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Ethanol Fermentation by High-Stress-Tolerance Aquatic Yeasts and Their Mutants

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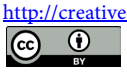


Abstract

Bioethanol is thought to be a renewable source of energy, because the biomasses used to make ethanol, such as sugar cane and its residual substance, molasses, are resources that can be continuously produced. But the practical use of ethanol to replace fossil fuels or atomic energy has been limited, because the production efficiencies of ethanol in relation to its substrates are not so high. Thus, for industrial production of the bioethanol, yeast fermentation would ideally be carried out in biomasses containing more highly concentrated carbohydrates. However, the environmental stresses in highly concentrated cultures might weaken the yeast's physiological activities. From various kinds of aquatic yeast with stress tolerance, *Torulaspora derbrueckii* F2-11 and *Wicherhamomyces anomalus* AN2-64 were selected as candidates for high-sugar-tolerance yeasts as they showed remarkable growth in the YPD + sorbitol (600 g/L) medium at 25˚C for 120 hrs. When the amounts and kinds of sugar alcohols in the cells of the two strains were measured in cultures containing 20 g/L or 400 g/L of D-glucose, maltose, or sucrose, the main two sugar alcohols that accumulated as the sugar concentration increased were glycerol and arabitol. Mutation by ethyl methanesulfonate of the parent strains *T. derbrueckii* F2-11 and *W. anomalus* AN2-64 induced mutants F2-11M or AN2-64M, which showed higher sugar, heat, and ethanol tolerances than their respective parents. Ethanol productivities and sugar assimilation activities of the mutants were also higher than those of the parents in the 25% (v/v) molasses.

Keywords

Ethanol Fermentation, Stress Tolerance, Aquatic Yeast, Mutant, Ethyl Methanesulfonate (EMS)

1. Introduction

Yeasts are eukaryotic microorganisms that have been used for millennia in a wide range of industries including food production and liquor brewing [1]. Recently, bioethanol has been attracting attention as a route to obtaining a low-carbon circular economy; namely, it can substitute for fossil fuels, which cause serious global warming, and atomic energy, which carries enormous safety concerns and is less portable. Bioethanol is thought to be a renewable energy source, because the biomasses used to make it, such as sugar cane and its residual substance, molasses, can be continuously produced [2] [3]. Several review articles about bioethanol production by yeasts have been published [4] [5], but its actual use replacing fossil fuels or atomic energy has been limited, because ethanol production from substrates is not very efficient. For industrial production of bioethanol, yeast fermentation would ideally be carried out in biomasses containing more highly concentrated carbohydrates. However, highly concentrated cultures can produce environmental stresses such as accumulated salts, sugars, or ethanol, which weaken the physiological activities of most yeasts and reduce ethanol productivity [6] [7]. Recently, marine yeasts with stress tolerance against salt water (about 3% w/v NaCl) were isolated and applied to the improvement of bioethanol production [8] [9]. We also have been studying various kinds of stress-tolerant yeasts from non-marine aquatic environments: we isolated thermotolerant fermentative yeasts from hot spring drainage [10] [11] [12] and produced bioethanol from highly concentrated saccharified suspensions of water hyacinth [13] [14], paper shredder scrap [15], and seaweed [16] [17] [18]. We also characterized salt-tolerant yeasts [19], and those tolerant to other stressors [20] [21].

In industrial-scale bioethanol production from molasses, the sugar concentration is often more than 40% (w/v) [22]; the yeasts are required to have considerable fermentative ability even under such high stress pressures. Bloomberg *et al.* noted that yeast *Saccharomyces cerevisiae* accumulated sugar alcohols, mainly glycerol, postulating it as a compatible solute in cells to improve their stress tolerance under various high-stress pressures [23].

In this study, we surveyed our microbial library [21] for fermentative yeast strains known to have higher stress tolerances, and assayed the amounts and kinds of accumulated sugar alcohols serving as compatible solutes in the selected yeasts. For further study, we mutated the yeast strains with ethyl methanesulfonate (EMS) and compared the bioethanol production from molasses of the parents and their mutants.

2. Materials and Methods

2.1. Aquatic Yeast Strains Used

Our previous study identified among the 1028 yeast strains isolated from aquatic environments in Japan and bioethanol productions, 31 strains that demonstrated high stress tolerance [21]. Among these, we selected for this study 6 strains or species demonstrating high salt tolerance (NaCl 10% - 15%): *Candida glabrata* TC18, *Lachancea kluyveri* F2-67, *Pichia kudriavzevii* AN1-13, *Saccharomyces* cf. *cerevisiae/paradoxus* H28, *Torulaspora derbrueckii* F2-11, and *Wicherhamomyces anomalus* AN2-64.

