT2* with *bleR* encoding the zeocin resistance gene was performed [35]. Primers were designed at approximately 1000 bp upstream and downstream from the coding region of each target gene (Table 2). Transporter genes, *LAC12* and *KHT2*, and *bleR* were amplified by PCR using the genomic DNA of the parental strain, DMKU 3-1042 and pSH65 [36] DNA as templates, respectively, with the corresponding primer sets (Table 2). The PCR products were fused with pUC19 [28] by In-Fusion cloning (TaKaRa, Japan) according to the manual provided by the supplier. Using the resultant pUC-LAC12-bleR and pUC-KHT2-bleR as templates, fused DNA fragments

of *LAC12-bleR* and *KHT2-bleR*, respectively, were amplified by PCR and introduced into SY14 by the LiAc method [37]. Transformants were then obtained on 1% YAYC agar plates containing 100  $\mu$ g/mL of zeocin after incubation for 6 h at 37 °C. Finally, isolated transformants were used for glucose and cellobiose fermentation tests.

Table 2. Primers used in study.

Name	<b>Sequence</b> 5′ > 3′
pUC19-up	GATCCCCGGGTACCGAGCTC
pUC19-down	GATCCTCTAGAGTCGACCTG
KHT2-up	CGGTACCCGGGGATCAAATTTTTCCACCCCGCTCT
KHT2-down	TGTGTGGGGGGATCCCTGCCACATTTTAGAGGCCTT
LAC12-up	CGGTACCCGGGGATCAATTTTCCCCCCACTGA
LAC12-down	TGTGTGGGGGGATCCCTTGATGTGAAGAATACTGGT
bleR-up	GGGATCCCCCACACACCATA
bleR-down	GGACTCTAGACCATCGCGTACACGCGTCTGTACAG

#### 3. Results

3.1. Mutagenesis of K. marxianus DMKU3-1042 and Screening of Strains with High Cellobiose-Fermenting Activity

After being treated by ultraviolet irradiation as described in Materials and Methods, cells were spread on YAYC plates and incubated at 37 °C for 48 h to 72 h. Among the colonies that appeared, large colonies (96 to 144) were selected and subjected to fermentation tests. Consequently, a strain, named SY5-1-17, that showed the highest activities for cellobiose consumption and ethanol production, was selected.

Next, the SY5-1-17 strain was subjected to the same treatment by ultraviolet irradiation as described above followed by plate screening after shaking culture in YAYC medium and a fermentation test, and a strain named SY5-1-17-40, that had the highest activities for cellobiose consumption and ethanol production, was obtained. We repeated the same procedure to obtain SY8, SY10 and SY12. SY12 was further subjected to the same procedure except for supplementation of the YAYC medium for liquid and plate cultures with 1  $\mu$ M DSF. As a result, acquired SY13 showed a higher growth rate than that of SY12 in YAY medium supplemented with cellobiose, but its ability for consumption of cellobiose was still weak (Figure 1a,b). SY13 was therefore similarly mutagenized and screened with the YAYC medium supplemented with 1  $\mu$ M DSF and 0.1% 2DOG. The resultant strain, SY14, showed significantly enhanced cellobiose consumption and ethanol production in YAYC medium compared to that of SY13 (Figure 1).

Further UV mutagenesis with SY14 and screening with three different media, YAYC medium supplemented with (i) 1  $\mu$ M DSF, 0.5% 2DOG and 4  $\mu$ g/mL CTZ, (ii) 1  $\mu$ M DSF and 0.5% 2DOG and (iii) 10  $\mu$ M DSF and 0.1% 2DOG, were performed, and SY15-1, SY15-2 and SY15-3, respectively, were obtained. The fermentation abilities of the strains were then compared with those of the parental strain in YAYC medium at 40 °C under a shaking condition. SY15-1, SY15-2 and SY15-3 similarly showed better growth than that of the parental strain and showed much higher abilities for cellobiose assimilation and ethanol production than those of the parental strain (data not shown). The representative strain, SY15-2, was further compared with SY14, and SY15-2 showed the similar cellobiose assimilation ability to that of SY14 and better ethanol production ability (Figure 1). The results suggest that the three mutant strains have excellent cellobiose-fermenting ability compared to the former mutant strains and the parental strain.



