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Saccharomyces cerevisiae KNU5377 with Multiple Stress Tolerance and its Potential as a Worldwide On-site Industrial Strain for Alcohol Fermentation

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Saccharomyces cerevisiae KNU5377 was examined to assay the recovering capacity against heat and other stressors. Along with a particular fermentation ability that is able to produce ethanol even at high temperature such as 40°C with a comparable rate to the fermentation at 33°C, this strain also exhibited higher viability than a reference strain owing to its own thermotolerance that conferred the survival after the severe heat shock at 60°C for 30 minutes. Furthermore, this strain showed outstanding tolerances against H_2O_2, ethanol and some chemical compounds. But, especially due to the thermotolerance, this strain has been suspected of other species of yeast. However, ITS (internally transcribed spacer) 1 and 2 sequencing data confirmed this strain was a typical strain of *S. cerevisiae*. The outstanding tolerances to various environmental stressors indicate this *S. cerevisiae* KNU5377 is enough to use both as an on-site potential strain for world-wide alcohol fermentation industry and as a model strain for researches into the routes to acquire the tolerance to various stressors.

Key wods: Stress tolerance, *Saccharomyces cerevisiae*, ITS sequence

Saccharomyces cerevisiae has several industrial applications for the production of alcohol, baker's yeast, and so on. *S. cerevisiae* is one of domesticated microorganisms for various purposes, especially ethanol fermentation, and thousand years of use have resulted in development of several strains optimized for these specific applications. The different *S. cerevisiae* strains have different patterns of by-products and secondary metabolite production. Although strain selection during thousands of years has resulted in screening *S. cerevisiae* strains with extraordinarily high ethanol productivity and ethanol tolerance, there are still some problems (defective) of *S. cerevisiae* that are far from optima from an on-site industrial point of view. In the classical applications of *S. cerevisiae*, three major problems can be encountered, including glucose repression of utilization of mixed carbon sources, glycerol formation under anaerobic conditions, and crab tree effect (i.e. production of ethanol under aerobic conditions)[3,15].

In addition, industrial stress conditions give also major problems for economical uses of *S. cerevisiae*. For example, physical or chemical stressors including heat, ethanol, pressure, heavy metals and oxidative stress would cause the decrease of products by damaging the industrial strain. Therefore, if cells can acquire the capacities to endure various stresses, the productivity would be also increased.

For this purpose, in this study, the stress tolerance was targeted for strain development of yeast and for revealing how this strain could acquire some tolerance especially against heat, ethanol, H_2O_2 and other chemicals[5,13]. Fortunately, *S. cerevisiae* KNU5377 (abbreviated as KNU5377 afterwards), originally named as F38-1, was isolated and identified as a thermotolerant strain that could produce alcohol at high temperature like 40°C, with the comparable rate to 30°C[8,9]. The validity for using this strain as a target for the development and as a research source for stress tolerance was here examined on the limit of heat tolerance and relatively high tolerance against various other stressors by comparing with the reference cells, *S. cerevisiae*

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ATCC 24858 and W303.

Yeast cells are often treated at lethal temperature in order to determine the thermotolerance level onto the liquid and solid media and so to know their viability. Here the KNU5377 and reference strains are compared with each other onto the solid media, YPD agar media (agar 1.8%, yeast extract 1%, peptone 2%, and dextrose 2%). After overnight mother culture on the YPD agar media, it was copied by replicating into fresh agar media. The replica were then treated at each condition of various stress and cultured at 30°C for overnight. As shown in Fig. 1, the KNU5377 strain showed higher viability at every condition, including 30 minutes treatment at 60°C, than two reference strains. Especially, even after treatment for 5 hours at 53°C (data not shown), the KNU5377 only had formed colonies. Laboratory *S. cerevisiae* can not synthesize proteins at over 43°C[12], which is the temperature limit for protein synthesis and also causes an exponential cell death[16]. The cell death would be dependent on the lethal temperature and the duration of treatment as well as on the intrinsic thermotolerance of the examining cells. In other words, if there is no induction of thermotolerance via the mild pre-treatment before treating the cells at the lethal temperature, the determinate factors for cell survival will be confined to the constitutive characteristics of the cells. The characteristics are surely strain-specific that can be originated from natural mutations of the cell. Therefore, the viability at 60°C suggests that the KNU5377 strain should be constitutively thermotolerant.