2.2. Selection of High Sugar-Tolerant Yeasts from the 6 Strains

The 6 species strains described above were respectively precultured in 10 mL of modified YPD liquid medium (D-glucose, 180 g/L; peptone, 20 g/L; yeast extract, 10 g/L) at 25˚C for 48 hrs. Each was cultured in 10 mL of YPD (D-glucose, 20 g/L; peptone, 20 g/L; yeast extract, 10 g/L) + sorbitol (600 g/L) liquid medium at 25˚C for 120 hrs. The growth curve of each culture was detected by BIO-PHOTORECORDER TN-2612 (temperature gradient incubator, Advantec Toyo Kaisha, Ltd., Japan).

2.3. Assay of Accumulated Sugar Alcohols as Compatible Solutes in the Yeast Cells

The amount and kinds of accumulated sugar alcohols as compatible solutes in the selected yeasts were measured. Each strain was cultured in 40 ml of yeast nitrogen base (YNB) without amino acids (Becton, Dickinson and Company, USA) + sugar (D-glucose, sucrose, or maltose, 20 g/L or 400 g/L) at 25˚C for 48 hrs. The growing yeast was centrifuged at 3000 rpm for 5 min, and the precipitate was washed with saline. The centrifugation/washing procedure was conducted three times, and the cells were obtained as a pellet. A portion of the pellet was freeze-dried by Freeze Dryer DC801 (Yamato Scientific Co., Ltd., Japan), and the dry weight of the cells was measured. The cell pellet was ground at 4˚C for 30 min by Bug Crasher GM-01 (TAITEC Co., Japan), centrifuged at 10,000 rpm for 10 min, and filtered through a Millipore membrane filter (pore size; 0.22 μm, MERK & Co., USA). The amounts and kinds of sugar alcohols in the filtrate were measured using HPLC with a Shimadzu Shim pack SPR-PB column (250 mm × 7.8 mm) at a column temperature of 80˚C and a detection wavelength of 340 nm using RID10A detector (Shimadzu Co., Japan). Elution was performed with distilled water for HPLC (FUJIFILM Wako Pure Chemicals Co, Japan). Each sample injection volume was 20 μL, the flow rate was 0.5 mL/minute, and the detection time was within 60 min. The ratios of the accumulated amounts of sugar alcohols (mg) to those of dried yeast cells (g) were calculated.

2.4. Mutation with Ethyl Methanesulfonate and Isolation of Higher Sugar Tolerant Mutants

Mutations were induced in the above two high-sugar-tolerance yeasts by expo-

sure to ethyl methanesulfonate (EMS). The procedure was as follows: each strain was cultured in yeast minimum medium (D-glucose, 20 g/L + YNB without amino acids, 6.7 g/L) at 25˚C for 48 hrs. One mL of the culture was added to 30 μL of EMS (Tokyo Chemical Industry Co. Ltd., Japan) and gently shaken at 25˚C for 1 hr. The yeast suspension with EMS was neutralized with the same volume of 10% (w/v) sodium thiosulfate, followed by repeated centrifugation and washing with saline. The EMS-treated yeast was cultured in 10 mL of YMM + sorbitol (800 g/L) at 25˚C for 120 hrs, spread on an agar plate of the same medium, and cultured at 25˚C for 120 hrs. The rapidly growing colonies were identified, and the mutant of each strain with the highest sugar tolerance was isolated.

2.5. Tests for Stress-Tolerance of the Mutants

The mutants described above were tested for various stress tolerances. First, both the parents and the mutants were precultured in 10 mL of YMM + sorbitol (800 g/L) at 25˚C for 120 hrs, spread on agar plates of the same medium, and cultured at 25˚C for 120 hrs. The numbers of grown colonies of parents and mutants were compared. Second, both parents and mutants were precultured in 10 mL of YMM at 25˚C for 48 hrs, spread on agar plates of YPD medium, and cultured at 36˚C - 39˚C for 3 - 5 days to test their heat tolerance. Third, both parents and the mutants were precultured in 10 mL of YPD at 25˚C for 48 hrs, and 100 μL of each culture was inoculated into test tubes containing inverted Durham pipes and 10 mL of YPD + ethanol (10% - 20% v/v). The fermentation was conducted in a test tube covered with aluminum cap for 2 weeks at 25˚C to test their ethanol tolerance by visualizing their CO_2 production.