**Figure 1.** Cellobiose fermentation ability of mutants obtained from *K. marxianus* DMKU 3-1042. Mutants obtained from *K. marxianus* DMKU 3-1042 and the parental strain were grown in 30 mL of YAYC (**a**–**c**) or YAYD (**d**–**f**) medium at 40 °C under a shaking condition at 160 rpm. (**a**,**d**) Cell growth was determined by measuring cell density at OD<sub>660</sub> and concentrations of cellobiose (**b**), glucose (**e**) and ethanol (**c**,**f**) in the culture medium were determined by HPLC as described in Materials and methods. Error bars indicate standard deviation of three independent experiments.

To further analyze the abilities of mutant strains for cellobiose utilization, BGL activities in SY13, SY14 and SY15 were compared using cell extracts from cells grown in YAY medium supplemented with cellobiose or glucose for 24 h (Figure 2) because the assimilation of cellobiose was very active at 24 h (Figure 1b). BGL activities of the three mutants in the cellobiose medium were almost the same and were higher than BGL activity of the parental strain, suggesting that a mutation(s) in SY13 is responsible for increased BGL activity and the transfer of BGL activity to SY14 and SY15-2, and that the parental strain can express BGL to some extent in a cellobiose-containing medium. In the glucose medium, BGL activities of all strains were significantly lower than those in the cellobiose medium, probably due to glucose repression (see below), and BGL activities of the three mutants were higher than that of the parental strain.



**Figure 2.** Measurement of  $\beta$ -glucosidase activity. Cells were grown in YAYC medium (**a**) or YAYD medium (**b**) for 24 h, and crude extracts were prepared and subjected to enzyme assays as described in Materials and Methods.

#### 3.2. Mutation Points of Mutant Strains

Differences in fermentation parameters (Figure 1) suggest that mutations that accumulated in the processes from SY13 to SY14 and from SY14 to SY15 are responsible for the significant increase in cellobiose-fermenting ability. Next-generation sequencing was performed to detect the mutations in these mutant strains, and putative mutation points in protein-coding regions are listed in Table S1; insertion or deletion of amino acids and frame shifts are shown in Figure 3. The numbers of such mutation points in SY10, SY13, SY14 and SY15-2 were 12, 19, 26 and 28, respectively. All such mutations in SY13 and SY14 were found in SY14 and SY15s, respectively (Figure 3), suggesting high sequencing accuracy. Mutations that were gained by SY14 in the background of mutations in SY13 were found in HGT1 for a high-affinity glucose transporter, NGL1 for RNA exonuclease, LAC12 for lactose permease, KHT2 for hexose transporter 2, RAG1 for a low-affinity glucose transporter, MEC1 for serine/threonine-protein kinase and KLMA\_80374 for an uncharacterized protein. Some of these mutations might contribute significantly to the increased cellobiose utilization and ethanol production in SY14 compared to those of SY13. On the other hand, mutations gained in the process from SY14 to SY15-2 were in RCY1 for recyclin-1 and CCT4 for T-complex protein 1 subunit delta. Most of the mutations found in SYs were missense mutations in coding regions, but those in HGT1, AMN1 and RGT1 were nonsense mutations. However, there is no reasonable evidence to explain the relationship between mutations in RCY1 and CCT4 and high cellobiose assimilation capacity.

# 3.3. Glucose-Fermenting Abilities of Mutant Strains

Since at least three genes, *HGT1*, *KHT2* and *RAG1*, for transporters that are able to uptake glucose [38–40] were found to have either nonsense or missense mutations in SY14 and SY15-2, their glucose-fermenting abilities were examined in YAYD medium (Figure 1). As expected, SY14 and SY15-2 showed large reductions of glucose utilization and ethanol production. These findings are consistent with the observation that SY14 showed slow growth at 12 h (but not at 24 h) on YAY plates supplemented with 0.2% or 2% glucose compared to the growth of SY13 or the parental strain (Figure 4). Therefore, the reduced glucose utilization ability seemed to be consistent with the low level of growth.



