fuels

MDPI

Article

Mutants with Enhanced Multi-Stress Tolerance of *Kluyveromyces marxianus* and Their Ability for Ethanol Fermentation

Noppon Lertwattanasakul [1], Sornsiri Pattanakittivorakul [2], Sukanya Nitiyon [3], Minenosuke Matsutani [4], Akihiro Oguchi [5], Katsushi Hirata [5], Tomoyuki Kosaka [2,5,6], Savitree Limtong [1,7] and Mamoru Yamada [2,5,6,*]

[1] Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand; fscinple@ku.ac.th (N.L.); fscistl@ku.ac.th (S.L.)
[2] Graduate School of Science and Technology for Innovation, Yamaguchi University, Yamaguchi 753-8515, Japan; sornkiri@gmail.com (S.P.); tkosaka@yamaguchi-u.ac.jp (T.K.)
[3] Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok 10900, Thailand; sukanyaniti@gmail.com
[4] NODAI Genome Research Center, Tokyo University of Agriculture, Tokyo 156-8502, Japan; mm207299@nodai.ac.jp
[5] Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan; 23akhm@gmail.com (A.O.); katsushi.hirata@hugp.com (K.H.)
[6] Research Center for Thermotolerant Microbial Resources, Yamaguchi University, Yamaguchi 753-8515, Japan
[7] Biodiversity Center, Kasetsart University, Bangkok 10900, Thailand
* Correspondence: m-yamada@yamaguchi-u.ac.jp

Abstract: *Kluyveromyces marxianus* is an attractive thermotolerant yeast species for ethanol production because of its ability to utilize various carbon sources as a fermentation substrate. The use of thermotolerant microorganisms enables the performance of high-temperature ethanol fermentation, which has several advantages, including the reduction of cooling costs and minimization of contamination risks. To improve *K. marxianus* for ethanol fermentation under stress conditions, two strains, DMKU 3-1042 and DMKU 3-118, were adapted for heat resistance and resistance to toxic substances in pulp wastewater from a paper mill, respectively, resulting in the generation of KMR1042 and KMR118, respectively. Both adapted mutants exhibited clumpy clusters of cells as pseudo-hyphae and altered colony morphology, and their sedimentation speeds were much faster than those of the corresponding parent strains. The two mutants showed stronger tolerance to various stresses and higher performance for ethanol production than those of the corresponding parent strains at high temperatures or in the presence of toxic substances. Genome sequencing analysis revealed that both mutants had disruption of the same gene, *SWI5*, despite adaptation under different stress conditions, suggesting that the formation of pseudo-hyphae is a common strategy of *K. marxianus* for coping with stresses.

Keywords: *Kluyveromyces marxianus* DMKU 3-1042; *Kluyveromyces marxianus* DMKU 3-118; stress-resistant mutant; pseudo-hyphae formation; *SWI5*

Citation: Lertwattanasakul, N.; Pattanakittivorakul, S.; Nitiyon, S.; Matsutani, M.; Oguchi, A.; Hirata, K.; Kosaka, T.; Limtong, S.; Yamada, M. Mutants with Enhanced Multi-Stress Tolerance of *Kluyveromyces marxianus* and Their Ability for Ethanol Fermentation. *Fuels* **2023**, *4*, 469–483. https://doi.org/10.3390/fuels4040029

Academic Editor: Javier Ereña

Received: 7 August 2023
Revised: 27 October 2023
Accepted: 23 November 2023
Published: 30 November 2023

1. Introduction

Fossil energy sources, such as oil and coal, are being increasingly depleted, resulting in increases in oil prices and impacts on energy security and economic development. The use of fossil fuels is causing global warming due to CO_2 emissions. Therefore, renewable, sustainable, and low-cost energy alternatives to fossil fuels are needed [1]. Bioethanol is one of the clean and sustainable alternatives. However, the supply of starch-based biomass, which is relatively easy to convert to ethanol, is approaching its limit. There is a need for the use of cellulosic biomass, but the high cost of pretreatment and saccharifying enzymes is a problem. High-temperature fermentation (HTF) to produce bioethanol is attracting attention because it can reduce the costs of cooling, saccharifying enzymes used in simultaneous saccharification and fermentation, and equipment [2]. Thermotolerant

fermenting microorganisms are essential for HTF, but stable fermentation at high temperatures requires the use of microorganisms that are resistant not only to temperature but also to toxic substances present in the fermentation broth and have high ethanol productivity.

Some thermotolerant and ethanologenic yeasts have been isolated and used to produce ethanol from biomass. For instance, a newly isolated thermotolerant yeast strain, *Pichia kudriavzevii* (*Issatachenkia orientalis*) IPE100, produced ethanol with a theoretical yield of 85% per g of glucose at 42 °C [3]. A thermotolerant methylotrophic yeast, *Hansenula polymorpha* (*Ogataea augusta*), is capable of ethanol production from glucose, xylose, and cellobiose at elevated temperatures of 37 °C and higher [4]. *Candida glabrata* has been reported to have high stress tolerance to both acid and high temperatures, in addition to effective ethanol production capability [5]. The thermotolerant *S. cerevisiae* TJ14 strain produced 40 g L^{-1} of ethanol from 161 g L^{-1} of paper sludge organic material containing 66% (*w*/*w*) glucan in an SSF process at 42 °C using a cellulase produced by the filamentous fungus *Acremonium cellulolyticus* [6].

Kluyveromyces marxianus, which is one of the most thermotolerant yeasts, has several useful characteristics for 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 [7–11]. *K. marxianus* DMKU 3-1042 has been extensively studied for elucidating its thermotolerance and developing HTF [12–15].

During the fermentation process, yeast survival and ethanol production are affected by various types of stress, including high ethanol concentrations, high temperatures, high sugar concentrations, and acids produced by contaminated bacteria. Yeast cells, therefore, need to deal with and overcome such multiple stresses simultaneously [10].

In this study, in order to obtain a robust yeast strain that is resistant to stresses present in the fermentation environment, we adapted *K. marxianus* DMKU 3-1042 and DMKU 3-118 to different conditions: exposure to a high temperature and exposure to pulp wastewater, respectively. A high temperature tends to generate reactive oxygen species (ROS) via an increase in mitochondrial membrane fluidity [16], and ROS cause damage to DNA, lipids, and proteins [17]. Pulp wastewater contains toxic chemicals, including acids, furfural, and hydroxymethyl furfural, in addition to hexoses (such as glucose and mannose) and pentoses (such as xylose and arabinose) [18]. Surprisingly, both adapted mutants shared a similar phenotype, formation of pseudo-hyphae, suggesting that the formation of pseudo-hyphae is a common strategy for resistance to stresses. Genome sequencing analysis revealed a key gene responsible for the different stress tolerances. The results of this study may lead to the identification of targets for genetic engineering of *K. marxianus* to improve its tolerance to various stresses. The mutant strains obtained were found to be resistant to various stresses and have higher ethanol productivity than that of the corresponding parent strains under stress conditions. Therefore, the mutant strains are expected to be used for more stable HTF to produce ethanol and may also be applicable for the stable production of other useful materials.

2. Materials and Methods

2.1. Yeast Strains and Media

The yeast strains used in this study were *K. marxianus* strains DMKU 3-1042 and DMKU 3-118 [7] and their derivatives, KMR1042 and KMR118. Cells were grown in YPD medium (10 g L^{-1} of yeast extract, 20 g L^{-1} of peptone, and 20 g L^{-1} of glucose) at 30 °C for pre-culture and at 30 °C and 45 °C for culture under a shaking condition at 160 rpm.

2.2. Screening of Mutants

To obtain mutants that are resistant to stresses, two strategic approaches were used for two *K. marxianus* strains, DMKU 3-1042 and DMKU-118. For *K. marxianus* DMKU 3-1042, cells were spread on about 100 YPD agar plates and incubated at 45 °C for 12 days. During incubation, all plates were sealed by parafilm to prevent the agar plates from drying out.

The temperature and incubation time were chosen because we noticed that colonies on YPD agar plates turned from white to brown after about 10 days at 45 °C, presumably causing cell damage. Only one white colony was found in this screening, which was used for further experiments. For *K. marxianus* DMKU 3-118, cells were adapted in pulp wastewater (Nippon Paper Industries CO., Ltd., Tokyo Japan) by serial subculture 52 times at 37 °C. Then, 5 g L^{-1} of polypeptone, 2 g L^{-1} of yeast extract, and 1 g L^{-1} of KH_2PO_4 were added to pulp wastewater, and the pH was adjusted to 5.8. A half volume of *K. marxianus* cells grown in 3 mL of YPD medium at 37 °C for 24 h was harvested and resuspended in 3 mL of adjusted pulp wastewater and then incubated at 37 °C for 24 h under a shaking condition of 160 rpm. The temperature was chosen because cell viability had greatly reduced at temperatures above 37 °C, probably due to the toxicity of the pulp wastewater. After repeating this process and observing colony morphology, a colony different from the wild type appeared on the 52nd attempt and was selected.