2.6. Ethanol Fermentation by the Yeasts in Molasses

Molasses (ALISHAN (Japan) Co. Ltd.) was used as the substrate. We assayed sugar consumptions and ethanol productivities of both the parents and their mutants after the fermentation of molasses diluted by distilled water. Each yeast was aerobically precultured at 25˚C for 48 hours in 10 mL of modified YPD medium. The growing yeast was centrifuged at 3000 rpm for 5 min, and its precipitate was washed with saline. The centrifugation/washing procedure was conducted three times, and the cells were obtained as a pellet. Then, 0.3 g of the yeast pellet was anaerobically cultured for 192 hours at 25˚C in 10 mL of 25% (w/v) or 50% (w/v) diluted molasses using the Anaero Pack System (Mitsubishi Gas Chemical Co., Japan). The sugar consumptions and ethanol productions by the yeasts were determined at specified time points with the Sucrose/D-glucose/D-Fructose F-kit (Roche Diagonotics) and Ethanol F-kit (Roche Diagonotics) as the same method of [21].

3. Results

3.1. Selection of High Sugar-Tolerant Yeasts from 6 Strains

Figure 1 shows growth curves of the 6 strains—*C. glabrata* TC18, *L. kluyveri*

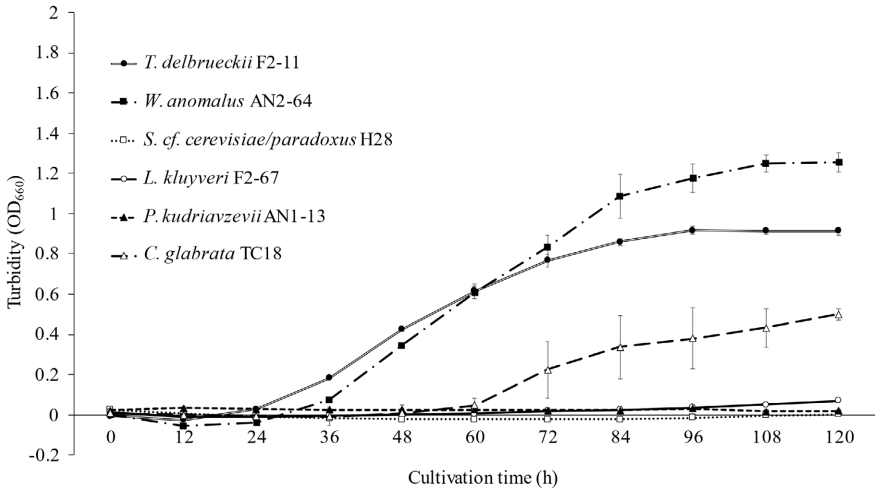


Figure 1. Growth curves of the 6 strains: *Candida glabrata* TC18, *Lachancea kluyveri* F2-67, *Pichia kudriavzevii* AN1-13, *Saccharomyces* cf. *cerevisiae/paradoxus* H28, *Torulaspora derbrueckii* F2-11, and *Wicherhamomyces anomalus* AN2-64. Graphs shows mean values of triplicate trials and their standard deviations (SDs).

F2-67, *P. kudriavzevii* AN1-13, *S.* cf. *cerevisiae/paradoxus* H28, *T. derbrueckii* F2-11, and *W. anomalus* AN2-64—in the YPD + sorbitol (600 g/L) medium at 25˚C for 120 hrs. *L. kluyveri* F2-67, *P. kudriavzevii* AN1-13, and *S.* cf. *cerevisiae/paradoxus* H28 showed little growth and apparently did not have sugar tolerance under these conditions. *C. glabrata* TC18 showed low growth after 60 hrs incubation and thus seemed to have a moderate sugar tolerance. Both *T. derbrueckii* F2-11 and *W. anomalus* AN2-64 showed high growth during 36 - 120 hrs incubation and were selected as the candidates for high sugar tolerance.

3.2. Amounts and Kinds of Accumulated Sugar Alcohols as Compatible Solutes in *T. derbrueckii* F2-11 and *W. anomalus* AN2-64

Figure 2(a) shows the amounts and kinds of accumulated sugar alcohols in *T. derbrueckii* F2-11 cultures containing 20 or 400 g/L of D-glucose, maltose, or sucrose. In all conditions, the main two kinds of sugar alcohol detected were glycerol and to a lesser extent, arabitol. The amount of glycerol at the end of the culture period increased from about 160 to 290 (mg/g cells) in the yeast as the glucose in the culture medium increased from 20 to 400 g/L. In the same manner, the final amount of glycerol significantly increased in the yeast with increasing maltose or sucrose 20 to 400 g/L in the medium. The amounts of arabitol also increased under the same conditions; however, the major compatible solute in *T. derbrueckii* F2-11 was glycerol.