**Figure 3.** Mutations in mutants and their transfer from SY10 to its descendants. Mutations that cause amino acid substitutions, insertion or deletion of amino acids and frame shifts in SYs are shown. The relative abilities of cellobiose assimilation and ethanol production from cellobiose in SYs are shown at the top.



**Figure 4.** Growth of SY13 and SY14 on YAY agar plates containing glucose, lactose or cellobiose. Growth of SY13, SY14 and the parental strain (WT) on YAY agar plates supplemented with 0.2% glucose, 2% glucose, 0.2% lactose, 2% cellobiose or 2% cellobiose were compared. The procedure for the spotting test is described in Materials and Methods. The plates after spotting were incubated at 40 °C and photographs were taken at 12 h (**a**) and 24 h (**b**).

# 3.4. Introduction of KHT2 and LAC2 Genes into SY14

Based on the findings of mutations in HGT1, KHT2 and RAG1 in SY14 and S15-2, we assumed that the intracellular concentration of glucose was low enough to lead to a glucose repression-free state, which in turn would lead to the expression of genes for cellobiose utilization. This assumption was tested by the introduction of wild-type KHT2 into the genome of SY14, which was expected to increase the intracellular concentration of glucose. The cell growth on glucose and cellobiose utilization of the KHT2-introduced strain, SY14-KHT2-bleR, were then compared with those of SY14 (Figure 5). SY14-KHT2bleR showed larger colonies on YAYD plates and significantly reduced cellobiose utilization in YAYC medium. Therefore, these results suggest that the reduction of intracellular glucose concentration causes an increase in cellobiose utilization ability. The missense mutation of LAC12 for a lactose transporter and probably a cellobiose transporter [22] in SY14 and SY15-2 might enhance the uptake of cellobiose. When growth on YAY plates supplemented with 0.2% lactose, 2% lactose, 0.2% cellobiose or 2% cellobiose was compared, however, no significant difference between SY13 and SY14 was observed (Figure 4), suggesting that the missense mutation of LAC12 in SY14 changes neither the cellobiose-uptake activity nor the lactose-uptake activity. Consistently, SY14-LAC12-bleR showed similar cellobiose assimilation to that of SY14.



**Figure 5.** Cellobiose and glucose fermentation abilities of SY14 and SY14 derivatives into which *KHT2-bleR* or *LAC12-bleR* was introduced. SY14, SY14-KHT2-bleR, SY14-LAC12-bleR and the parental strain were grown in 30 mL of YAYC (**a–c**) or YAYD (**d–f**) medium at 40 °C under a shaking condition at 160 rpm. (**a,d**) Cell growth was determined by measuring cell density at OD<sub>660</sub>. Concentrations of cellobiose (**b**), glucose (**e**) and ethanol (**c**,**f**) in the culture medium were determined by HPLC as described in Materials and Methods. Error bars indicate standard deviation of three independent experiments.

# 3.5. Co-Culture of SY15-2 and the Parental Strain in a Medium Supplemented with Cellobiose and Glucose

Although SY14 and SY15-2 gained enhanced cellobiose fermentation ability, both had impaired glucose uptake and weakened glucose fermentation ability. Towards future applications of mutants for cellulosic biomass, co-culture of SY15-2 and the parental strain in YAYC medium supplemented with glucose was examined (Figure 6). The combination of SY15-2 and the parental strain consumed both cellobiose and glucose and produced the corresponding amount of ethanol, while only SY15-2 and only the parental strain consumed mainly cellobiose and glucose, respectively. Low levels of ethanol in SY15-2 may be due to the glucose repression in the medium supplemented with glucose. The pattern of cellobiose and glucose and glucose and glucose and the mutant strain utilized cellobiose thereafter.