2.3. Preparation of Genomic DNA, Genome Sequencing, and Determination of Mutations

The genomic DNA of mutated strains was isolated, as described previously [19], from cultured cells and further purified using a Genomic-tip 20 kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genome library construction for KMR118, KMR1042, and DMKU 3-118 was performed using a Twist 96-plex library preparation kit (Twist Bioscience, San Francisco, CA, USA) according to the manufacturer's instructions. Genome sequencing of the three strains was performed using the Illumina NextSeq 1000 and Illumina HiSeq 2500 platforms (Hokkaido System Science Co., Ltd., Hokkaido, Japan), respectively. Genome sequencing was performed by a massively parallel sequencer (MiSeq; Illumina KK, Tokyo, Japan), as reported previously [20]. The sequenced reads were selected by a quality score higher than the Phred score of 30 and were trimmed 12 bases from the 50 end and 20 bases from the 30 end. Truncated reads of less than 150 bases or with ambiguous nucleotides were not used for further analysis.

The reference genome sequence for genome mapping analysis of *K. marxianus* DMKU 3-1042 (GenBank acc. No: AP012213–AP012221) was downloaded from the NCBI ftp site: https://ftp.ncbi.nlm.nih.gov/ (accessed on 28 March 2020). The Illumina sequence reads of these strains were mapped to the reference sequences using BWA with default parameters [21]. All single-nucleotide polymorphisms (SNPs) and indels were detected using the Genome Analysis Toolkit (GATK) [22,23]. Critical mutations, including mutations of *KLMA_50490* in KMR118 and KMR1042, were confirmed by the Sanger method [24]. The 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)).

2.4. Cell Morphology and Sedimenting Ability

For observation of cell morphology, cells were grown in 30 mL of YPD medium at 30 °C for 18 h under a shaking condition of 160 rpm, harvested by low-speed centrifugation, washed once with saline, suspended in saline, and subjected to observation under a microscope (E600, Nikon, Tokyo, Japan) at ×400 magnification. For testing the sedimenting ability, cells were grown in 30 mL of YPD medium at 30 °C or 45 °C for 18 h under a shaking condition of 160 rpm, harvested by low-speed centrifugation, washed once with 0.1 M EDTA, and suspended in 0.1 M EDTA to adjust OD_{660} to 40 and 80 units for the mutant and wild-type strains, respectively. The ratio of OD_{660} of the mutant and wild-type strains was based on a standard curve of cell dry weight and OD_{660}. The sedimenting test was started (0 min) after the cell suspension in a vial had been mixed by a vortex mixer. The sedimenting abilities were compared by taking photos at 5 min, 10 min, and 20 min. DMKU 3-1042 was mainly used as a control because DMKU 3-1042 and DMKU 3-118 showed nearly the same rates of sedimentation. The rate of sedimentation indicates the level of cell numbers in the pseudo-hyphae. If a mutant sediments quickly, it indicates that pseudo-hyphae are formed by many cells.

2.5. Analysis of Stress Tolerance

To prepare an inoculum, cells were grown at 30 °C for 18 h at 160 rpm in YPD medium, harvested, washed with distilled water, and resuspended in distilled water. The cell suspension was adjusted to an OD_{660} of 1.0 and then 10-fold sequentially diluted and spotted onto YPD agar plates supplemented with individual chemical compounds of (1) 1 g L^{-1} of vanillin, (2) 100 g L^{-1} or 250 g L^{-1} of glucose, (3) 60 g L^{-1} or 90 g L^{-1} of ethanol, (4) 1 g L^{-1} or 2 g L^{-1} of acetic acid, (5) 15 mM or 20 mM of furfural, and (6) 4 mM, 8 mM, or 16 mM of hydrogen peroxide (H_2O_2). The concentrations of stress chemicals were chosen based on previous stress studies on *K. marxianus* [9,25]. Glucose was used as a carbon source because it is the main sugar derived from most biomass. To minimize the effect of temperature on cell growth, the plates were incubated at 30 °C for 48 h. In addition, the effect of temperature was examined on YPD agar plates using the inoculum, prepared as described above. The plates were incubated at 30 °C, 48 °C, or 49 °C for 48 h. Each experiment was performed at least three times and similar results were obtained.

2.6. Analysis of Ethanol Fermentation Ability

To investigate the ethanol fermentation ability of mutants and the corresponding parents, YP medium (10 g L^{-1} of yeast extract and 20 g L^{-1} of peptone) supplemented with 160 g L^{-1} of glucose was used. Cells were precultured in 30 mL of YPD medium in a 100 mL Erlenmeyer flask sealed with aluminum foil at 30 °C under a shaking condition at 160 rpm for 18 h. The preculture was inoculated to 30 mL of fresh YP medium supplemented with 160 g L^{-1} of glucose in a 100 mL Erlenmeyer flask at the initial OD_{660} of 0.1, and incubation was carried out at the appropriate temperature under a shaking condition at 100 rpm. Samples were taken at the indicated times until 36 h or 48 h of incubation. Yeast growth and concentrations of glucose, ethanol, and acetate were determined. To examine the effects of mixed chemical compounds on growth and ethanol fermentation ability, cells precultured as described above were grown in a YP medium containing 20 g L^{-1} or 100 g L^{-1} of glucose, 0.5 g L^{-1} of vanillin, 1 g L^{-1} of acetic acid, and 10 mM of furfural under a shaking condition at 40 °C. The concentrations of the three toxic substances were chosen because preliminary experiments showed that both parental strains of the mutants were able to grow at 40 °C in YPD medium containing 1 g L^{-1} of vanillin, 2 g L^{-1} of acetic acid, or 20 mM of furfural, but not in YPD medium containing the same concentrations of all three.

Yeast growth was determined by measuring the optical density at 660 nm on a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). To determine glucose and ethanol concentrations in culture media, cultures were collected and centrifuged at 14,000 rpm for 5 min. The supernatant was passed through a 0.45 μm filter (Pall Corporation, Tokyo, Japan) and then subjected to quantitative analysis of glucose, ethanol, and acetate on a high-performance liquid chromatography (HPLC) system (Hitachi, Tokyo, Japan), consisting of a Hitachi Model D-2000 Elite HPLC system Manager, column oven L-2130, pump L-2130, auto-sampler L-2200, and RI detector L-2490, equipped with a GL-C610-S gel pack column (Hitachi) in the mode of 0.5 mL/min eluent of deionized water at 60 °C.

2.7. Sequence Data Deposition

Illumina sequence reads of the KMR118 and KMR1042 strains and the DMKU 3-118 strain were deposited in the DDBJ Sequence Read Archive. The DRR accession numbers for KMR118, KMR1042, and DMKU 3-118 are DRR495714, DRR495716, and DRR495715, respectively.

3. Results

3.1. Stress-Resistant Mutants from K. marxianus Strains DMKU 3-1042 and DMKU 3-118

To improve the performance of *K. marxianus* for ethanol fermentation under a multi-stress condition, two wild-type strains of *K. marxianus*, DMKU 3-1042 and DMKU 3-118,

were exposed to different stresses: high temperature and drying by evaporation of water on agar plates and toxic substances in pulp wastewater, respectively. Our previous observations revealed that the colony color of DMKU 3-1042 after incubation for several days at 45 °C on YPD agar plates changed from white to dark brown in color, leading to cell death. Therefore, we spread *K. marxianus* DMKU 3-1042 cells on YPD agar plates (~100 colonies per plate) and incubated them at 45 °C for 12 days. A white colony, named KMR1042, was obtained after the incubation and was used for further experiments. On the other hand, the strain DMKU 3-118 was serially sub-cultured in pulp wastewater 52 times and an adapted strain, named KMR118, was obtained. Macroscopic observation revealed that the colony edges of both mutants on YPD plates were rough, and microscopic observation revealed that the two mutants formed pseudo-hyphae in YPD media (Figure 1).

