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A *Kluyveromyces marxianus* 2-deoxyglucose-resistant mutant with enhanced activity of xylose utilization

Suprayogi,[1,2] Minh T. Nguyen,[1,3] Noppon Lertwattanasakul,[4] Nadchanok Rodrussamee,[5] Savitree Limtong,[4] Tomoyuki Kosaka,[6] MamoruYamada[1,6*]

[1]Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi Univ., Ube, Japan.
[2]Dept. of Agroindustrial Technology, Fac. of Agriculture Technology, Brawijaya Univ., Malang, Indonesia.
[3]Dept. of Microbiology, Fac. of Environment, Vietnam National Univ. of Agriculture, Hanoi, Vietnam. [4]Dept. of Microbiology, Fac. of Science, Kasetsart Univ., Bangkok, Thailand. [5]Dept. of Biology, Fac. of Science, Chiang Mai Univ., Chiang Mai, Thailand. [6]Dept. of Biological Chemistry, Fac. of Agriculture, Yamaguchi Univ., Yamaguchi, Japan

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Summary. Thermotolerant ethanologenic yeast Kluyveromyces marxianus is capable of fermenting various sugars including xylose but glucose represses to hamper the utilization of other sugars. To acquire glucose repression-defective strains, 33 isolates as 2-deoxyglucose (2-DOG)-resistant mutants were acquired from about 100 colonies grown on plates containing 2-DOG, which were derived from an efficient strain DMKU 3-1042. According to the characteristics of sugar consumption abilities and cell growth and ethanol accumulation along with cultivation time, they were classified into three groups. The first group (3 isolates) utilized glucose and xylose in similar patterns along with cultivation to those of the parental strain, presumably due to reduction of the uptake of 2-DOG or enhancement of its export. The second group (29 isolates) showed greatly delayed utilization of glucose, presumably by reduction of the uptake or initial catabolism of glucose. The last group, only one isolate, showed enhanced utilization ability of xylose in the presence of glucose. Further analysis revealed that the isolate had a single nucleotide mutation to cause amino acid substitution (G270S) in RAG5 encoding hexokinase and exhibited very low activity of the enzyme. The possible mechanism of defectiveness of glucose repression in the mutant is discussed in this paper. [**Int Microbiol** 18(4):235-244 (2015)]

Keywords: *Kluyveromyces marxianus* · glucose repression · 2-deoxyglucose-resistant mutants · ethanol fermentation on xylose · thermotolerant yeast

Introduction

Compared to *Saccharomyces cerevisiae,* which is used for ethanol fermentation industries, *Kluyveromyces marxianus* has advantageous potentials in application for ethanol production. First, *K. marxianus* is thermotolerant and is able to efficiently produce ethanol at around 40°C [9,10,18]. It is thus applicable for high-temperature fermentation as an economical fermentation, enabling reduction in cooling cost, efficient simultaneous saccharification and fermentation, reduction in contamination, and stable fermentation even in tropical countries [2,3,18]. Second, the yeast can assimilate various sugars, including xylose, arabinose, sucrose, raffinose and inulin, in addition to several hexoses [16,28]. This broad spectrum in sugar assimilation capability is beneficial for the conversion to ethanol of biomass including various sugars. Third, the yeast has relatively weak glucose repression of the utilization of sucrose [16] and thus is highly preferable to biomass such as sugar cane juice, which contains glucose,

*Corresponding author: M. Yamada
Applied Molecular Bioscience
Graduate School of Medicine
Yamaguchi University
Ube, 753-8515 Japan
Tel. +81-839335869. Fax +81-839335869
E-mail: m-yamada@yamaguchi-u.ac.jp

fructose and sucrose as main sugars. Despite such beneficial properties, some crucial problems in the use of *K. marxianus* remain to be solved. One problem is glucose repression of the utilization of other sugars including xylose [28], which is a principal constituent in hemicellulose for second-generation biofuels [21].

2-Deoxyglucose (2-DOG) is a convenient reagent for screening of mutants defective in glucose repression [22,33]. It is a stable glucose analogue that is taken up into cells by hexose transporters and phosphorylated but cannot be fully metabolized. Accumulation of 2-deoxyglucose-6-phosphate in cells interferes with carbohydrate metabolism by inhibiting the activities of glycolytic enzymes including phosphoglucose isomerase and hexokinase [7,32,38]. In general, it is assumed that the biological effects of 2-DOG are the consequence of a block in carbohydrate catalysis, implying that 2-DOG-treated cells are unable to metabolize glucose and stop growing as a result of a lack of energy and metabolic intermediates [26].