**Figure 6.** Co-culture of SY15-2 and the parental strain in the medium supplemented with cellobiose and glucose. The same volumes of *K. marxianus* DMKU3-1042 and SY15-2 pre-cultures were inoculated at a total inoculum size of 0.1% into 2% glucose and 2% cellulose medium at 40 °C under a shaking condition at 160 rpm. As a control, the *K. marxianus* DMKU3-1042 pre-culture or the SY15-2 pre-culture was inoculated at an inoculum size of 0.1%. At the times indicated, the concentrations of glucose (**a**), cellobiose (**b**) and ethanol (**c**) in the medium were determined by HPLC as described in Materials and Methods. Error bars indicate standard deviation of three independent experiments.

# 4. Discussion

The conversion of cellulosic biomass to fuel is an important issue in breaking away from fossil fuels and, in particular, the enzymes used to decompose cellulose are much more expensive than the enzymes used to convert starchy biomass. Consequently, the production cost of ethanol is high. In order to solve this problem, attempts have been made to breed enzyme-producing strains [41–43] or to produce cellulolytic enzymes in fermenting yeast [44,45]. The purpose of this study was to increase the expression of enzyme genes inherent in yeast, and, as a result, we succeeded in developing strains that are able to produce one of the three main enzymes required for cellulose degradation.

In this study, we developed cellobiose-fermenting mutants via UV mutagenesis from thermotolerant *K. marxianus* DMKU3-1042 as a parental strain. Mutagenesis was performed at each step in the development process, and the cellobiose-fermentation abilities of mutants isolated from each step were compared with that of the parental strain. Since a significant difference in cellobiose utilization activity was observed between SY13 and SY14, we focused on the genetic difference between SY13 and its descendants to understand the mechanism for enhancement of cellobiose-fermenting ability. Measurements of BGL activity in the mutants and the parental strain suggested that BGL is expressed in a cellobiose-containing medium and that mutations in SY14 (but not mutations in SY13) caused no increase in its expression. Among the mutations detected in SY14 but not in SY13, those in LAC12, HGT, KHT2 and RAG1 had been thought to be responsible for the increase in cellobiose-fermenting ability. Results of further experiments suggested that the mutation in LAC12 hardly enhances cellobiose utilization. On the other hand, the mutations in HGT, *KHT2* and *RAG1* may be responsible for cellobiose utilization. It is assumed that glucose from cellobiose that is hydrolyzed outside cells is imported via glucose transporters, HGT, KHT2 and/or RAG1, to repress the expression of genes related to cellobiose utilization. Taken together, our findings suggest that K. marxianus has the potential to ferment cellobiose but that the expression of genes for cellobiose utilization is negatively regulated by glucose repression.

There is a *LAC12-CEL2* cluster on the genome of *K. marxianus*, which may be involved in the utilization of cellobiose, and Lac12 and Cel2 may function as a transporter and a hydrolyzing enzyme, respectively [22]. Considering the results in this study, it is inferred

that *LAC12*, *CEL2* or both genes are regulated by glucose repression and that when glucose repression is released, cellobiose is imported by Lac12 and hydrolyzed by Cel2 and the resulting glucose is converted to ethanol.

The mutants obtained in this study seem to avoid glucose repression by mutations of genes for glucose transporters. However, these mutants have a disadvantage of reduced glucose-fermenting ability due to their weak glucose uptake activity. It may be possible to avoid glucose repression, but disruption of genes for glucose repression regulators such as MIG1 and RAG5 may not be suitable because it also influences glucose uptake or metabolism [17]. More preferable mutants may have mutations at elements for binding of glucose repression regulators in genes for cellobiose utilization.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fuels3020015/s1. Figure S1: Schematic procedure for the development of cellobiose-fermenting mutants from *K. marxianus* DMKU3-1042. Table S1: Mutations in protein-coding regions of isolated mutants.