Due to this surprising thermotolerance, this strain was often suspected that it is not a *S. cerevisiae*. But it was well identified as a typical strain of *Sacchromyces cerevisiae* [8]. As shown in Table 1, the ITS (internally transcribed spacers) 1 and 2 sequencing data proved to have the

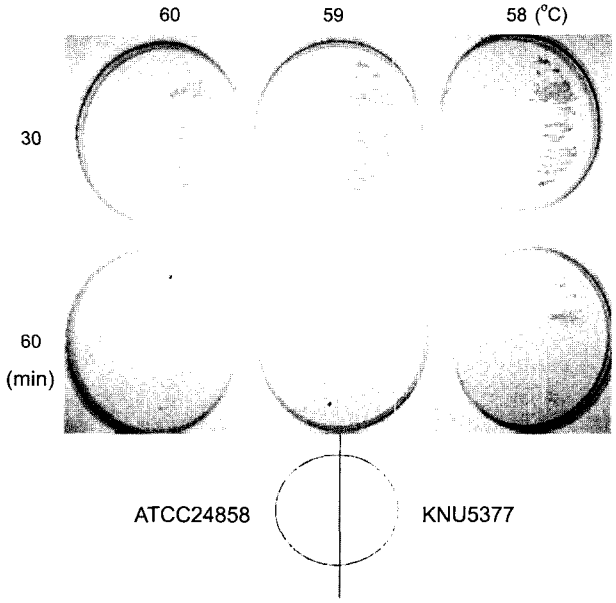

Fig. 1. Thermotolerance assay of *S. cerevisiae* KNU5377 and ATCC 24858 on the YPD agar medium. Overnight-cultured mother plates were replicated onto fresh YPD agar plates. The daughter plates were then treated at each temperatures and times, 58 to 60°C for 30 and 60 minutes, in dry oven. After the treatment, the plates were transferred and incubated for overnight at 30°C. The left and right half of each plate denotes *S. cerevisiae* ATCC 24858 and KNU5377, respectively, and the perpendicular figures express treatment time at given temperatures.

Table 1. Internally transcribed spacer (ITS) 1 and 2 sequence

(A) ITS 1 sequence

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aagaaatttaataattttgaaaatggatttttttgttttggcaagagcatg  50
agagcttttactgggcaagaagacaagagatggagagtccagccgggcct  100
gcgcttaagtgcgcggtcttgctaggcttgtaagtttctttcttgctatt  150
ccaaactgtgagagatttctgtgcttttgttataggacaattaaaaccgt  200
ttcaatacaacacactgtggagttttcatatctttgcaacttttttctttg  250
ggcattcgagcaatcggggcccagaggtaacaaacacaaacaattttatt  300
tattcattaaatttttgtcaaaaacaagaattttcgtaactggaaatttt  350
aaaatattaa  360
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(B) ITS 2 sequence

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ccttctcaaacattctgtttggtagtgagtgatactctttggagttaact  50
tgaaattgctggccttttcattggatgttttttttccaaagagaggttt  100
ctctgcgtgcttgaggtataatgcaagtacggtcgttttaggttttacca  150
actgcggctaatctttttttatactgagcgtattggaacgttatcgataag  200
aagagagcgtctaggcgaacaatgttcttaaagt  234
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GeneBank accession numbers of ITS1 and ITS2 sequence is AY013705 and AY013706, respectively. The sequences were analyzed and determined by our request to Korean Collection Type Cultures. The 5'- and 3'- ends for sequencing were originated from the sequences of *Saccharomyces cerevisiae* IFO 10217 (GeneBank accession no. D89886).

identities of 99% and 95% in each sequences, respectively. Refer to their GeneBank Accession Number of AY 013705 and AY 103706 for ITS 1 and 2 genes of *S. cerevisiae* KNU5377, respectively.

Furthermore it was found that this thermotolerant strain possessed also other stress tolerances for some chemical compounds, ethanol and oxidative stress. To examine the ethanol tolerance of yeast strains, the exponentially growing cells (about 10^5 cells/ml) were treated by adding absolute ethanol to the cell suspension to make a final ethanol concentration of 20% (v/v). During the treatment period for 120 minutes, cells were withdrawn at intervals of 10 minutes and spotted immediately onto the YPD agar plate. As considered the capacity of colony-forming after overnight incubation of the plates, it was determined that KNU5377 had the higher ethanol tolerance than the reference strains (Fig. 2). The ATCC 24858 strain was informed as an ethanol-tolerant strain at the description of ATCC (American Type Culture Collection) catalog, and was also revealed to moderately thermotolerant strain in this study. While the KNU5377 could survive after the treatment of 20% ethanol for 120 minutes, the ethanol tolerance of the reference strains, W303 and ATCC 24858, was much lower than that of KNU5377. Consequently, the thermotolerant KNU5377 strain also showed the higher ethanol tolerance than the references, implying KNU5377 has constitutive characteristics that confer the high tolerances simultaneously against the stressors including ethanol and heat.