Figure 2(b) shows amounts and kind of accumulated sugar alcohols in *W. anomalus* AN2-64 cultures containing 20 g/L or 400 g/L of D-glucose, maltose, or sucrose. In all conditions, the main two kinds of sugar alcohol detected were glycerol and arabitol. The final amount of glycerol increased from 35 to 110 (mg/g cells) in the yeast as glucose in the medium increased from 20 to 400 g/L.

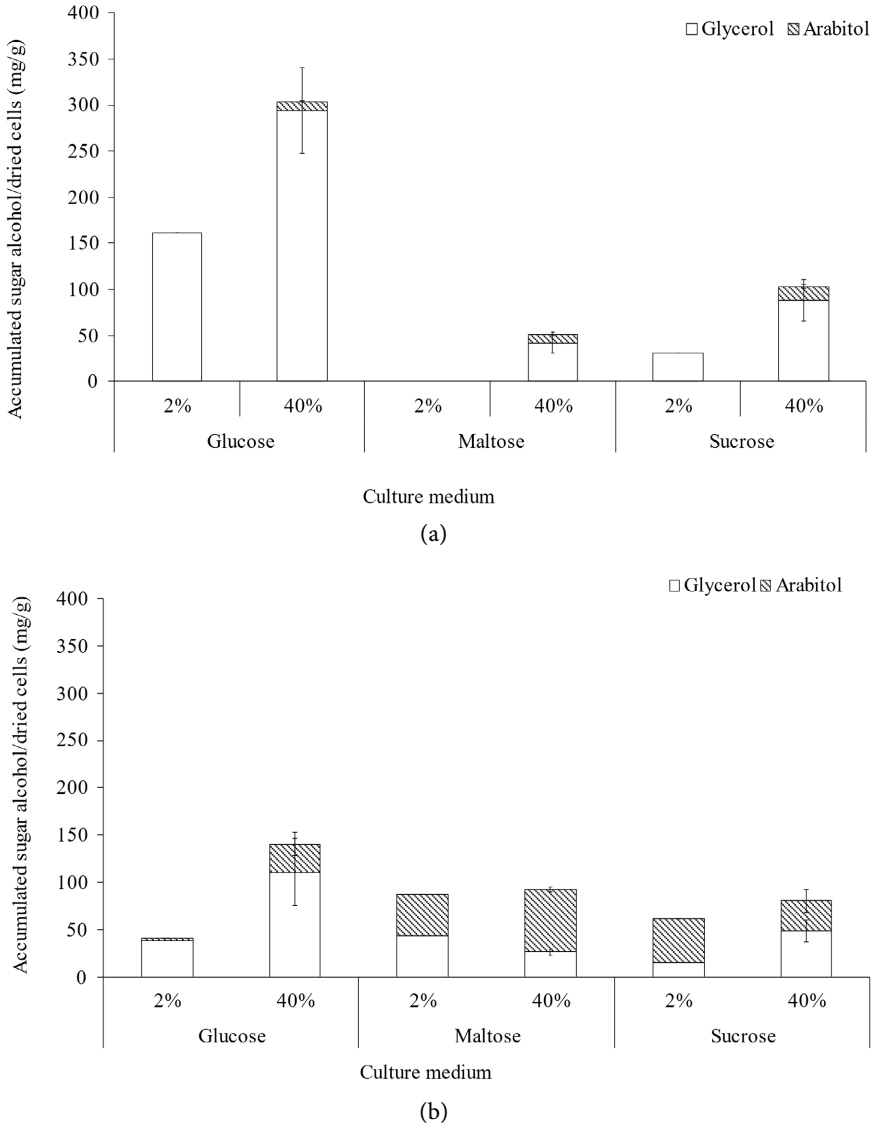


Figure 2. (a) Amounts and kinds of accumulated sugar alcohols in *Torulaspora derbrueckii* F2-11 in the cultivation containing 20 g/L or 400 g/L of D-glucose, maltose, or sucrose Bar graphs show mean values of triplicate trials and their SDs. (b) Amounts and kind of accumulated sugar alcohols in *Wicherhamomyces anomalus* AN2-64 in the cultivation containing 20 g/L or 400 g/L of D-glucose, maltose, or sucrose Bar graphs show mean values of triplicate trials and their SDs.

On the other hand, arabitol increased about 35 to 72 (mg/g cells) as the maltose in the medium increased from 20 to 400 g/L. Thus, the major compatible solutes in *W. anomalus* AN2-64 were glycerol and arabitol.

3.3. Comparison of Stress-Tolerance between the Parents and Their Mutants

The high-sugar-tolerance mutants F2-11M and AN2-64M were isolated by selecting rapidly growing colonies from the cultures of their parents, *T. derbrueckii* F2-11 or *W. anomalus* AN2-64, respectively.