In order to acquire glucose repression-defective strains, isolation and characterization of 2-DOG-resistant mutants from *K. marxianus* DMKU 3-1042 as one of the most thermotolerant strains, which was isolated via an enrichment culture method with samples collected in Thailand [18], were performed. The isolated 2-DOG-resistant mutants were characterized and classified by several experiments and eventually one mutant was found to have a significantly enhanced activity of xylose utilization. This mutant may be a preferable candidate for ethanol fermentation from biomass containing mixed sugars including glucose. This study thus provided not only a mutant with enhanced activity of xylose utilization in *K. marxianus* but also its metabolic characteristics of conversion of xylose to ethanol under the condition of coexistence of glucose.

Materials and methods

Strains and media. The thermotolerant *Kluyveromyces marxianus* DMKU 3-1042 strain isolated in Thailand [18] as a strain that was isolated by an enrichment culture and its derivatives obtained in this study. Pre-culture was carried out in YPGal medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l galactose) for preparation of the inoculum. To examine sugar utilization ability and cell growth, YP medium (10 g/l yeast extract and 20 g/l peptone) supplemented with 20 g/l of glucose (Glc), galactose (Gal) or xylose (Xyl), designated as YPD, YPGal or YPXyl, respectively, was used. YP medium supplemented with 20 g/l Glc and 20 g/l of one of the other sugars was used to examine the effect of glucose on utilization ability of other sugars. YP medium was used for general experiments.

Cultivation conditions and spotting test. Cells were pre-cultured in 5 ml of YPGal medium at 30°C under a shaking condition at 160 rpm overnight. The pre-culture was inoculated into a 300-ml Erlenmeyer flask containing 100 ml fresh medium at an initial optical density at 660 nm (OD_{660}) value of 0.1, and cultivation was performed at 30°C under a shaking condition at 160 rpm for an appropriate time. In experiments for spotting tests, pre-cultured cells were washed with deionized water, suspended in deionized water at 1×10^7 cells/ml, 10-fold sequentially diluted, and then spotted onto agar plates of YPD, YPGal and YPXyl with or without 0.1% 2-DOG. The plates were incubated at 30°C for 48 h.

Screening and phenotype characterization of 2-DOG-resistant mutants. To screen 2-DOG-tolerant mutants, cells of *K. marxianus* DMKU 3-1042 were grown in 5 ml YPD at 30°C overnight under a shaking condition at 160 rpm, collected by low-speed centrifugation, suspended in 1 ml sterilized water, spread on Yeast Nitrogen Base plates containing 2% Xyl and 0.1% 2-DOG (YNB + 2% Xyl + 0.1% 2-DOG), and incubated at 30°C for 3 days. Colonies on the plates were re-streaked on YPD, YNB + 2% Xyl + 0.1% 2-DOG, YNB + 2% Gal + 0.1% 2-DOG and YNB + 2% Ara + 0.1% 2-DOG and incubated at 30°C for 3 days. The colonies that were able to grow well on the four different plates were selected as 2-DOG-resistant mutants. Growth of all mutants was further examined on YP plates containing different types of sugars; Glc, Gal, Xyl, Gal + 0.1% 2-DOG and Xyl + 0.1% 2-DOG, and on YNB plates containing different concentrations of Glc (0.02, 0.2 and 2%) and the presence of antimycin A. YNB medium was used only for examinations on the first screening of 2-DOG-resistant mutants and effects of glucose concentration on cell growth and antimycin A.

Analytical methods. Cell density was measured turbidimetrically at 660 nm using a spectrophotometer (U-2000A, Hitachi Japan). Cultures were sampled and subjected to low-speed centrifugation. The supernatant was frozen and kept at –20ºC until the end of fermentation (96 h) and then analyzed together by using HPLC. Quantitative analysis of sugars, ethanol, glycerol and xylitol was performed by high-performance liquid chromatography (Hitachi Model D-2000 Elite HPLC System Manager) as described previously [28]. A GL-C610-S gel pack column (Hitachi) was used together with a refractive index detector (Model L-2490) at 60°C with 0.3 ml/min eluent of deionized water.

Determination of oxidized NAD^+ and reduced NADH concentrations. Cells were pre-cultured in 5 ml of YPGal medium at 30°C under a shaking condition at 160 rpm overnight. The pre-culture was inoculated into a 300-ml Erlenmeyer flask containing a 100-ml fresh YP medium containing 2% Xyl and 2% Glc, at an initial OD_{660} value of 0.1, and cultivation was performed at 30°C under a shaking condition at 160 rpm for an appropriate time. A sample of the culture (10^6 cells/ml) was subjected to centrifugation at 4°C. The cell pellet was washed with a cold phosphate-buffered saline. Intracellular NAD^+ and NADH concentrations were determined by using EnzyChrom NAD^+/NADH Assay Kit (E2ND-100) (BioAssay Systems, USA) according to the instruction manual of the supplier and the reaction mixture was measured in a Powerscan HT microplate reader (DS Pharma Biomedical, Osaka, Japan).