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# References

- 1. Bayer, E.A.; Chanzy, H.; Lamed, R.; Shoham, Y. Cellulose, cellulases and cellulosomes. *Curr. Opin. Struct. Biol.* **1998**, *8*, 548–557. [CrossRef]
- Henrissat, B.; Davies, G. Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* 1997, 7, 637–644. [CrossRef]
- Klarskov, K.; Piens, K.; Ståhlberg, J.; Høj, P.B.; Beeumen, J.V.; Claeyssens, M. Cellobiohydrolase I from *Trichoderma reesei*: Identification of an active-site nucleophile and additional information on sequence including the glycosylation pattern of the core protein. *Carbohydr. Res.* 1997, 304, 143–154. [CrossRef]
- 4. Xu, Q.; Singh, A.; Himmel, M.E. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. *Curr. Opin. Biotechnol.* **2009**, *20*, 364–371. [CrossRef] [PubMed]
- Christian, P.K. Systems biological approaches towards understanding cellulase production by *Trichoderma reesei*. J. Biotechnol. 2013, 163, 133–142.
- 6. Saloheimo, M.; Kuja-Panula, J.; Ylösmäki, E.; Ward, M.; Penttilä, M. Enzymatic properties and intracellular localization of the novel *Trichoderma reesei* beta-glucosidase BGLII (*cel1A*). *Appl. Environ. Microbiol.* **2002**, *68*, 4546–4553. [CrossRef]
- Herpoël-Gimbert, I.; Margeot, A.; Dolla, A.; Jan, G.; Mollé, D.; Lignon, S.; Mathis, H.; Sigoillot, J.-C.; Monot, F.; Asther, M. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. *Biotechnol. Biofuels.* 2008, 23, 18. [CrossRef]
- Berlin, A.; Maximenko, V.; Gilkes, N.; Saddler, J. Optimization of enzyme complexes for lignocellulose hydrolysis. *Biotechnol. Bioeng.* 2007, 97, 287–296. [CrossRef]
- 9. Baba, Y.; Sumitani, J.-I.; Tani, S.; Kawaguchi, T. Characterization of *Aspergillus aculeatus* β-glucosidase 1 accelerating cellulose hydrolysis with *Trichoderma* cellulase system. *AMB Express.* **2015**, *24*, 3. [CrossRef]
- 10. Dekker, R.F. Kinetic, inhibition, and stability properties of a commercial beta-D-glucosidase (cellobiase) preparation from *aspergillus niger* and its suitability in the hydrolysis of lignocellulose. *Biotechnol. Bioeng.* **1986**, *28*, 1438–1442. [CrossRef]
- 11. Limtong, S.; Sringiew, C.; Yongmanitchai, W. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour. Technol.* **2007**, *98*, 3367–3374. [CrossRef] [PubMed]
- 12. Goshima, T.; Tsuji, M.; Inoue, H.; Yano, S.; Hoshino, T.; Matsushika, A. Bioethanol production from Lignocellulosic biomass by a novel *Kluyveromyces marxianus* strain. *Biosci. Biotechnol. Biochem.* **2013**, 77, 1505–1510. [CrossRef] [PubMed]