Oxidative stress can be also occurred by the heat stress, and besides the adaptive responses can be varied according to the kind of stressors like as menadione, H_2O_2 and other oxidative stressors[6]. Among these oxidative stressors, hydrogen peroxide was used in this experiment by adding final 35 mM concentration of hydrogen peroxide (H_2O_2) into the media. The tolerance was examined with the same method as the ethanol tolerance assay. As shown in Fig. 3, after oxidative treatment for 2 hours, KNU5377 showed the

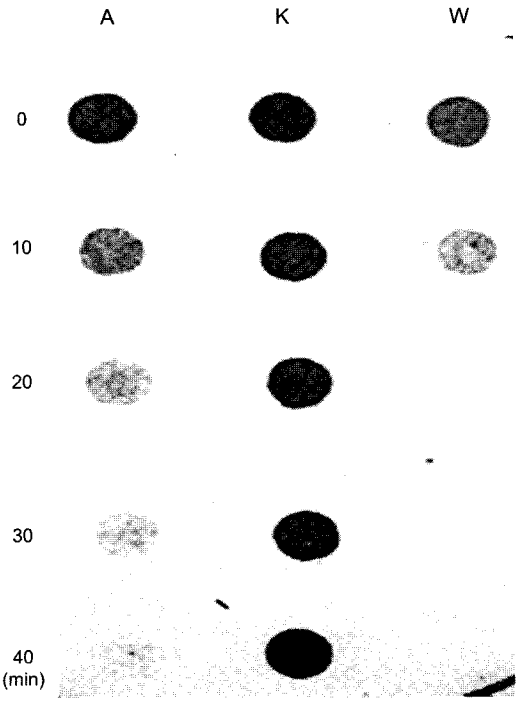


Fig. 2. The comparison of test strains on the ethanol tolerance. Exponentially growing *S. cerevisiae* KNU5377 and reference strains (10^5 cells/ml) were shocked for 2 hours in the YPD media containing 20% (v/v) final concentration of ethanol. Cells were picked up at intervals of 10 minutes and immediately spotted 5 ul of the cell suspension onto the YPD agar plates, and then incubated at 30°C for overnight. In this figure, data were illustrated up to 40 minutes. Abbreviated characters, A, K, and W indicate *S. cerevisiae* ATCC 24858, KNU5377, and W303, respectively.

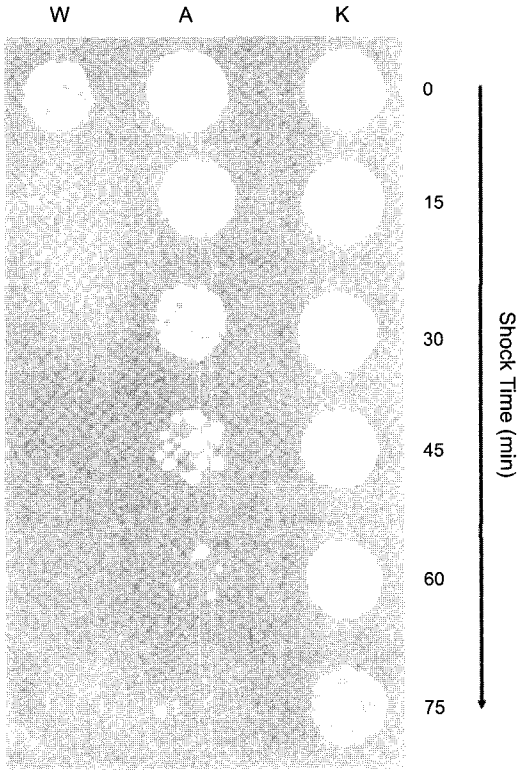
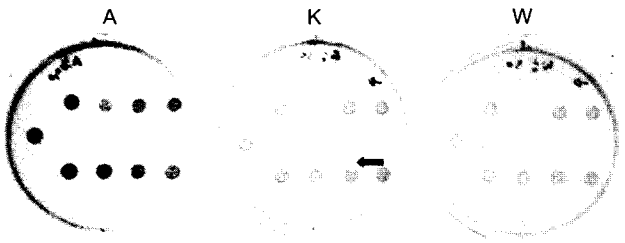