Preparation of cell extracts. 2-DOG-resistant no. 23 mutant and the parental strains were pre-cultured in 5 ml YPGal at 30°C overnight under a shaking condition at 160 rpm. The pre-culture was inoculated into a 300-ml Erlenmeyer flask containing a 100-ml fresh YP medium containing 2% Xyl and 2% Glc at an initial OD_{660} value of 0.1, and cultivation was performed at 30°C under a shaking condition at 160 rpm. Cells in the exponential growth phase were collected by centrifugation (Himac CR20 Hitachi) at 5000 rpm and 4°C for 10 min and washed twice with 10 mM potassium phosphate buffer (pH 7.0). The washed cells were re-suspended in the same buffer. The cells suspension was passed through a French press (Aminico, USA) at 1000 psi twice and centrifuged at 4°C for 10 min at 9000 rpm to remove cells

debris. The supernatant was further centrifuged at 4°C for 1 h at 44,000 rpm by using a micro-ultracentrifuge (Himac CS 100GXL Hitachi) to remove membrane fractions. The resultant supernatant was used as cell extracts for enzyme assays.

Enzyme assays. Hexokinase activity was measured spectrophotometrically by coupling the reaction to G6-P dehydrogenase [6]. The assay was performed in a reaction mixture containing 0.24 M triethanolamine (pH 7.5), 5.3 mM ATP, 4 mM D-fructose, 0.72 mM $NADP^+$, 5 mM $MgCl_2$, and 2 U/ml G6-P dehydrogenase from *Leuconostoc* sp. (Oriental yeast, Japan) by using a spectrophotometer (U-2000A Hitachi, Japan). Glucokinase activity was determined by the same assay except that 4 mM D-glucose was used as a substrate instead of D-fructose. Protein content was determined by the Lowry method [20].

Nucleotide sequencing and alignment. From cells of 2-DOG resistant no. 23 mutant and parental strains that had been grown in YPD medium, genomic DNA was prepared as described [29]. PCR amplification was performed using primeSTAR DNA polymerase (Takara BIO, Japan) with PCR primers listed in Table 1. The amplification condition was as follows: one cycle of 10 s denaturation at 98°C, 30 cycles of 10 s denaturation at 98°C, 5 s annealing at 60°C and 2.5 min extension at 72°C and 1 cycle of 5 min extension at 72°C. DNA fragments were purified using a QIAquick PCR purification kit (QIAGEN) and subjected to nucleotide sequencing [30] by using ABI Prism 310 (Perkin Elmer, USA). DNA sequences determined were subjected to BLAST analysis [1]. Alignment of amino acid sequences was performed using a clustalW [36].

Table 1. Primers used in this study

Name	Sequence 5′ → 3′
RAG5-I5′[a]	5′CTGTTGCCAGTTGCCAGTTGC3′
RAG5-I3′	5′GGCTGGTGGCTTCTTTGGACC3′
RAG5-II5′	5′CAAGGAACAACTAGTTAAGC3′
RAG5-II3′	5′ATCTTGTTTTGGGAGGCTGGG3′
RAG5-III5′	5′AGTTGTTCTGGTCAAGTTGGG3′
RAG5-III3′	5′AACCGGAAGTCATCTTTTCG3′
RAG5-IV5′	5′CAAGATGGGTATCATCATTGG3′
RAG5-IV3′	5′TCCTTCAAAGCTTGAGCAGCC3′
RAG5-V5′	5′CCATACGTCATGGACACCACC3′
RAG5-V3′	5′TGAGCGATCGTGAATGAATGTC3′

[a]Primers were designed according to the *RAG5* nucleotide sequence [17].

Results

Screening and phenotype characterization of 2-DOG-resistant mutants. About 100 colonies that appeared on YNB plates containing 2% Xyl and 0.1% 2-DOG at the first screening were subjected to re-streaking on the same plates, and 33 independent isolates were obtained. To confirm 2-DOG resistance of the isolates, spotting tests were carried out on YPGal and YPXyl plates supplemented with 2-DOG by using YPD or YPGal and YPXyl without 2-DOG plates as controls (Fig. 1). All 33 isolates exhibited sufficient growth at 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} fold dilutions on both YPGal and YPXyl plates supplemented with 2-DOG, suggesting that they are resistant to 2-DOG, but the parental strain hardly grew in the presence of 2-DOG. Consequently, the 33 isolates were further analyzed as 2-DOG-resistant mutants.

It was found that the 33 mutants formed colonies of different sizes on YPD plates (Fig. 1). Mutants of no. 1, 5, 9 and 23 as well as the parental strain formed larger colonies than did other mutants on YPD plates at 10^{-3} and 10^{-4} fold dilutions. On the other hand, all mutants exhibited colonies of almost the same sizes on YPGal and YPXyl plates.