- Nitiyon, S.; Keo-Oudone, C.; Murata, M.; Lertwattanasakul, N.; Limtong, S.; Kosaka, T.; Yamada, M. Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL-21. *Springerplus* 2016, 27, 185. [CrossRef] [PubMed]
- Saini, P.; Beniwal, A.; Kokkiligadda, A.; Vij, S. Evolutionary adaptation of *Kluyveromyces marxianus* strain for efficient conversion of whey lactose to bioethanol. *Process. Biochem.* 2017, 62, 69–79. [CrossRef]
- Kosaka, T.; Nakajima, Y.; Ishii, A.; Yamashita, M.; Yoshida, S.; Murata, M.; Kato, K.; Shiromaru, Y.; Kato, S.; Kanasaki, Y.; et al. Capacity for survival in global warming: Adaptation of mesophiles to the temperature upper limit. *PLoS ONE* 2019, 14, e0215614.
- 16. Rodrussamee, N.; Lertwattanasakul, N.; Hirata, K.; Suprayogi; Limtong, S.; Kosaka, T.; Yamada, M. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1573–1586. [CrossRef]
- 17. Nurcholis, M.; Murata, M.; Limtong, S.; Kosaka, T.; Yamada, M. *MIG1* as a positive regulator for the histidine biosynthesis pathway and as a global regulator in thermotolerant yeast *Kluyveromyces marxianus*. *Sci. Rep.* **2019**, *9*, 9926. [CrossRef]
- Lertwattanasakul, N.; Kosaka, T.; Hosoyama, A.; Suzuki, Y.; Rodrussamee, N.; Matsutani, M.; Murata, M.; Fujimoto, N.; Suprayogi; Tsuchikane, K.; et al. Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: Complete genome sequence and transcriptome analyses. *Biotechnol. Biofuels*. 2015, 18, 47. [CrossRef]
- Tingle, M.A.; Halvorson, H.O. A comparison of β-glucanase and β-glucosidase in *Saccharomyces lactis*. *Biochim. Biophys. Acta* 1971, 250, 165–171. [CrossRef]
- Rubio-Texeira, M.; Arévalo-Rodríguez, M.; Lequerica, J.; Lequerica, L.; Polaina, J. Lactose utilization by Saccharomyces cerevisiae strains expressing Kluyveromyces lactis LAC genes. J. Biotechnol. 2001, 84, 97–106. [CrossRef]
- Sadie, C.J.; Rose, S.H.; Den Haan, R.; Van Zyl, W.H. Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 2011, 90, 1373–1380. [CrossRef] [PubMed]
- Varela, J.A.; Puricelli, M.; Ortiz-Merino, R.A.; Giacomobono, R.; Braun-Galleani, S.; Wolfe, K.H.; Morrissey, J.P. Origin of Lactose Fermentation in *Kluyveromyces lactis* by Interspecies Transfer of a Neo-functionalized Gene Cluster during Domestication. *Curr. Biol.* 2019, 29, 4284–4290. [CrossRef] [PubMed]
- Khan, S.; Singhal, S.; Mathur, T.; Upadhyay, D.J.; Rattan, A. Antifungal potential of disulfiram. *Nippon Ishinkin Gakkai Zasshi* 2007, 48, 109–113. [CrossRef] [PubMed]
- Nguyen, M.T.; Lertwattanasakul, N.; Rodrussamee, N.; Limtong, S.; Kosaka, T.; Yamada, M. A *Kluyveromyces marxianus* 2deoxyglucose-resistant mutant with enhanced activity of xylose utilization. *Int. Microbiol.* 2015, 18, 235–244.
- 25. Orikasa, Y.; Mikumo, D.; Ohwada, T. A 2-Deoxyglucose-Resistant Mutant of Saccharomyces cerevisiae Shows Enhanced Maltose Fermentative Ability by the Activation of *MAL* Genes. *Foods* **2018**, *7*, 52. [CrossRef]
- 26. Kaewbanjong, J.; Wan, S.H.P.; Boonme, P. Clotrimazole microemulsion and microemulsion-based gel: Evaluation of buccal drug delivery and irritancy using chick chorioallantoic membrane as the model. J. Pharm. Pharmacol. 2017, 69, 1716–1723. [CrossRef]
- Nonklang, S.; Abdel-Banat, B.M.; Cha-aim, K.; Moonjai, N.; Hoshida, H.; Limtong, S.; Yamada, M.; Akada, R. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. *Appl. Environ. Microbiol.* 2008, 74, 7514–7521. [CrossRef]
- Yanisch-Perron, C.; Vieira, J.; Messing, J. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 1985, 33, 103–119. [CrossRef]
- 29. Watanabe, T.; Watanabe, I.; Yamamoto, M.; Ando, A.; Nakamura, T. A UV-induced mutant of *Pichia stipitis* with increased ethanol production from xylose and selection of a spontaneous mutant with increased ethanol tolerance. *Bioresour. Technol.* **2011**, *102*, 1844–1848. [CrossRef]
- 30. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, 193, 265–275. [CrossRef]
- 31. Sambrook, J.; Russell, D.W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2008; Volume 1.
- 32. Akuzawa, S.; Nagaoka, J.; Kanekatsu, M.; Kanesaki, Y.; Suzuki, T. Draft Genome Sequence of Oceanobacillus picturae Heshi-B3, Isolated from Fermented Rice Bran in a Traditional Japanese Seafood Dish. *Genome Announc.* **2016**, *4*, e01621-15. [CrossRef]
- Hirokawa, M.; Suzuki, A.; Miyauchi, A. Thyroid Fine-Needle Aspiration and Smearing Techniques. *VideoEndocrinology* 2018, 5, ve.2018.0119. [CrossRef] [PubMed]
- Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 1977, 74, 5463–5467. [CrossRef] [PubMed]
- Lertwattanasakul, N.; Suprayogi; Murata, M.; Rodrussamee, N.; Limtong, S.; Kosaka, T.; Yamada, M. Essentiality of respiratory activity for pentose utilization in thermotolerant yeast *Kluyveromyces marxianus* DMKU 3-1042. *Antonie Van Leeuwenhoek* 2013, 103, 933–945. [CrossRef] [PubMed]
- Guldener, U.; Heinisch, J.; Kohler, G.J.; Voss, D.; Hegemann, J.H. A second set of *loxP* marker cassettes for Cre- mediated multiple gene knockouts in budding yeast. *Nucl. Acids Res.* 2002, *30*, e23. [CrossRef] [PubMed]
- 37. Antunes, D.F.; de Souza, C.G., Jr.; de Morais, M.A., Jr. A simple and rapid method for lithium acetate-mediated transfor- mation of *Kluyveromyces marxianus* cells. *World J. Microbiol. Biotechnol.* **2000**, *16*, 653–654. [CrossRef]
- Billard, P.; Menart, S.; Blaisonneau, J.; Bolotin-Fukuhara, M.; Fukuhara, H.; Wesolowski-Louvel, M. Glucose uptake in Kluyveromyces lactis: Role of the HGT1 gene in glucose transport. J. Bacteriol. 1996, 178, 5860–5866. [CrossRef]

