JARQ 52 (2), 137 - 142 (2018) https://www.jircas.go.jp

REVIEW
Selection and Development of Stress-tolerant Yeasts for Bioethanol Production

Toshihide NAKAMURA[1]* and Jun SHIMA[2]

[1] Food Research Institute, National Agriculture and Food Research Organization (NARO) (Tsukuba, Ibaraki 305-8642, Japan)
[2] Faculty of Agriculture, Ryukoku University (Otsu, Shiga 520-2123, Japan)

Abstract
Yeast is the major microorganism used to produce ethanol by fermentation. Because fermentation environments are stressful, the fermentation ability of yeast depends greatly on the yeast's stress tolerance. Stress-tolerant yeasts can produce ethanol in a harsh environment. During the fermentation process of ethanol production, yeasts can be exposed to various stresses, including high temperature, hyperosmolarity, and/or inhibitory substances. The isolation of yeasts tolerant to these stresses and the clarification of their tolerance mechanisms are important for the development of stress-tolerant yeasts for efficient ethanol production. This review focuses on the stress tolerance of yeasts and the use of self-cloning-based gene modification to enhance yeast stress tolerance. We first conducted a search for stress-tolerant yeasts among our stock strains. We also identified stress tolerance-related genes using a *Saccharomyces cerevisiae* gene deletion strain collection. We then investigated the effects of stress tolerance-related gene overexpression on yeast growth and ethanol production.

Discipline: Biofuel
Additional key words: environmental stresses, fermentation, molecular breeding, *Saccharomyces cerevisiae*

Introduction

The massive use of fossil fuels causes anthropogenic climate change, and there is widespread fear about the eventual complete depletion of fossil fuels (Höök & Tang 2013). The production and utilization of bioethanol are of great interest from the viewpoint of fossil fuel substitution. Bioethanol is ethanol made from biomass (raw material of plant origin), which is considered a clean energy source that does not increase atmospheric carbon dioxide when it burns, because the released carbon dioxide was originally fixed by existing plants. Several studies on bioethanol production using various types of biomass as a raw material are ongoing (Kim & Dale 2004, Sanchez & Cardona 2008).

Although there are many methods of producing ethanol from biomass, the first step entails preparation of a sugar solution by grinding and/or decomposition (saccharification), after which ethanol is produced by microbial fermentation (Fig. 1). Sugarcane juice and molasses, two by-products of sugar production, are the primary fermentation substrates used for bioethanol production (Akbas & Stark 2016). Corn starch has also been used recently as a biomass material for industrial ethanol production.

The bioethanol production process creates a harsh environment for yeast (Shima & Nakamura 2015). For example, the fermentation heat generated with the progress of fermentation raises the temperature of the fermenter and imposes high temperature stress on yeast. In addition, biomass-derived sugar solutions often contain substances that inhibit yeast fermentation. Fermentation-inhibiting substances are mainly produced during biomass pretreatment (e.g., delignification) and subsequent saccharification (Fig. 1). Acid and/or heating treatments, in particular, lead to excessive decomposition; thus, sugar solutions derived from biomass (i.e., starch, cellulose, hemicellulose) often contain furan compounds (e.g., furfural, 5-hydroxymethylfurfural). In addition, phenolic compounds derived from lignin (e.g., vanillin, guaiacol, 4-hydroxybenzaldehyde) and weak acids (e.g., acetic acid, formic acid) may be produced in some cases. These substances not only inhibit yeast growth but also inhibit ethanol productivity, which is a major cause of low ethanol conversion efficiency. Yeasts are also dehydrated by the high concentration of salts generated by

*Corresponding author: e-mail nakamrto@affrc.go.jp
Received 16 March 2017; accepted 14 July 2017.