To further analyze the Glc utilization ability of the 33 mutants, their growth on YNB plates containing different concentrations of Glc was compared (Fig. 2A–C). Mutants of no. 1, 5, 9 and 23 as well as the parental strain sufficiently grew on 0.2% Glc, but other mutants hardly grew at that low concentration of Glc. All 33 mutants, however, grew well on 2% Glc. These findings suggested that the 33 mutants except for no. 1, 5, 9 and 23 are defective in Glc uptake or in its initial catabolism.

To examine the effect of the respiratory inhibitor antimycin A on cell growth of 2-DOG-resistant mutants, growth of the mutants was examined on YNB plates containing 5 μM antimycin A at 30°C (Fig. 2D). Mutants of no. 5 and 9 grew well like the parental strain in the presence of antimycin A, and mutants of no. 1 and 23 showed weaker growth than that of the parental strain. In contrast, other mutants did not grow in the presence of antimycin A. These results and the growth phenotype on 0.2% Glc (Fig. 2B) suggest that mutants no. 1, 5, 9 and 23 can support growth on glucose on a fermentative basis, which means that respiration is not induced at this low concentration of glucose, and the uptake of 2 g/l glucose into the cells maintains a high enough Glc-6-phosphate concentration inside the cells as to signal a cell growth by fermentation activity. Whereas, the defective growth of other mutants in the presence of the respiratory inhibitor may be due to insufficient metabolic activity of Glc for growth under a fermentation condition.

Cell growth and sugar consumption of 2-DOG-resistant mutants in YP medium containing mixed sugars of Glc and Xyl. To determine whether the 2-DOG-resistant mutants had acquired the phenotype of glucose repression-defective mutants, their Xyl utilization was examined in the presence of Glc. The growth of mutants was examined in YP medium containing both Glc and Xyl,

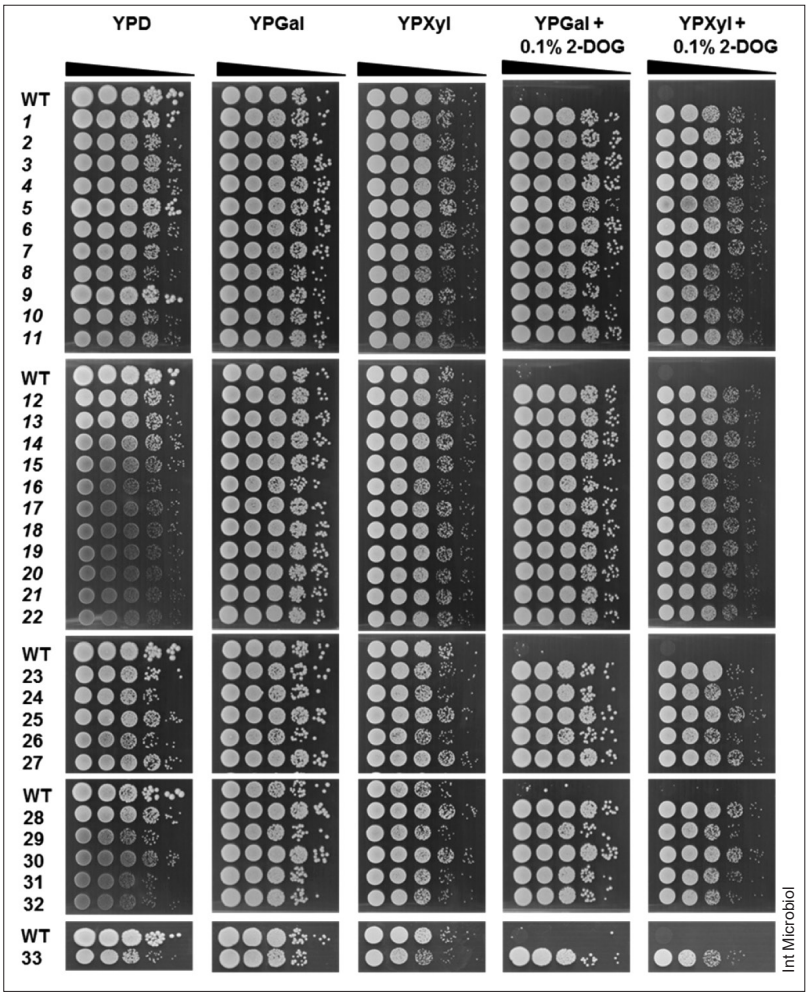

Fig. 1. Spotting test of 2-DOG-resistant isolates. Cells were grown in 2% YPGal medium at 30°C overnight and subjected to a spotting test as described in Materials and methods. After spotting (left to right, 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} fold dilutions) on 2% YPD, 2% YPGal, 2% YPXyl, 2% YPGal containing 0.1% 2-DOG, and 2% YPXyl containing 0.1% 2-DOG, plates were incubated at 30°C for 48 h.

and several factors including concentrations of Glc, Xyl and ethanol in the medium as well as turbidity of the medium were determined. According to the patterns of consumption of both sugars, cell growth and ethanol accumulation along with cultivation time, the 33 mutants were classified into three groups and representative mutants are shown in Fig. 3.