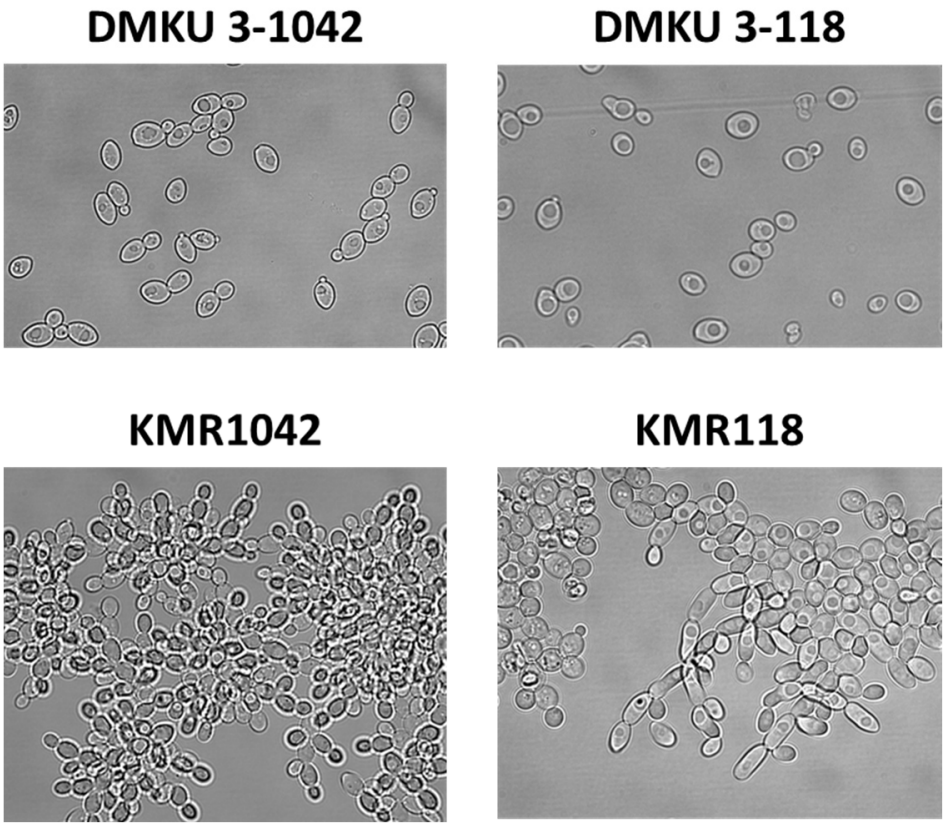

Figure 1. Morphological observation of adapted mutants and their parents. After growing in YPD medium at 30 °C for 18 h under a shaking condition of 160 rpm, the cells were subjected to morphological observation at ×400 magnification. Cell preparation and other details are described in the Section 2.

3.2. Sedimenting Ability of KMR1042 and KMR118

In the DMKU 3-1042 and DMKU 3-118 background, KMR1042 and KMR118 exhibited a defect in cell separation, resulting in clumpy clusters of cells and altered colony morphology. To quantify the cell separation defects, we tested the cell sedimenting ability of both mutants. Two different concentrations of cells that were grown at 30 °C and 45 °C were tested. As a result, both cells were rapidly sedimented compared to the sedimentation of the corresponding parent cells, and the sedimentation rates of both mutants were almost the same, and sedimentation was almost completed within 10 min (Figure 2).

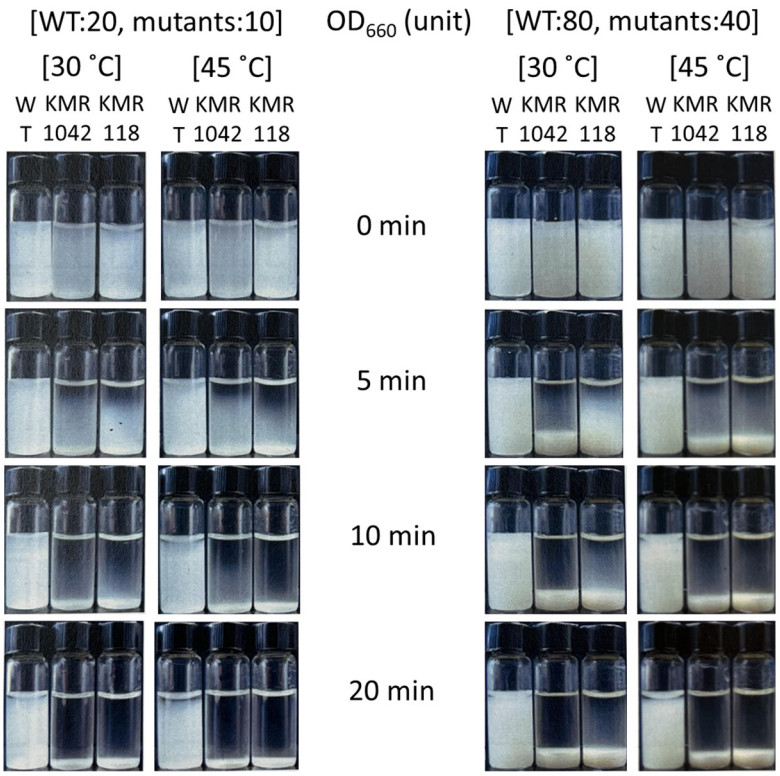

Figure 2. Sedimenting ability of adapted mutants and their parents. After growing in YPD medium at 30 °C or 45 °C for 18 h under a shaking condition of 160 rpm, the cells were subjected to a sedimentation test. Cell preparation and other details are described in the Section 2.

3.3. Stress Tolerance of KMR1042 and KMR118

During a fermentation process, yeast cells are generally exposed to various stresses, including heat stress, osmotic stress, acid stress, and ethanol stress, all of which have severe effects on cell viability and ethanol production [26,27]. In addition, cells during HTF tend to generate oxidative stress [28]. The degree of stress tolerance of the two mutants, KMR1042 and KMR118, was thus compared with that of the corresponding parents. At 48 °C and 49 °C, both mutants had much better growth than that of their parents (Figure 3). For osmotic stress, the two mutants and their parents showed similar levels of growth in the presence of 100 g L^{-1} of glucose, but the growth of both mutants was better than that of their parents in the presence of 250 g L^{-1} of glucose. For ethanol stress, KMR1042 and its parent showed similar levels of growth in the presence of 60 g L^{-1} and 90 g L^{-1} of ethanol. However, the mutant KMR118 showed a level of growth similar to that of its parent in the presence of 60 g L^{-1} of ethanol but grew much better than its parent in the presence of 90 g L^{-1} of ethanol. For oxidative stress, the two mutants grew in the presence of 8 mM of H_2O_2, but only KMR1042 could grow in the presence of 16 mM of H_2O_2. It is known that toxic substances, including acetic acid, furfural, and vanillin, that are generated in the process of hydrolysis of lignocellulosic materials, prevent the growth or fermentation ability of microorganisms [29,30]. Cells were thus spotted onto YPD agar plates supplemented with 1 g L^{-1} of vanillin or 1 g L^{-1} and 2 g L^{-1} of acetic acid or 15 mM and 20 mM of furfural, and the effects of the toxic substances on cell growth were evaluated after incubation at 30 °C for 48 h (Figure 3). The two mutants showed better growth than that of their parents. These findings suggest that KMR1042 and KMR118 are more suitable for ethanol fermentation under the conditions of various stresses.