Figure 3 compares the number of colonies of parent and mutant strains when grown on agar plates containing high concentrations of sugars. Both *T. derbruckii* F2-11 and F2-11M were cultured on agar plates of YMM + sorbitol (800 g/L) at 25˚C for 120 hrs. The number of F2-11M colonies was about 8.9×10^6 (CFU/mL) and that of F2-11 was about 2.7×10^6, with the former being 3.3-fold higher than the latter. The number of AN2-64M colonies was about 8.5×10^6 and that of the AN2-64 was about 2.3×10^6, with the former being 3.7-fold higher than the latter. The sugar tolerance of the mutants was found to be higher than that of the parents.

Next, the 4 strains were grown on YPD plates at 36˚C - 39˚C to test their heat tolerance. All grew well at 36˚C while no strains grew at 39˚C (data not shown). Figure 4 shows a photograph of the growing colonies of F2-11, the F2-11M, AN2-64, and AN2-64M at 37˚C and 38˚C. The mutants showed higher growth than the parents at both 37˚C and 38˚C. The heat tolerance of the mutants was found to be higher than that of the parents.

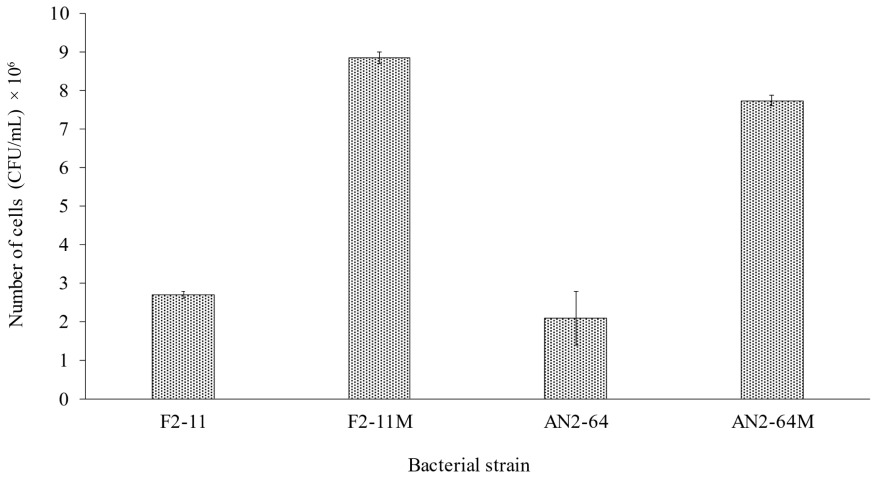


Figure 3. Comparison of number of growing colonies between the parents and their mutants on agar plates containing high concentration sugar. Bar graphs show the means of triplicate trials and their SDs.

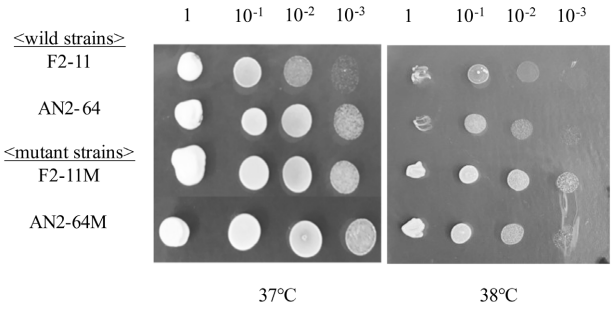


Figure 4. Thermotolerance of F2-11, F2-11M, AN2-64, and AN2-64M at 37˚C and 38˚C. Each strain was precultured in 10 mL of YMM at 25˚C for 48 hrs, and the grown cells were diluted to 10^{-3}, 10^{-2}, 10^{-1}, or 1 by physiological saline. Two-μL portions were spread on agar plates of YPD medium and cultured at 36˚C - 39˚C for 3 - 5 days to testing their heat tolerance.

Table 1 shows fermentation tests of the parents and the mutants in YPD + ethanol, 10% - 20% (v/v). Growth of F2-11 was high in ethanol, 10% (v/v), moderate in 15% (v/v), and absent in 20% (v/v). Growth of the AN2-64 was high in ethanol 10% (v/v) and moderate in ethanol 15% - 20% (v/v). On the other hand, the mutants F2-11M and AN2-64M showed high growth in ethanol, 10% - 20% (v/v). The ethanol tolerance of the mutants was found to be higher than that of the parents.