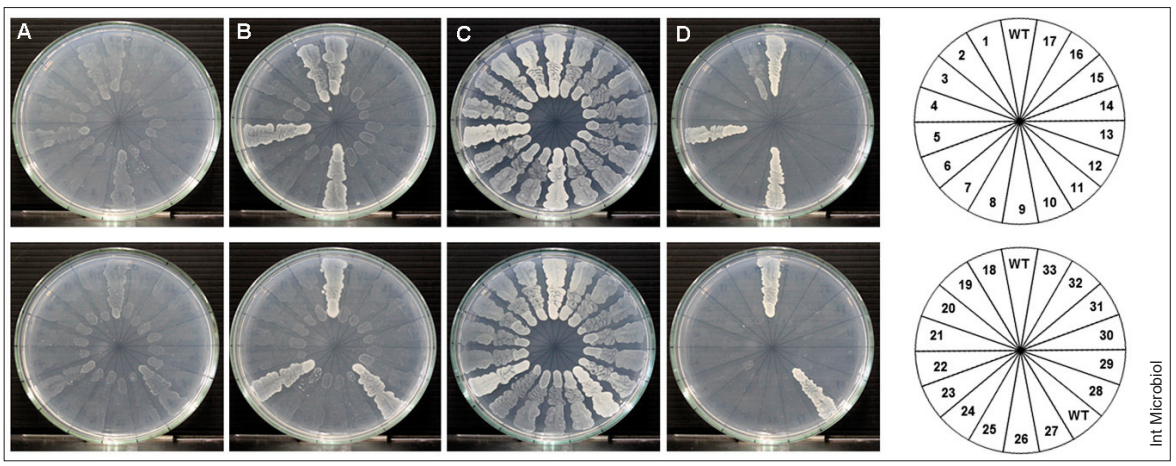

Fig. 2. Growth of 2-DOG-resistant mutants at different concentrations of Glc and effect of antimycin A. Cells were grown in 2% YPGal medium at 30°C overnight and streaked on YNB plates containing different concentrations of Glc. The plates were then incubated at 30°C for 48 h. (**A**) 0.02% Glc; (**B**) 0.2% Glc; (**C**) 2% Glc; (**D**) 2% Glc + 5 μM antimycin A.