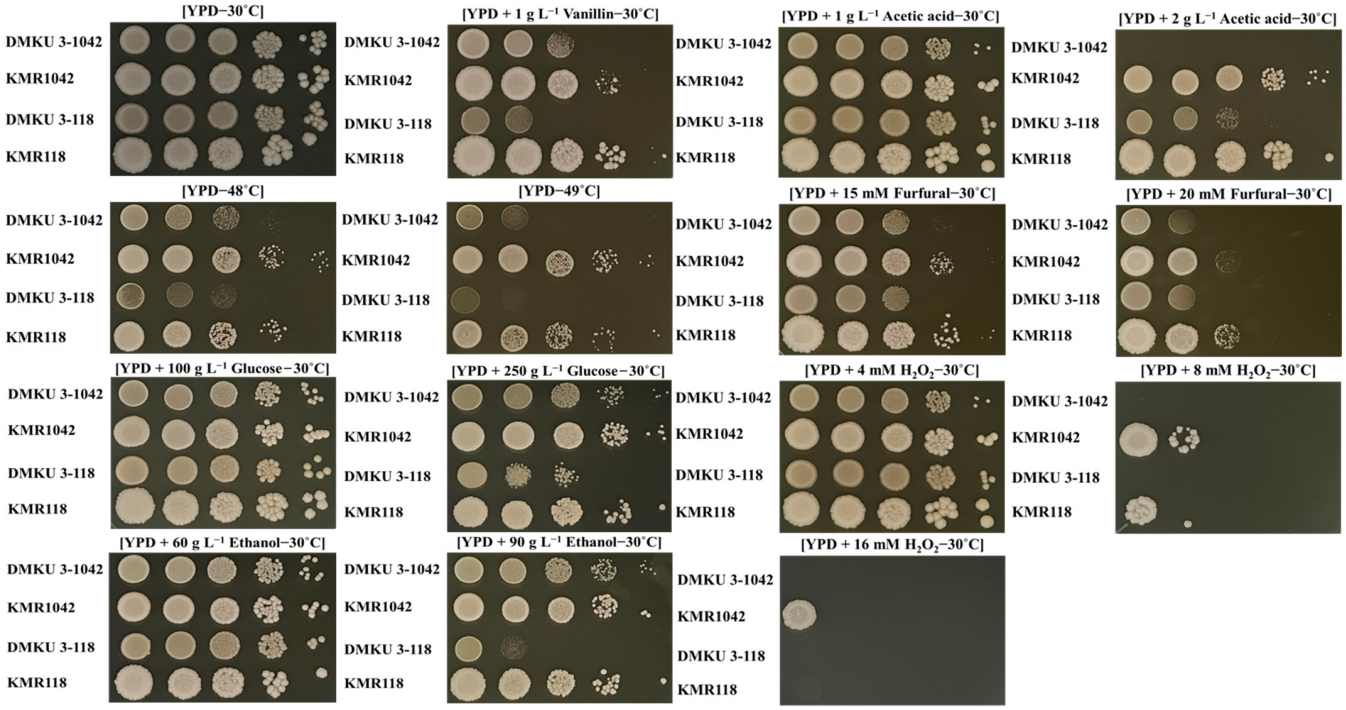

Figure 3. Tolerance of adapted mutants and their parents to various stresses. After growing in YPD medium at 30 °C for 18 h under a shaking condition of 160 rpm, the cells were subjected to a stress tolerance test. Cell preparation and other details are described in the Section 2.

3.4. Ethanol Fermentation Abilities of KMR1042 and KMR118 under Stress Conditions

To examine the fermentation abilities of KMR1042 and KMR118 at high temperatures, the cells were grown in a YP medium containing 160 g L^{-1} of glucose under a shaking condition at 45 °C, and the cell growth and concentrations of glucose and ethanol in the medium were compared with those of the parents (Figure 4). The temperature and sampling times were chosen based on the fermentation evaluation used with other mutants [25]. The turbidity of all strains tested increased with the decrease in the glucose concentration, but the turbidity of the two mutants was much lower than that of their parents. The lag phase was found in the first 6 h in all strains. Ethanol levels became nearly maximal at 24 h of incubation, except in the case of DMKU 3-118, for which the ethanol level was nearly maximal around 30 h. Both mutants showed higher ethanol concentrations with higher ethanol productivities than those of the parents (Table 1). Maximum ethanol concentrations produced by the KMR1042 and KMR118 strains were 51.3 ± 3.4 g L^{-1} and 52.3 ± 2.5 g L^{-1}, respectively, which were 5% and 37% higher, respectively, than those produced by their corresponding parents. KMR1042 produced slightly less acetate than that produced by its parent, while KMR118 produced more acetate than that produced by its parent.

Table 1. Comparison of growth and fermentation parameters in YP containing 160 g L^{-1} of glucose at 45 °C among two adapted strains and their corresponding parents.

Strains	Cultivation Time (h)	Dry Cell Weight (g L^{-1})	Acetate Accumulation (g L^{-1})	Ethanol Production (g L^{-1})	Increased Ethanol (%)	Ethanol Productivity (g L^{-1} h^{-1})	Ethanol Yield (g g^{-1})
DMKU 3-1042	24	64.5 ± 0.5	1.8 ± 0.0	48.7 ± 0.9	-	2.0 ± 0.0	0.3 ± 0.0
KMR1042	24	27.4 ± 1.7	1.6 ± 0.1	51.3 ± 3.4	5	2.1 ± 0.1	0.3 ± 0.0
DMKU 3-118	24	60.1 ± 1.5	1.1 ± 0.1	38.1 ± 3.1	-	1.6 ± 0.1	0.2 ± 0.0
KMR118	24	31.2 ± 2.2	1.7 ± 0.1	52.3 ± 2.5	37	2.2 ± 0.1	0.3 ± 0.0

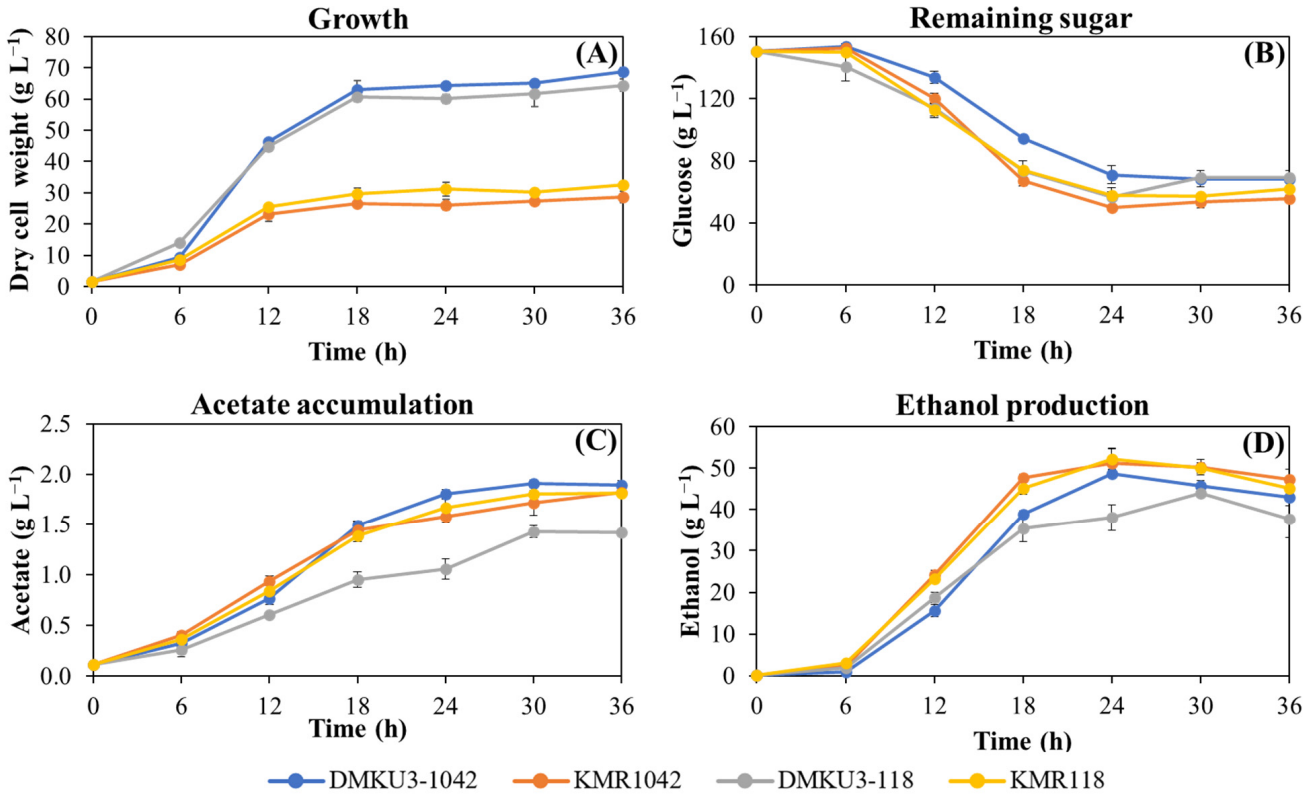

Figure 4. Growth (**A**) and ethanol fermentation of adapted mutants and their parents at 45 °C. Cells precultured in YPD medium at 30 °C for 18 h were inoculated into YP medium containing 160 g L^{-1} of glucose and cultured under a shaking condition of 160 rpm at 45 °C. Samples were taken at the times indicated, and glucose (**B**), acetate (**C**), and ethanol (**D**) concentrations were determined as described in the Section 2. Error bars represent ±SD of values from experiments performed in triplicate.

The fermentation abilities of KMR1042 and KMR118 were also examined in the presence of toxic substances, according to a previous study in which other strains were used [25]. Cells were grown in a YP medium containing 20 g L^{-1} of glucose, 0.5 g L^{-1} of vanillin, 1 g L^{-1} of acetic acid, and 10 mM of furfural, or in a YP medium containing 100 g L^{-1} of glucose, 0.5 g L^{-1} of vanillin, 1 g L^{-1} of acetic acid, and 10 mM of furfural under a shaking condition at 40 °C (Figure 5). In the case of 20 g L^{-1} of glucose with toxic substances, the growth of KMR1042 and KMR118 was similar to that of their parent counterparts, though their turbidity levels were lower than those of their parents. Ethanol accumulation of both mutants started earlier than that of their parents. On the other hand, in the case of 100 g L^{-1} of glucose with toxic substances, both mutants showed much faster growth and ethanol accumulation than those of their parents. Maximum ethanol concentrations produced by the KMR1042 and KMR118 strains were 9.9 ± 0.6 g L^{-1} and 7.5 ± 0.1 g L^{-1}, respectively, which were 320% and 750% higher, respectively, than those produced by their corresponding parents (Table 2). These findings and those described above suggest that KMR1042 and KMR118 are robust strains against various stresses and may have a potential for ethanol fermentation at high temperatures or in the presence of toxic substances for industrial applications.