Fig. 3. The comparison of test strains on the H_2O_2 tolerance. Exponentially growing cells were treated at various concentrations of H_2O_2 (0 to 35 mM) for 2 hours. After spotting the treated cells on YPD agar plates, they were incubated for overnight and compared the colony forming capacity that indicated the tolerance or viability of each strain. Abbreviated characters, A, K, and W indicate *S. cerevisiae* ATCC 24858, KNU5377, and W303, respectively.

relatively higher tolerance than other references in the similar pattern as the case of ethanol tolerance. It is generally known that over 4 mM concentration of H_2O_2 is lethal to the laboratory *S. cerevisiae* strains[1]. The 35 mM concentration of H_2O_2 applied in this study must be unbelievably lethal to this yeast. But this KNU5377 could survive despite treatment at this concentration of H_2O_2. Therefore, these constitutive characteristics of KNU5377 must be adequate to overcome the oxidative stress like this extreme condition, which may be often occurred in the industrial applications.

On the other hand, the tolerance of KNU5377 to chemical compounds including organic solvent like benzene was not so different from the reference strains. Among the 15 compounds examined in this study, KNU5377 showed superior tolerance only to benzonitrile, one of toxic cyanide compounds named as phenyl cyanide (C_6H_5CN) (Fig. 4). This experiment should be further expanded to examine the tolerance of this strain by using more organic solvents, and then the relationships between the many organic solvents and yeast tolerance should be analyzed. However, this result on its tolerance to benzonitrile suggests a possibility that their different tolerance against benzonitrile should be related just to the constitutive characteristics that confer the high tolerance to ethanol, H_2O_2 and heat. That is, it seems



5	4	3	2	1
10	9	8	7	6
15	14	13	12	11

A : S. *cerevisiae* ATCC24858
K : S. *cerevisiae* KNU5377
W : S.*cerevisiae* W303

Fig. 4. Tolerance to chemical compounds of *S. cerevisiae* KNU5377 and the reference strains. Total 15 compounds, which were illustrated by numbers 1 to 15 in the square box of figure, were used in this experiment: 1, dimethysulfoxide; 2, N,N-dimethylformamide; 3, nitromethane; 4, glycerol; 5, butylalcohol; 6, aniline; 7, benzonitrile; 8, chloroform; 9, benzene; 10, n-hexane; 11, p-xylene; 12, m-xylene; 13, cyclohexane; 14, isooctane; 15, ethylacetate. Exponentially growing cells were treated by the final 10% (v/v) concentration of the compounds for 30 minutes with vigorous shaking, and then 10-fold diluted cells were spotted on the YPD agar media. Abbreviated characters, A, K, and W indicate *S. cerevisiae* ATCC 24858, KNU5377, and W303, respectively.

to be sure that this characteristic phenotype of KNU5377 might be a clue for revealing the mechanisms related to the stress tolerance.

The major cause for multiple stress tolerance has been often mentioned as a result of the functional overlap of stress responses in the concept of acquired tolerance that is often occurred by mild pre-treatment of a given stressor[2, 11,12,14]. However, as shown in the case of KNU5377, the yeast tolerance to stress must be due to the constitutive characteristics, at least partly, because lethal stress was directly applied to the cell without inducing the acquired tolerance via the pre-treatment with the mild stress condition that is non-lethal. As one of the trials for revealing the intrinsic differences, the microarray analysis can be used to reveal the genetic background of the intrinsically thermotolerant *S. cerevisiae*[4]. A lot of recent studies for yeast breeding are being performed and also will continue to attempt like present studies in future by using various methods[6,7,10]. To reveal the constitutive characteristics of KNU5377 should be one of the most important things to know the mechanism of multi-stress tolerance of this KNU5377 yeast strain. In addition, it can be suggested that this *S. cerevisiae* KNU5377 is enough to use as model strain for the researches on the routes for acquiring the high intrinsic tolerance of yeast, and also this strain must be a potential as a worldwide on-site industrial strain for alcohol fermentation as of now.

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