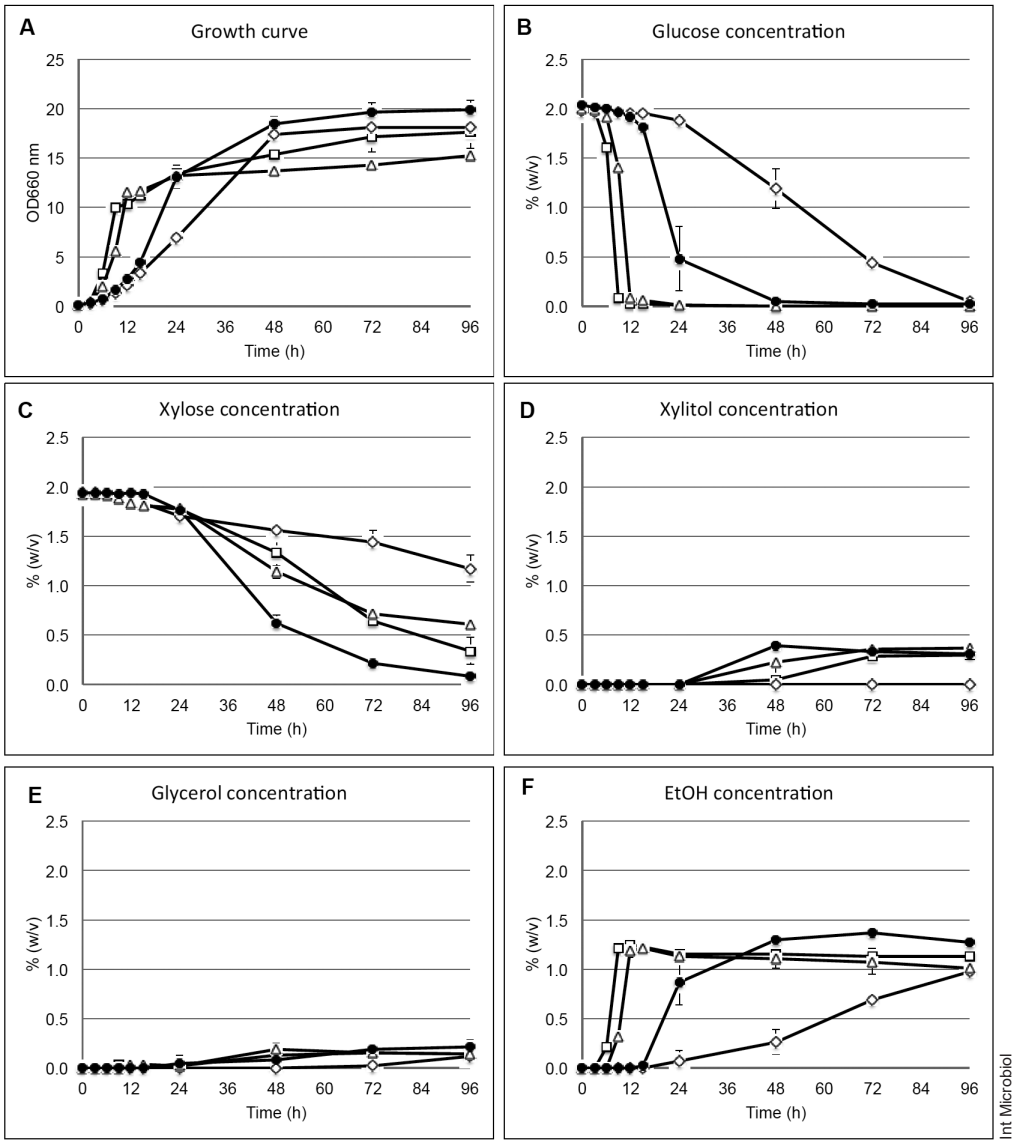

Fig. 3. Growth and sugar consumption of 2-DOG-resistant mutants in YP medium containing Glc and Xyl. Cells were grown in 2% YPGal medium at 30°C overnight, transferred to a fresh YP medium containing 2% Xyl and 2% Glc, and cultivated at 30°C under a shaking condition at 160 rpm for 96 h. No. 3 mutant (open diamonds), no. 5 mutant (open triangles), no. 23 mutant (closed circles) and their parental strain (open squares) were compared by measuring OD_{660} (**A**) and concentrations of Glc (**B**), Xyl (**C**), xylitol (**D**), glycerol (**E**), and ethanol (**F**) in the medium. Data presented were averages of triplicate experiments and error bars indicate the standard deviations.

One group consisting of no. 1, 5 and 9 mutants (no. 5 as a representative) showed similar patterns of the four parameters of turbidity (OD_{660}), Glc, Xyl and ethanol to those of the parental strain (Fig. 3A–C and F). This type of mutant started to utilize Xyl after depletion of Glc in the medium. Even after depletion of Glc, their turbidity increased (Fig. 3A–C), but ethanol level was slightly reduced during cultivation. The second group consisting of other mutants except for no. 23 (no. 3 as a representative) showed extremely slow Glc and Xyl consumption rates, and ethanol accumulation in the medium was observed after 24 h (Fig. 3B,C and F). This type of mutant could utilize Glc and Xyl slowly and simultaneously. No. 23 mutant as the third group showed enhancement of Xyl utilization and delay of Glc utilization compared to those of the parental strain (Fig. 3B,C). Notably, both sugars were utilized together after 15 h, indicating suppression of glucose repression. A phenotype similar to that of no. 23 has been reported in an *S. cerevisiae* 2-DOG-resistant mutant that is

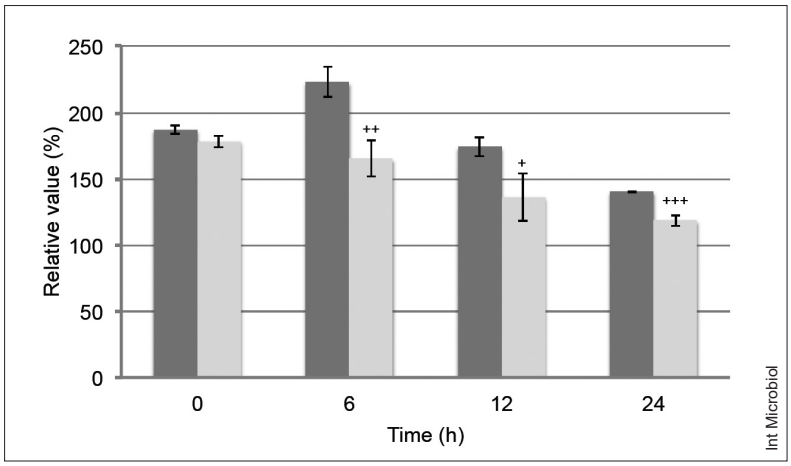

Fig. 4. Relative NAD^+/NADH values in 2-DOG-resistant no. 23 mutant in YP medium containing Glc and Xyl. Cells were grown in 2% YPGal medium at 30°C overnight, transferred to a fresh YP medium containing 2% Xyl and 2% Glc, and cultivated at 30°C under a shaking condition at 160 rpm. At the times indicated, cells were harvested and concentrations of NAD^+ and NADH were determined as described in Materials and methods. The relative NAD^+/NADH values of no. 23 mutant (light grey columns) were compared with those of the parental strain (dark grey columns). Data presented were averages of triplicate experiments and error bars indicate the standard deviations. Statistical analysis was performed between no. 23 mutant and the parental strain: +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$.