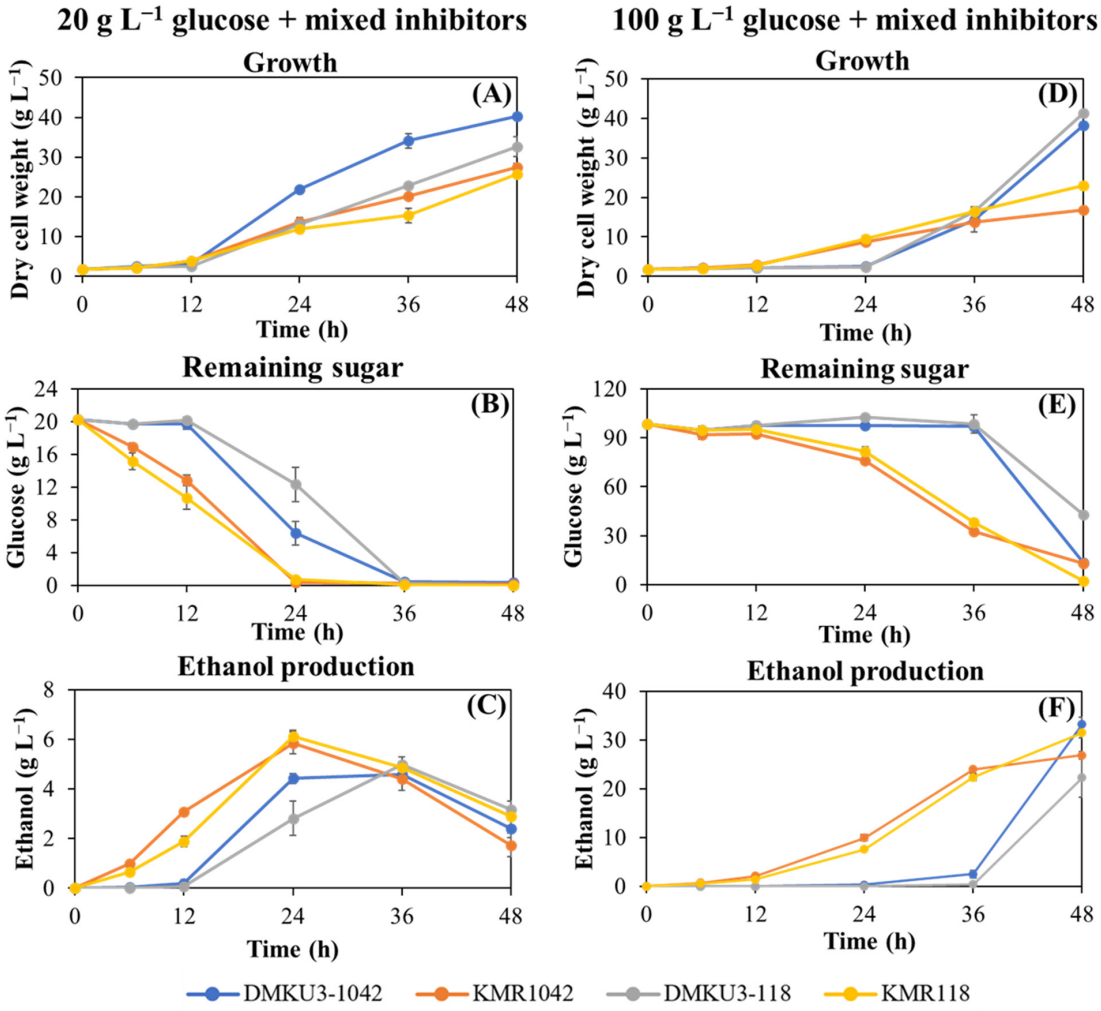

Figure 5. Growth (**A**,**D**) and ethanol fermentation of adapted mutants and their parents under multi-stress conditions. Cells precultured in YPD medium at 30 °C for 18 h were inoculated into YP medium containing 20 g L^{-1} or 100 g L^{-1} of glucose supplemented with 0.5 g L^{-1} of vanillin, 1 g L^{-1} of acetic acid, and 10 mM of furfural and cultured under a shaking condition of 160 rpm at 40 °C. Samples were taken at the times indicated, and glucose (**B**,**E**) and ethanol (**C**,**F**) concentrations were determined as described in the Section 2. Error bars represent ±SD of values from experiments performed in triplicate.

Table 2. Comparison of growth and fermentation parameters in YP containing 20 or 100 g L^{-1} in the presence of mixed inhibitors at 40 °C among two adapted strains and their corresponding parents.

Strains	Cultivation Time (h)	Dry Cell Weight (g L^{-1})	Acetate Accumulation (g L^{-1})	Ethanol Production (g L^{-1})	Increased Ethanol (%)	Ethanol Productivity (g L^{-1} h^{-1})	Ethanol Yield (g g^{-1})
YP containing 20 g L^{-1}							
DMKU 3-1042	24	21.8 ± 0.4	1.1 ± 0.0	4.4 ± 0.2	-	0.2 ± 0.0	0.2 ± 0.0
KMR1042	24	13.7 ± 1.1	1.1 ± 0.2	5.8 ± 0.4	32	0.2 ± 0.0	0.3 ± 0.0
DMKU 3-118	24	13.0 ± 0.5	1.1 ± 0.1	2.8 ± 0.7	-	0.1 ± 0.0	0.1 ± 0.0
KMR118	24	12.0 ± 1.1	1.2 ± 0.0	6.1 ± 0.2	118	0.3 ± 0.0	0.3 ± 0.0
YP containing 100 g L^{-1}							
DMKU 3-1042	24	2.5 ± 0.1	1.5 ± 0.4	0.3 ± 0.0	-	0.0 ± 0.0	0.0 ± 0.0
KMR1042	24	8.8 ± 0.2	1.3 ± 0.0	9.9 ± 0.6	320	0.4 ± 0.0	0.1 ± 0.0
DMKU 3-118	24	2.3 ± 0.0	1.3 ± 0.0	0.1 ± 0.0	-	0.0 ± 0.0	0.0 ± 0.0
KMR118	24	9.5 ± 0.8	1.2 ± 0.1	7.5 ± 0.1	750	0.3 ± 0.0	0.1 ± 0.0

3.5. Analysis of Mutation Points of KMR1042 and KMR118

Genome sequencing was performed to identify the mutations of the two mutants (Table 3). As a result, KMR1042 and KMR118 were shown to have four and nine mutation points in protein-coding regions, respectively. The mutations in KMR1042 are a single-nucleotide polymorphism (SNP) in the coding region of *TVP38* for Golgi apparatus membrane protein Tvp38, causing a synonymous mutation, deletion of ten nucleotides in the coding region of *SWI5* for metallothionein expression activator, causing a frame-shift mutation, insertion of one nucleotide in the coding region of *KLMA_80154* for putative zinc metalloproteinase Yil108w, causing a frame-shift mutation, and an SNP in the coding region of *UPS2* for protein Msf1, causing a missense mutation. In KMR118, there are three synonymous mutations, three missense mutations, one frame-shift mutation, one nonsense mutation, and one six-nucleotides insertion. The synonymous mutations occur in *SRV2* for adenylyl cyclase-associated protein, *KLMA_10608* for hypothetical protein, and *HPR1* for THO complex subunit Hpr1. Of the three missense mutations, two occur in *HKR1* for herpes_gp2 and one occurs in *KLMA_70080* for conserved hypothetical protein. The frame-shift and nonsense mutations occur in *HKR1* and *SWI5*, respectively. The six-nucleotides insertion occurs in *IXR1* for HMGB-UBF_HMG-box-containing protein, causing two amino acids' insertion. Interestingly, both mutants were found to have mutations in the same gene, *KLMA_50490*, for the metallothionein expression activator as an orthologue of *SWI5* in *S. cerevisiae*. The *KLMA_50490* mutations in KMR1042 and KMR118 caused a frame-shift mutation and a nonsense mutation, respectively, by the insertion of ten nucleotides and nucleotide substitution, respectively. Since there are no other overlapping gene mutations and disruption of the *SWI5* homologue causes formation of pseudo-hyphae in *S. cerevisiae* (see Section 4), the mutations of *KLMA_50490* shared by the two mutants may be responsible for exhibiting a similar colony morphology, pseudo-hyphae formation, and tolerance phenotype against multi-stress. The shared disruption mutations may also contribute to the significant increase in ethanol fermentation at high temperatures. Since the two mutants had nearly identical phenotypes, it is possible that additional mutations in KMR1042 and KMR118 contributed little to the phenotypes.