3.4. Sugar Assimilation and Ethanol Fermentation of the Yeasts from 25% (v/v) Molasses

By diluting molasses with distilled water, a 25% (v/v) solution whose sugar components were sucrose (104.0 g/L), D-fructose (33.4 g/L), and D-glucose (24.8 g/L) was prepared.

Figure 5(a) shows the sugar components of 25% (v/v) molasses before and after yeast fermentation. Before fermentation, the total sugars in 25% (v/v) molasses were about 162 g/L. Residual sugars after fermentation were about 75 g/L by F2-11, 71 g/L by F2-11M, 100 g/L by AN2-64, and 88 g/L by AN2-64M. Sugar assimilation activities of the mutants were higher those of the parents in the 25% (v/v) molasses.

Figure 5(b) shows ethanol production by yeast fermentation from 25% (v/v) molasses. The ethanol amounts produced were 32 g/L by F2-11 and 35 g/L by F2-11M; 26 g/L by AN2-64 and 33 g/L by AN2-64M. The ethanol productions of the mutants in 25% (v/v) molasses were also higher than those of the parents. Under these conditions, the sugar assimilation activity of each yeast was found to be highly related to its ethanol productivity.

3.5. Ethanol Fermentation of the Yeasts from 50% (v/v) Molasses

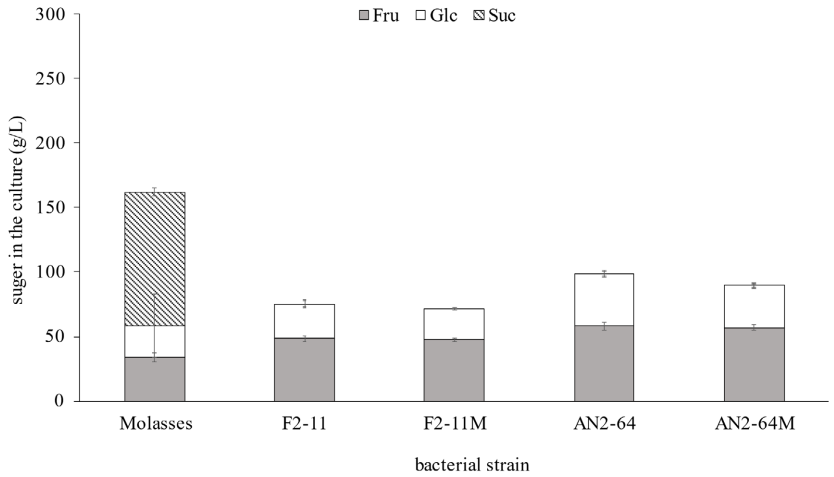
By diluting molasses with distilled water, a 50% (v/v) solution whose sugar components were sucrose (210.2 g/L), D-fructose (52.7 g/L), and D-glucose (53.5 g/L) was prepared.

Figure 6 shows the ethanol productivity of each yeast during fermentation from 50% (v/v) molasses over the span of 192 hrs. Ethanol was produced about 77 g/L at 48 hrs, and 94 g/L at 120 - 192 hrs by F2-11. It was produced at about 27 g/L at 48 hrs, 43 g/L at 120 hrs and 48 g/L at 192 hrs by the F2-11M. Ethanol

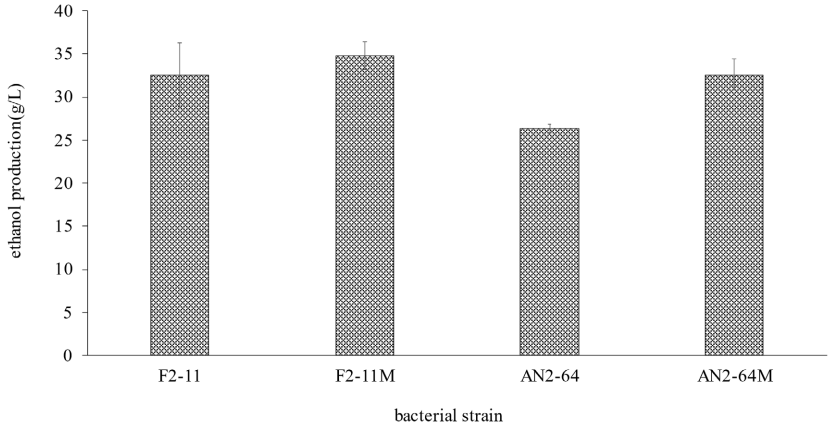
Table 1. Fermentation tests of the parents and the mutants. (ethanol [% (v/v)])

		10	15	20
wild strains	F2-11	++	+	−
	AN2-64	++	+	+
mutant strains	F2-11M	++	++	++
	AN2-64M	++	++	++

−: no fermentative, +: moderate fermentative, ++: high fermentative



(a)



(b)

Figure 5. (a) Sugar components of 25% (v/v) molasses before and after yeast fermentation. Bar graphs show mean values of triplicate trials and their SDs. (b) Ethanol production by yeast fermentation from 25% (v/v) molasses. Bar graphs show mean values of triplicate trials and their SDs.