able to assimilate both sugars of Glc and Xyl together [14]. Concentrations of ethanol, glycerol and xylitol in the medium increased after 24 h, and the ethanol level was more than that of the parental strain after 48 h (Fig. 3D–F). The ethanol yield of no. 23 mutant was 0.50 g EtOH/g sugars at 24 h, 0.39 g EtOH/g sugars at 48 h, 0.37 g EtOH/g sugars at 72 h and 0.33 g EtOH/g sugars at 96 h. This mutant showed significantly higher ethanol production than that of the parental strain at 48 h ($P < 0.05$), 72 h ($P < 0.01$) and 96 h ($P < 0.05$) (Fig. 3F).

NAD^+ and NADH concentrations in no. 23 mutant in YP medium containing mixed sugars of Glc and Xyl. Since no. 23 mutant showed a delay of Glc consumption and almost parallel utilization of Xyl with Glc in contrast to the parental strain, it was guessed to be due to the limitation of NAD^+ for Glc utilization in the mutant. To obtain the clue of the glucose repression-defective phenotype on the mutant, intracellular NAD^+ and NADH concentrations were measured and the relative NAD^+/NADH values between no. 23 mutant and the parental strain were compared (Fig. 4). The experiments were performed under the same condition used in Fig. 3. The ratio of NAD^+ to NADH concentrations in the mutant was lower than that of the parental strain at all times tested, whereas the relative amount of NAD^+ was gradually reduced after about 6 h. The ratio of NAD^+ to NADH in the mutant was 1.6, 1.4 and 1.2 at 6 h, 12 h and 24 h, respectively, and that in the parental strain was 2.2, 1.7 and 1.4 at 6 h, 12 h and 24 h, respectively. Therefore, it may be possible that the relatively low level of NAD^+ in no. 23 mutant caused its slow utilization of Glc.

Weak hexokinase activity in no. 23 mutant. Two main mechanisms for resistance to 2-DOG have been reported to be either the defect of glucose phosphate kinase [19] or the induction of glucose-6-phosphate phosphatase activity [13]. We thus measured activities of hexokinase and glucokinase in no. 23 mutant since *K. marxianus* DMKU 3-1024 bears these two kinases. Cells were grown under the same condition

Table 2. Hexokinase and glucokinase activities of 2-DOG-resistant no. 23 mutant

Substrate	Enzyme	Specific activity (Umg^{-1})		
		DMKU3-1042	No. 23	*P* value
Fructose	Hexokinase	0.497 ± 0.027	0.031 ± 0.003	$P < 0.001$
Glucose	Hexokinase and glucokinase	0.711 ± 0.044	0.220 ± 0.026	$P < 0.001$

Fig. 5. Alignment of amino acid sequences of hexokinases in *Kluyveromyces marxianus, K. lactis* and *Saccharomyces cerevisiae*. The primary sequences of hexokinases of *K. marxianus* KmRag5p (line 1), *K. lactis* KlRag5p (line 2), *S. cerevisiae* ScHxk2p (line 3) and *S. cerevisiae* ScHxk1p (line 4) were aligned. Stars represent conserved amino acid residues. A vertical arrow represents Gly-270, which was substituted to be serine in no. 23 mutant.

used in Fig. 3 and were harvested at the mid-log phase for the enzyme assay. As shown in Table 2, the specific activity of fructose kinase corresponding to hexokinase of no. 23 mutant was 16 times lower than that of the parental strain. Whereas, the specific activity of glucose kinase corresponding to glucokinase in the no. 23 mutant was 3 times lower than that of the parental strain. These results suggest that no. 23 mutant has a defective hexokinase enzyme but retains glucokinase activity almost equivalent to that of the parental strain.

Determination of a mutation site on no. 23 mutant. Due to almost no activity of hexokinase in no. 23 mutant, the nucleotide sequence of *RAG5* encoding the enzyme was determined. As a result, there was one nucleotide mutation from G to A at the 270th codon in *RAG5,* causing G270S substitution. Interestingly, G270 is conserved in hexokinases (Fig. 5) and is located close to the substrate-binding site [5,15]. The location of the substitution site might match with the fact that there was a trace amount of hexokinase activity in the no. 23 mutant.

Discussion

In this study, we attempted to isolate glucose repression-defective strains by screening 2-DOG-resistant mutants in *K. marxianus* as promising yeast for economical bioethanol production from various types of biomass [28]. We compared the growth abilities of the mutants isolated at different concentrations of Glc and in the presence of antimycin A and

the consumption patterns of mixed sugars of Xyl and Glc, and we consequently classified them into three groups.