Table 3. List of mutation sites in *K. marxianus* mutant strains, KMR1042 and KMR118.

Locus_Tag	Gene	Product	Reference Genome (NCBI Ref Seq Acc. No.)	Position	REF	ALT	AA Sub
KMR1042							
KLMA_10406	TVP38	Golgi apparatus membrane protein TVP38	AP012213.1	858,831	C	T	Val158Val
KLMA_50490	SWI5	metallothionein expression activator	AP012217.1	1,055,034	TACCAC CATTA	T	Asn320fs [(a)]
KLMA_50562	UPS2	protein MSF1	AP012217.1	1,204,779	C	T	Asp37Asn
KLMA_80154	-	putative zinc metalloproteinase YIL108W	AP012220.1	339,946	C	CG	Ala660fs [(a)]
KMR118							
KLMA_10572	SRV2	adenylyl cyclase-associated protein	AP012213.1	1,202,978	G	A	Pro65Pro
KLMA_10608	-	hypothetical protein	AP012213.1	1,272,508	G	T	Thr373Thr
KLMA_20191	HPR1	THO complex subunit HPR1	AP012214.1	431,928	C	A	Ile202Ile
KLMA_30667	HKR1	herpes_gp2	AP012215.1	1,429,635	C	G	Pro158Ala
KLMA_30667	HKR1	herpes_gp2	AP012215.1	1,429,636	C	A	Pro158His
KLMA_30667	HKR1	herpes_gp2	AP012215.1	1,429,661	A	AC	Asp168fs [(a)]
KLMA_50490	SWI5	metallothionein expression activator	AP012217.1	1,053,571	G	T	Tyr810stop
KLMA_60329	IXR1	HMGB-UBF_HMG-box-containing protein	AP012218.1	691,365	A	AGCTTGG	Ala173_ Gln174ins [(a)]
KLMA_70080	-	conserved hypothetical protein	AP012219.1	159,037	G	A	Ala417Thr

[(a)] fs: frame-shift mutation; ins: insertion.

4. Discussion

Evolutionary adaptation has contributed to improved substrate utilization efficiency [31–34], functional implementation of alternative pathways [35,36], and increased tolerance to toxic substances [33,37] in various organisms. In this study, we performed adaptation of two *K. marxianus* strains under two different stress conditions to establish much more stable HTF, and we obtained one mutant from each adaptation. Remarkably, both adapted mutants (KMR1042 and KMR118) exhibited similar phenotypes despite the different adaptation conditions, forming pseudo-hyphae (Figure 1) and sedimenting much faster than the respective parent strains (Figure 2). The latter phenotype may be useful for cell removal from fermentation broths in industrial applications. Both mutants also showed tolerance to multi-stress, including inhibitors, and achieved high ethanol production at high temperatures (Figure 5 and Table 2). Interestingly, analysis of the mutants revealed that adaptation to one type of stress increases tolerance to other types of stress [38]. KMR1042 and KMR118 adapted mainly to temperature and toxic substances in pulp wastewater, respectively, but both were resistant to high temperatures and various inhibitors (Figure 3). This phenomenon allows us to speculate that different stresses induce common cellular responses, such as modulation of energy metabolism, expression of common protective proteins (such as heat shock proteins), and production of small protective molecules (compatible solutes, such as glycerol and trehalose). Moreover, different stress responses may share certain factors to induce common responses. This important part of the stress response is referred to as the yeast general or environmental stress response [39].

The relationship between pseudo-hyphae formation and stress tolerance, which was observed in both mutants obtained (Figures 1 and 3), is very interesting. Single-cell organisms, such as yeasts, are known to be capable of differentiation and forming specialized cell types that exist either as individuals or as constituents of organized multicellular populations [40]. Cell differentiation to opposite mating types and switching from yeast form to filamentous form (hyphae or pseudo-hyphae) are examples of individual yeast cell differentiation. The spatial positioning of cells within the filamentous structure allows the formation of gradients of metabolites, signaling molecules, and waste products, all of which may participate in cell diversification and specialization. It is possible that such differentiation in the filamentous structure enhances stress tolerance.

Yeast cells are generally exposed to various stresses in the culture environment, and the effects of these stresses appear to be additive (or synergistic) [25]. In this study, the ethanol fermentation ability of KMR1042 and KMR118 in the presence of mixed inhibitors, including vanillin, acetic acid, and furfural, was improved compared to that of their respective parents (Figure 5), but the ethanol yield when 100 g L^{-1} of glucose was used as a substrate was much lower than that when 20 g L^{-1} of glucose was used (Table 2), probably due to the osmotic pressure caused by glucose. Ethanol produced is also a stress, and when combined with other stresses can increase stress levels and cause a decrease in glucose consumption. Acetic acid is a main by-product, which increases at high temperatures in *K. marxianus* DMKU 3-1042 [25], and its accumulation becomes a stress. The acetate concentration of the two mutants, KMR1042 and KMR118, was found to be lower than that of the respective parents in YP containing 100 g L^{-1} of glucose with mixed inhibitors, but this was not the case with 20 g L^{-1} of glucose with mixed inhibitors (Table 2). In YP containing 160 g L^{-1} of glucose without mixed inhibitors, the acetate accumulation of KMR1042 and that of KMR118 were lower and higher than that of their parents, respectively (Table 1). The higher accumulation of acetate in KMR118 may be due to higher production of ethanol, which is converted to acetic acid, under the tested conditions.

The two mutants acquired in this study have disrupted mutations in the same gene, *KLMA_5049*, which is an orthologue of *SWI5* in *S. cerevisiae*, and mutations in the same gene may be responsible for the similar phenotypes, including the formation of pseudo-hyphae, as described above. In *S. cerevisiae*, Ace2p, which shares 37% identity with Swi5p, regulates the expression of several genes during the late M and early G1 stages of the mitotic cell cycle [41,42]. Targets of Ace2p are predominantly expressed in daughter cells

and include *CTS1* for chitinase and genes for proteins involved in separation from the mother cells [41,43,44]. Deletion of *ACE2* results in increased pseudo-hyphal growth and invasion of agar [45,46]. On the other hand, *SWI5* in *S. cerevisiae* encodes a transcription factor that activates the transcription of genes expressed at the M/G1 boundary and in the G1 phase of the cell cycle [47–49]. The DNA-binding domain of Swi5p is very similar to that of Ace2p [47], and both bind the same DNA sequences in vitro with similar affinities to regulate a shared set of genes in vivo [48,49]. However, the two regulators have distinct domains responsible for promoter-specific transcription activation and distinct promoter specificities in some genes; for example, transcription of the HO endonuclease gene is activated by Swi5p but not by Ace2p, and *CTS1* is activated by Ace2p but not by Swi5p.

Ace2p and Swi5p are conserved in species closely related to *S. cerevisiae*, including the opportunistic pathogenic yeast *Candida glabrata* [50]. The *Candida albicans* genome, however, contains only a single gene orthologue (*C. albicans ACE2*), the product of which is equidistant from both the *S. cerevisiae* proteins [51]. Deletion of *ACE2* in *C. albicans* results in gross attenuation of virulence of the fungus in a mouse model of disseminated infection [51]. In contrast, deletion of *ACE2* in *C. glabrata* results in hypervirulence of the mutant strain in neutropenic mice [52] and in alterations in the expression of more than 60 proteins [53]. Mutants of *C. albicans*, *C. glabrata*, and *S. cerevisiae* with disruptions of *ACE2* and of *C. glabrata* and *S. cerevisiae* with disruptions of *SWI5* showed a clumping phenotype [54].

However, there has been no study on these genes in *K. marxianus*. A search in the NCBI database revealed that there is only one gene, *KLMA_50490,* for a protein that has high identities with *S. cerevisiae* Swi5p and Ace2p, 34% and 30% identities, respectively, in *K. marxianus*, and thus the *KLMA_50490*-encoded protein is an orthologue of *S. cerevisiae* Swi5p. A single orthologue of *S. cerevisiae* Swi5p or Ace2p may be enough in some yeasts, including *Kluyveromyces lactis* [55] and *Candida* spp. [56]. The *SWI5* disruption was found in both mutants, which were adapted to different stress conditions, suggesting that the *SWI5* mutation is one of the strategies of *K. marxianus* to cope with various stresses.