- 39. Weirich, J.; Goffrini, P.; Kuger, P.; Ferrero, I.; Breunig, K.D. Influence of mutations in hexose-transporter genes on glucose repression in *Kluyveromyces lactis. Eur. J. Biochem.* **1997**, 249, 248–257. [CrossRef]
- 40. Prior, C.; Mamessier, P.; Fukuhara, H.; Chen, X.; Wesolowski-Louvel, M. The hexokinase gene is required for transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. *Mol. Cell. Biol.* **1993**, *13*, 3882–3889.
- Liu, P.; Lin, A.; Zhang, G.; Zhang, J.; Chen, Y.; Shen, T.; Zhao, J.; Wei, D.; Wang, W. Enhancement of cellulase production in *Trichoderma reesei* RUT-C30 by comparative genomic screening. *Microb. Cell. Fact.* 2019, 18, 81. [CrossRef]
- 42. Patyshakuliyeva, A.; Arentshorst, M.; Allijn, I.E.; Ram, A.F.; de Vries, R.P.; Gelber, I.B. Improving cellulase production by *Aspergillus niger* using adaptive evolution. *Biotechnol. Lett.* **2016**, *38*, 969–974. [CrossRef] [PubMed]
- 43. Davison, S.A.; Den Haan, R.; van Zyl, W.H. Heterologous expression of cellulase genes in natural *Saccharomyces cerevisiae* strains. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 8241–8254. [CrossRef] [PubMed]
- 44. Liu, Z.L.; Li, H.N.; Song, H.T.; Xiao, W.J.; Xia, W.C.; Liu, X.P.; Jiang, Z.B. Construction of a trifunctional cellulase and expression in *Saccharomyces cerevisiae* using a fusion protein. *BMC. Biotechnol.* **2018**, *18*, 43. [CrossRef] [PubMed]
- Hong, J.; Wang, Y.; Kumagai, H.; Tamaki, H. Construction of thermotolerant yeast expressing thermostable cellulase genes. J. Biotechnol. 2007, 130, 114–123. [CrossRef]