The three groups could be clearly distinguished when compared in liquid culture with YP medium containing mixed sugars of Glc and Xyl (Fig. 3). The first group appears to be similar to the parental strain in utilization timing and patterns of Glc and Xyl and in the accumulation profile of ethanol as well as cell growth. The 2-DOG resistance of the first group might be due to blockage of the import of 2-DOG into cells or enhanced export of the chemical agent. They seem to normally import and ferment Glc as indicated by growth in the presence of antimycin A, and they utilize Xyl after depletion of Glc, indicating retainment of the glucose repression of Xyl utilization like the parental strain.

The second group might be defective in hexose transporters or initial catabolism of Glc because the group exhibited remarkable retardation of Glc and Xyl utilization compared to that of the parental strain. The retardation of Xyl utilization suggests that hexose transporters are responsible for the Xyl import in *K. marxianus* as reported previously [11]. This group appears to utilize Glc and Xyl together presumably because the intracellular concentration of Glc is too low to evoke glucose repression.

One mutant numbered 23 as the third group exhibited a glucose repression-defective phenotype (Fig. 3). This 2-DOG mutant may have acquired the ability of Xyl uptake in the presence of Glc in the medium, though Glc consumption was retarded compared to that of the parental strain. Notably, the utilization of Xyl was much faster than that of the parental strain, and larger amounts of glycerol and xylitol than those in the case of the parental strain were accumulated in the medium. The mutant showed a relatively low ratio of NAD^+ to NADH concentrations compared to that of the parental strain (Fig. 4).

The low ratio may be responsible for the utilization of Xyl because its process contains the conversion reaction of NAD+ to NADH by xylitol dehydrogenase [26], and the limitation of NAD^+ may slow down the glycolysis process, which in turn leads the delay of Glc utilization. Such an effect of NAD^+ limitation on the Glc utilization has been reported in *S. cerevisiae* [37]. Alternatively, the delayed Glc assimilation could be the consequence of low hexokinase activity.

Further analysis revealed that no. 23 mutant exhibited almost no hexokinase activity and had a single nucleotide mutation to cause amino acid substitution (G270S) in *KmRAG5* encoding hexokinase. KmRag5p in *K. marxianus* shares 89.9% and 72.6% identities with KlRag5p in *K. lactis* [25] and with ScHxk2p in *S. cerevisiae* [34], respectively. Amino acid alignment revealed that G270 of KmRag5p corresponds to G271 of ScHxk2p and is located in one of conserved regions (Fig. 5), and the amino acid substitution of G271C promotes residue-residue interactions to cause slight changes of backbone conformation [5,15]. Interestingly, Gly271 in ScHxk2p is located close to the glucose-binding site. Therefore, it is assumed that the G270S substitution caused reduction of hexokinase activity in no. 23 mutant.

Considering the function of hexokinase as a transcriptional regulator for the glucose repression in *S. cerevisiae* [23,24,31], it may be likely that the amino acid substitution of G270S in hexokinase leads to a glucose repression-defective of no. 23 mutant to permit uptake of Xyl together with Glc. On the other hand, the 2-DOG resistant phenotype of the mutant may be due to reduction in hexokinase-catalyzed formation of 2-DOG-6-phosphate, which hampers glycolysis as an inhibitor of glucose phosphate isomerase [13].

The findings in the experiments using mixed sugars of Glc and Xyl in no. 23 mutant (Fig. 3) can be explained as follows. Due to the defect of glucose repression, Xyl was imported at the same time with Glc, and thus NAD^+ would quickly become insufficient for Glc catabolism by its conversion to NADH at the xylitol oxidation step [27], which in turn slows down the glycolysis process, consequently suppressing Glc uptake. The defect of hexokinase may also be responsible for the delay of Glc utilization to some extent. After around 15 h, the glycerol production pathway might be induced to supply NAD^+, which promotes glycolysis to import and catabolize Glc and to further provide NAD^+ by the ethanol production pathway. The amount of NAD^+ provided might be sufficient to assimilate both Glc and Xyl together. This assumption should be experimentally proved.

Unlike gene-engineered *S. cerevisiae* mutants in which foreign genes are introduced [4,8,12,35], *K. marxianus* strains improved by mutation breeding can be utilized unlimitedly as non-recombinants for the ethanol industry. Therefore, further improvement of no. 23 mutant to be a faster consumer of both sugars of Glc and Xly is expected.

We can conclude that *K. marxianus* is a highly competent yeast that can ferment various sugars including Xyl but bears glucose repression to inhibit the utilization of other sugars. This study was thus aimed at developing glucose repression-defective strains from a strongly thermotolerant *K. marxianus* DMKU 3-1042. Via the screening of 2-DOG-resistant mutants, one isolate, no 23 mutant, was acquired, which showed enhanced utilization ability of Xyl in the presence of Glc but delayed utilization of Glc, due to the *RAG*5 mutation that largely reduced hexokinase activity. This

mutant produced significantly higher ethanol than that of the parental strain after 48 h in the medium containing mixed sugars of Glc and Xyl.

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