Altering transcriptional programs is a major strategy for microorganisms to adapt to their surrounding environment. In *S. cerevisiae*, the environmental stress response involves about 900 genes for which expression is coordinately altered upon exposure to different types of stress [57]. Transcription analysis revealed that environmental stresses, including heat, hyperosmolarity, and oxidative stress, and starvation stress induce a very similar pattern of regulated genes in *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces pombe*, and *C. glabrata,* which live in different environments [58–60].

We have almost no idea about the downstream from the *SWI5* disruption to cause multi-stress resistance in *K. marxianus*. However, if, as in the case of *S. cerevisiae* Swi5p, *K. marxianus* Swi5p activates transcription of genes expressed at the M/G1 boundary and in the G1 phase of the cell cycle, it is possible that the dysfunction of Swi5p causes cell cycle arrest at G1, which in turn provides time for repairing the cellular damage caused by stresses. Further studies downstream of Swi5p should lead to an understanding of the mechanisms of multi-stress resistance, and that knowledge will be useful for the use of resulting mutants for stable HTF.

5. Conclusions

Thermotolerant and multi-stress-tolerant yeast strains are needed for stable HTF as low-cost fermentation. In this study, we thus carried out two different procedures, and acquired one robust mutant from each. Surprisingly, both mutants exhibited similar phenotypes, including formation of pseudo-hyphae and multi-stress resistance, and had disruption of the same gene encoding an orthologue of *S. cerevisiae* Swi5p. It is, therefore, assumed that disruption of the gene is an effective mutation for a robust phenotype. Both mutants showed higher ethanol production at high temperatures under multi-stress conditions than that of their parents, suggesting that they are candidates for stable HTF.

Further studies downstream of Swi5p should lead to an understanding of the mechanism of multi-stress tolerance.

Author Contributions: N.L. and M.Y. organized the study and wrote the original manuscript; A.O. and K.H. performed adaptation to isolate mutants; S.P., S.N., A.O. and K.H. performed experiments and analyzed data; M.M. performed genome analysis; T.K. and S.L. gave advice and support for this project; M.Y. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Special Coordination Funds for Promoting Science and Technology, Ministry of Education, Culture, Sports, Science, and Technology (MEXT), and the Advanced Low-Carbon Technology Research and Development Program, Japan Science and Technology Agency (JST). This work was partially performed as collaborative research in the Asian Core Program and in the Core-to-Core Program, which was supported by the Scientific Cooperation Program agreed by the Japan Society for the Promotion of Science (JSPS), the National Research Council of Thailand (NRCT), the Ministry of Science and Technology in Vietnam, the National University of Laos, the University of Brawijaya, and Beuth University of Applied Science, Berlin. The research visit of N.L. to Japan was supported by the Research Center for Thermotolerant Microbial Resources, Yamaguchi University.

Data Availability Statement: All data are reported in this manuscript.

Acknowledgments: We thank Kazunobu Matsushita, Toshiharu Yakushi, and Naoya Kataoka for their helpful discussion and the NODAI Genome Research Center, Tokyo University of Agriculture, for the sequencing and its analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bai, F.W.; Anderson, W.A.; Moo-Young, M. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* **2008**, *26*, 89–105. [CrossRef] [PubMed]
2. Lertwattanasakul, N.; Rodrussamee, N.; Kumakiri, I.; Pattanakittivorakul, S.; Yamada, M. Potential of thermo-tolerant microorganisms for production of cellulosic bioethanol. In *The Handbook of Biorefinery Research and Technology*; Bisaria, V., Ed.; Springer: Dordrecht, The Netherlands, 2023; pp. 1–30.
3. Kwon, Y.J.; Ma, A.Z.; Li, Q.; Wang, F.; Zhuang, G.Q.; Liu, C.Z. Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia orientalis*. *Bioresour. Technol.* **2011**, *102*, 8099–8104. [CrossRef] [PubMed]
4. Ryabova, O.B.; Chmil, O.M.; Sibirny, A.A. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotorophic yeast *Hansenula polymorpha*. *FEMS Yeast Res.* **2003**, *4*, 157–164. [CrossRef] [PubMed]
5. Watanabe, I.; Nakamura, T.; Shima, J. Characterization of a spontaneous flocculation mutant derived from *Candida glabrata*: A useful strain for bioethanol production. *J. Biosci. Bioeng.* **2009**, *107*, 379–382. [CrossRef] [PubMed]
6. Prasetyo, J.; Naruse, K.; Kato, T.; Boonchird, C.; Harashima, S.; Park, E.Y. Bioconversion of paper sludge to biofuel by simultaneous saccharification and fermentation using a cellulase of paper sludge origin and thermotolerant *Saccharomyces cerevisiae* TJ14. *Biotechnol. Biofuels* **2007**, *4*, 35. [CrossRef] [PubMed]
7. 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]
8. 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]
9. 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]
10. 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]
11. 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.
12. 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]
13. Lertwattanasakul, N.; Rodrussamee, N.; Suprayogi; Limtong, S.; Thanonkeo, P.; Kosaka, T.; Yamada, M. Utilization capability of sucrose, raffinose and inulin and its less-sensitiveness to glucose repression in thermotolerant yeast *Kluyveromyces marxianus* DMKU 3-1042. *AMB Expr.* **2011**, *1*, 20. [CrossRef]

14. 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 Leeuwenhoek* **2013**, *103*, 933–945. [CrossRef]
15. 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]
16. Tarrío, N.; García-Leiro, A.; Cerdán, M.E.; González-Siso, M.I. The role of glutathione reductase in the interplay between oxidative stress response and turnover of cytosolic NADPH in *Kluyveromyces lactis*. *FEMS Yeast Res.* **2008**, *8*, 597–606. [CrossRef]
17. Auesukaree, C. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. *J. Biosci. Bioeng.* **2017**, *124*, 133–142. [CrossRef]
18. Jönsson, L.J.; Alriksson, B.; Nilvebrant, N.-O. Bioconversion of lignocellulose: Inhibitors and detoxification. *Biotechnol. Biofuels* **2013**, *6*, 16. [CrossRef]
19. Sambrook, J.; Russell, D.W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2008; Volume 1.
20. 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] [PubMed]
21. Li, H.; Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics.* **2010**, *26*, 589–595. [CrossRef] [PubMed]
22. DePristo, M.A.; Banks, E.; Poplin, R.; Garimella, K.V.; Maguire, J.R.; Hartl, C.; Philippakis, A.A.; del Angel, G.; Rivas, M.A.; Hanna, M.; et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* **2011**, *43*, 491–498. [CrossRef] [PubMed]
23. Matsutani, M.; Matsumoto, N.; Hirakawa, H.; Shiwa, Y.; Yoshikawa, H.; Okamoto-Kainuma, A.; Ishikawa, M.; Kataoka, N.; Yakushi, T.; Matsushita, K. Comparative genomic analysis of closely related *Acetobacter pasteurianus* strains provides evidence of horizontal gene transfer and reveals factors necessary for thermotolerance. *J. Bacteriol.* **2020**, *202*, e00553-19. [CrossRef] [PubMed]
24. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463–5467. [CrossRef] [PubMed]
25. Pattanakittivorakul, S.; Tsuzuno, T.; Kosaka, T.; Murata, M.; Kanesaki, Y.; Yoshikawa, H.; Limtong, S.; Yamada, M. Evolutionary adaptation by repetitive long-term cultivation with gradual increase in temperature for acquiring multi-stress tolerance and high ethanol productivity in *Kluyveromyces marxianus* DMKU 3-1042. *Microorganisms* **2022**, *10*, 798. [CrossRef] [PubMed]
26. Gibson, B.R.; Lawrence, S.J.; Leclaire, J.P.; Powell, C.D.; Smart, K.A. Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol. Rev.* **2007**, *31*, 535–569. [CrossRef] [PubMed]
27. Puligundla, P.; Smogrovicova, D.; Obulam, V.S.R.; Ko, S. Very high gravity (VHG) ethanolic brewing and fermentation: A research update. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1133–1144. [CrossRef] [PubMed]
28. Zhang, M.; Shi, J.; Jiang, L. Modulation of mitochondrial membrane integrity and ROS formation by high temperature in *Saccharomyces cerevisiae*. *Electron. J. Biotechnol.* **2015**, *18*, 202–209. [CrossRef]
29. Mussatto, S.I.; Roberto, I.C. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. *Bioresour. Technol.* **2004**, *93*, 1–10. [CrossRef]
30. Behera, S.; Arora, R.; Nandhagopal, N.; Kumar, S. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renew. Sustain. Energy Rev.* **2014**, *36*, 91–106. [CrossRef]
31. Wisselink, H.W.; Toirkens, M.J.; Wu, Q.; Pronk, J.T.; van Maris, A.J.A. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. *Appl. Environ. Microbiol.* **2009**, *75*, 907–914. [CrossRef]
32. Kuyper, M.; Toirkens, M.J.; Diderich, J.A.; Winkler, A.A.; van Dijken, J.P.; Pronk, J.T. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res.* **2005**, *5*, 925–934. [CrossRef]
33. Guadalupe-Medina, V.; Almering, M.J.H.; van Maris, A.J.A.; Pronk, J.T. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl. Environ. Microbiol.* **2010**, *76*, 190–195. [CrossRef]
34. Koppram, R.; Albers, E.; Olsson, L. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnol. Biofuels.* **2012**, *5*, 32. [CrossRef]
35. Zelle, R.M.; Harrison, J.C.; Pronk, J.T.; van Maris, A.J.A. Anaplerotic role for cytosolic malic enzyme in engineered *Saccharomyces cerevisiae* strains. *Appl. Environ. Microbiol.* **2011**, *77*, 732–738. [CrossRef]
36. de Kok, S.; Nijkamp, J.F.; Oud, B.; Roque, F.C.; de Ridder, D.; Daran, J.-M.; Pronk, J.T.; van Maris, A.J.A. Laboratory evolution of new lactate transporter genes in a jen1 delta mutant of *Saccharomyces cerevisiae* and their identification as *ADY2* alleles by whole-genome resequencing and transcriptome analysis. *FEMS Yeast Res.* **2012**, *12*, 359–374. [CrossRef]
37. Adamo, G.M.; Brocca, S.; Passolunghi, S.; Salvato, B.; Lotti, M. Laboratory evolution of copper tolerant yeast strains. *Microb. Cell Fact.* **2012**, *11*, 1. [CrossRef]
38. Święciło, A. Cross-stress resistance in *Saccharomyces cerevisiae* yeast—New insight into an old phenomenon. *Cell Stress Chaperones* **2016**, *21*, 187–200. [CrossRef]