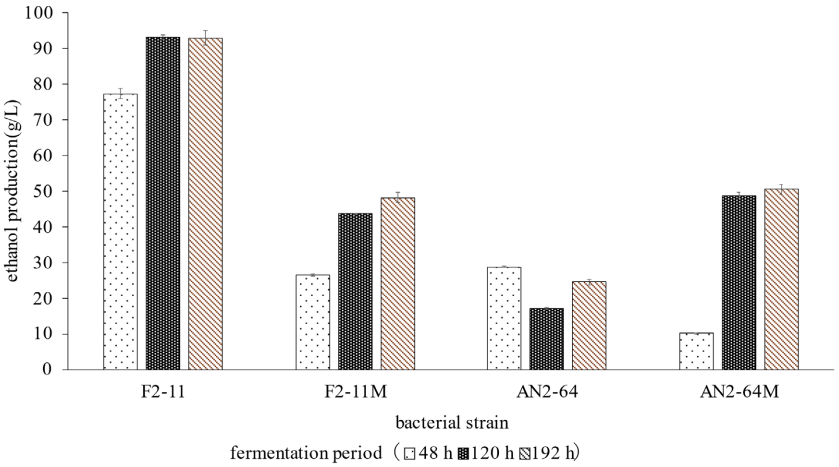


Figure 6. Ethanol productivity of each yeast during fermentation 0 - 192 hrs from 50% (v/v) molasses. Bar graphs show mean values of triplicate trials and their SDs.

productivity of the parent (F2-11) was found to be higher than that of the mutant (F2-11M). Ethanol was also produced about 17 - 29 g/L at 48 - 192 hrs by AN2-64. It was produced 10 g/L at 48 hrs, 49 g/L at 120 hrs, and 51 g/L at 192 hrs by the AN2-64M. Ethanol productivity of the mutant (AN2-64M) was found to be higher than that of the parent (AN2-64).

3.6. Sugar Assimilation of the Yeasts from 50% (v/v) Molasses

To elucidate the higher ethanol productivity by F2-11, we measured the changes of residual sugars in 50% (v/v) molasses during fermentation by F2-11 and F2-11M; results are shown in Figure 7. The total sugars in both cultures before fermentation were about 315 g/L. In F2-11, total residual sugars were about 102 - 124 g/L while residual sucrose was 0 g/L from 48 to 192 hrs. However, in F2-11M, the total residual sugars were about 147 - 225 g/L from 24 to 192 hrs, and the residual sucrose was 14 - 104 g/L from 48 to 120 hrs. Therefore, the assimilation activity of sucrose in F2-11M was found to decrease; this phenomenon was thought to have caused its decease of ethanol productivity from 50% (v/v) molasses.

4. Discussion

Mukherjee *et al.* reported an overview of non-conventional yeast species for different stress tolerance traits desirable in second-generation bioethanol production [24]. First, they identified several non-conventional osmotolerant yeast species using agar plates with increasing concentrations of glucose, fructose, and sorbitol (each ~600 g/L). Among the list of the osmotolerant yeast species were our two key parent yeast strains, *Torulaspora derbrueckii* and *Wicherhamomyces anomalus*. Second, three different salts (NaCl, KCl, and LiCl, 2500 - 2570 mM) were used to evaluate the yeasts' halotolerance. *T. derbrueckii* and *W. anomalus* were also in their list of halotolerant yeast species. Third, they identified