39. Gasch, A.P. The environmental stress response: A common yeast response to diverse environmental stresses. In *Yeast Stress Responses*; Hohmann, S., Mager, W.H., Eds.; Springer: Berlin/Heidelberg, Germany; New York, NY, USA, 2003; Volume 1, pp. 11–70.
40. Palkova, Z.; Vachova, L. Yeast cell differentiation: Lessons from pathogenic and non-pathogenic yeasts. *Semin. Cell Dev. Biol.* **2016**, *57*, 110–119. [CrossRef]
41. Doolin, M.T.; Johnson, A.L.; Johnston, L.H.; Butler, G. Overlapping and distinct roles of the duplicated transcription factors Ace2p and Swi5p. *Mol. Microbiol.* **2001**, *40*, 422–432. [CrossRef] [PubMed]
42. Simon, I.; Barnett, J.; Hannett, N.; Harbison, C.T.; Rinaldi, N.J.; Volkert, T.L.; Wyrick, J.J.; Zeitlinger, J.; Gifford, D.K.; Jaakkola, T.S.; et al. Serial regulation of transcriptional regulators in the yeast cell cycle. *Cell* **2001**, *106*, 697–708. [CrossRef] [PubMed]
43. Colman-Lerner, A.; Chin, T.E.; Brent, R. Yeast Cbk1 and Mob2 activate daughter-specific genetic programs to induce asymmetric cell fates. *Cell* **2001**, *107*, 739–750. [CrossRef] [PubMed]
44. Weiss, E.L.; Kurischko, C.; Zhang, C.; Shokat, K.; Drubin, D.G.; Luca, F.C. The *Saccharomyces cerevisiae* Mob2p-Cbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter cell-specific localization of Ace2p transcription factor. *J. Cell Biol.* **2002**, *158*, 885–900. [CrossRef]
45. King, L.; Butler, G. Ace2p, a regulator of Cts1 (chitinase) expression, affects pseudohyphal production in *Saccharomyces cerevisiae*. *Curr. Genet.* **1998**, *34*, 183–191. [CrossRef] [PubMed]
46. O'Conallain, C.; Doolin, M.T.; Taggart, C.; Thornton, F.; Butler, G. Regulated nuclear localization of the yeast transcription factor Ace2p controls expression of chitinase (CTS1) in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **1999**, *262*, 275–282. [CrossRef]
47. Dohrmann, P.R.; Butler, G.; Tamai, K.; Dorland, S.; Greene, J.R.; Thiele, D.J.; Stillman, D.J. Parallel pathways of gene regulation: Homologous regulators *SWI5* and *ACE2* differentially control transcription of HO and chitinase. *Genes. Dev.* **1992**, *6*, 93–104. [CrossRef] [PubMed]
48. Dohrmann, P.R.; Voth, W.P.; Stillman, D.J. Role of negative regulation in promoter specificity of the homologous transcriptional activators Ace2p and Swi5p. *Mol. Cell. Biol.* **1996**, *16*, 1746–1758. [CrossRef] [PubMed]
49. McBride, H.J.; Yu, Y.; Stillman, D.J. Distinct regions of the Swi5 and Ace2 transcription factors are required for specific gene activation. *J. Biol. Chem.* **1999**, *274*, 21029–21036. [CrossRef] [PubMed]
50. Dujon, B.; Sherman, D.; Fischer, G.; Durrens, P.; Casaregola, S.; Lafontaine, I.; De Montigny, J.; Marck, C.; Neuveglise, C.; Talla, E.; et al. Genome evolution in yeasts. *Nature* **2004**, *430*, 35–44. [CrossRef]
51. Kelly, M.T.; MacCallum, D.M.; Clancy, S.D.; Odds, F.C.; Brown, A.J.P.; Butler, G. The *Candida albicans* CaACE2 gene affects morphogenesis, adherence and virulence. *Mol. Microbiol.* **2004**, *53*, 969–983. [CrossRef]
52. Kamran, M.; Calcagno, A.M.; Findon, H.; Bignell, E.; Jones, M.D.; Warn, P.; Hopkins, P.; Denning, D.W.; Butler, G.; Rogers, T.; et al. Inactivation of transcription factor gene *ACE2* in the fungal pathogen *Candida glabrata* results in hypervirulence. *Eukaryot. Cell* **2004**, *3*, 546–552. [CrossRef]
53. Stead, D.; Findon, H.; Yin, Z.K.; Walker, J.; Selway, L.; Cash, P.; Dujon, B.A.; Hennequin, C.; Brown, A.J.P.; Haynes, K. Proteomic changes associated with inactivation of the *Candida glabrata ACE2* virulence-moderating gene. *Proteomics* **2005**, *5*, 1838–1848. [CrossRef]
54. MacCallum, D.M.; Findon, H.; Kenny, C.C.; Butler, G.; Haynes, K.; Odds, F.C. Different consequences of ACE2 and SWI5 gene disruptions for virulence of pathogenic and nonpathogenic yeasts. *Infect. Immun.* **2006**, *74*, 5244–5248. [CrossRef] [PubMed]
55. Bussereau, F.; Casaregola, S.; Lafay, J.F.; Bolotin-Fukuhara, M. The *Kluyveromyces lactis* repertoire of transcriptional regulators. *FEMS Yeast Res.* **2006**, *6*, 325–335. [CrossRef] [PubMed]
56. Mulhern, S.M.; Logue, M.E.; Butler, G. *Candida albicans* transcription factor Ace2 regulates metabolism and Is Required for filamentation in hypoxic conditions. *Eukaryot. Cell* **2006**, *5*, 2001–2013. [CrossRef] [PubMed]
57. Gasch, A.P.; Spellman, P.T.; Kao, C.M.; Carmel-Harel, O.; Eisen, M.B.; Storz, G.; Botstein, D.; Brown, P.O. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **2000**, *11*, 4241–4257. [CrossRef]
58. Chen, D.; Toone, W.M.; Mata, J.; Lyne, R.; Burns, G.; Kivinen, K.; Brazma, A.; Jones, N.; Bähler, J. Global transcriptional responses of fission yeast to environmental stress. *Mol. Biol. Cell* **2003**, *14*, 214–229. [CrossRef]
59. Enjalbert, B.; Nantel, A.; Whiteway, M. Stress-induced gene expression in *Candida albicans*: Absence of a general stress response. *Mol. Biol. Cell* **2003**, *14*, 1460–1467. [CrossRef]
60. Gasch, A.P. Comparative genomics of the environmental stress response in ascomycete fungi. *Yeast* **2007**, *24*, 961–976. [CrossRef]