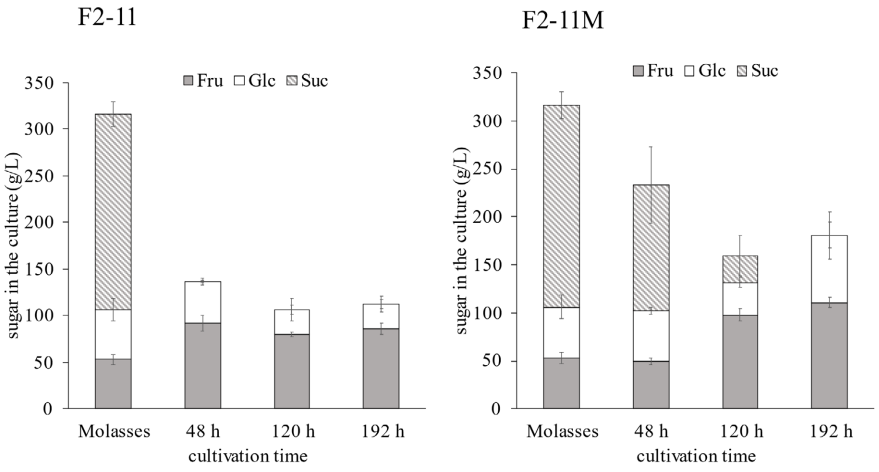


Figure 7. Change of residual sugars in 50% (v/v) molasses during fermentation by the F2-11 or the F2-11M. Bar graphs show mean values of triplicate trials and their standard deviation SDs.

the yeast species that grew at 41˚C; on that list of thermotolerant yeasts were *Candida glabrata*, *Pichia kudriavzevii*, *Saccharomyces cerevisiae*, and *W. anomalus*. Fourth, the "ethanol-tolerant" yeast species growing on 13% (v/v) ethanol included *S. cerevisiae*, *T. derbrueckii*, and *W. anomalus*. Fifth, a growth test on 5-hydroxymethylfurfural (5-HMF, 7 g/L) was performed, and the 5-HMF tolerant yeast species included *C. glabrata*, *Lachancea kluyveri*, *S. cerevisiae*, and *W. anomalus*. Therefore, all 6 representative yeast species used in this study were also included on the lists of non-conventional yeast species for different stress tolerance traits [24]. *T. derbrueckii* and *W. anomalus*, our two candidates for the high-sugar-tolerance yeasts in this study, were the most frequently listed in the report [24].

Both *T. delbrueckii* and *W. anomalus* are used for wine making in some countries [25] [26], suggesting that these yeasts have tolerance against high concentrations of alcohol. *W. anomalus*, also known as *Pichia anomala* and *Hansenula anomala*, is frequently related to spoilage or processing of food and grain products. Its capacity for growth on a wide range of carbon sources at low pH, under high osmotic pressure, and with little or no oxygen enables it to propagate in a wide range of environments [27]. It is a non-*Saccharomyces* wine yeast that contributes to the wine aroma through the production of volatile compounds. *T. delbrueckii* has also been isolated from several human bioprocesses including bread and wine industries, and is thought to be a spoilage yeast contaminating highly osmotic liquids [28].

As shown in **Figure 5(a)** and **Figure 5(b)**, both sugar assimilations and ethanol productions from 25% (v/v) molasses were higher by the mutants (F2-11M and AN2-64M) than by the parents (F2-11 and AN2-64). On the other hand, ethanol production from 50% (v/v) molasses was higher by the parent (F2-11) than by the mutant (F2-11M), as shown **Figure 6**. This phenomenon was explained to be caused by a decrease of sucrose assimilation activities by F2-11M, as shown in **Figure 7**. In general, sucrose is decomposed to D-glucose and D-fructose in a reaction catabolized by invertase secreted from yeasts; then, the monosaccharides are taken up by the cells and assimilated. Thus, the mutation with EMS was thought to cause an increase of stress tolerance for the yeasts but simultaneously a decrease of the amount of invertase secreted from the F2-11M. There have been several studies on *SUG* gene-defective yeast mutants; for instance, Carson *et al.* reported on mutants of *S. cerevisiae* that were unable to grow anaerobically on sucrose but still able to use glucose; they isolated these mutants and characterized their *SUG* genes mutation [29]. Therefore, it is thought to be indispensable for improving ethanol fermentation to isolate yeasts with different stress tolerance traits and without *SUG* related gene mutations.

5. Conclusion

Torulaspora derbrueckii F2-11 and *Wicherhamomyces anomalus* AN2-64 were selected as candidates for high-sugar-tolerance yeasts, because they showed high growth in the YPD + sorbitol (600 g/L) medium at 25˚C for 120 hrs. The amounts

and kinds of sugar alcohols in the strains in the cultures containing 20 g/L or 400 g/L of D-glucose, maltose, or sucrose were measured, and the main two sugar alcohols that accumulated with increasing sugar concentrations were glycerol and arabitol. Culture of the parents *T. derbrueckii* F2-11 or *W. anomalus* AN2-64 with EMS induced the mutants F2-11M or AN2-64M, respectively, which showed higher sugar, heat, and ethanol tolerances than those of the parents. Ethanol productivities of the mutants F2-11M and AN2-64 were 33 - 35 (g/L) with increase of the sugar assimilation activities, while those of the parent F2-11 and AN2-64 were 26 - 32 (g/L) in the 25% (v/v) molasses.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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