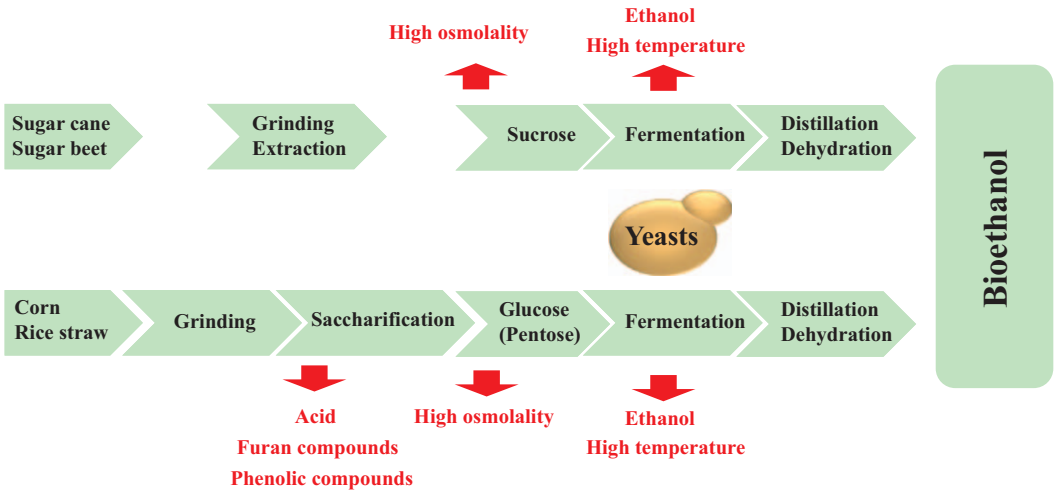

Fig. 1. Flow diagram of ethanol production from biomass
Factors that inhibit yeast fermentation are indicated in red.

Table 1. Screening of yeast strains tolerant to fermentation inhibitors

Fermentation inhibitor	Treatment	No. of tolerant strains	Growth limit of *S. cerevisiae* BY4743
Acetate	1.2% (v/v)	2	0.7% (v/v)
Lactate	9% (v/v)	1	2% (v/v)
Sulfate	pH 1.65*	2	pH 3.0*
Sodium sulfate	5% (w/v), pH 1.5*	3	5% (w/v), pH 4.5*
Sodium chloride	20% (w/v)	1	10%
Sodium hydroxide	126 mM, pH 12.0	3	pH 9.0
Furfural	60 mM	3	20 mM
5-hydroxymethylfurfural	60 mM	9	10 mM
Vanillin	40 mM	3	10 mM
Ethanol	15% (v/v)	8	10%
High temperature	48°C	3	39°C

*Sulfuric acid was added to adjust the pH

the neutralization of acids or alkalis used in the pretreatment and saccharification of cellulosic biomass, and the high osmolarity due to the high concentration of sugars produced by saccharification. Moreover, the accumulation of ethanol, the fermentation product, is another source of stress to yeasts.

Although yeasts have varied strategies to adapt to such stresses, when the stress conditions exceed the adaptation limits, the lifespan and functions of the yeasts are constrained. Since stress-tolerant yeasts are considered highly adaptive, their use is expected to improve ethanol production efficiency.

Screening of stress-tolerant yeasts

As many kinds of biomass are used for bioethanol production and the methods of converting biomass to sugar and ethanol vary greatly, the types of stress will depend on the sugar solution in a given system. We therefore screened yeast strains that are highly tolerant to conceivable stresses, in order to identify strains that more appropriately responsive to various sugar solutions. The stressors tested included ethanol, high temperature, high osmolarity (high salt and sugar concentrations), acids (lactate, acetate, and sulfate), alkalis, furan compounds (furfural and 5-hydroxymethylfurfural), and phenolic compounds (vanillin). We also investigated the ethanol productivity of yeasts, which is an important trait in ethanol production. We used yeast growth under stress conditions as an index of stress tolerance. All of the strains were subjected to a stepwise increase in stress for evaluating their tolerance. Prior to testing, yeasts were collected from flowers, fruits, soil, rotten trees, and water. Stress tolerance tests were conducted on approx. 1,700 strains, including laboratory stock strains and novel isolates from nature. This strategy allowed the identification of strains highly tolerant to the respective types of stress. Table 1 summarizes the number of strains that were tolerant to each stressor.

We then identified the species of strains that were highly tolerant to stress. We found that most strains with high ethanol productivity were *Saccharomyces cerevisiae*, while most strains with high stress tolerance were from another species. Some strains were tolerant to three fermentation inhibitors (furfural, 5-hydroxymethylfurfural, and vanillin). In addition, some strains were tolerant to multiple types of stress such as acid and high temperature, or salt and high temperature. Only a few strains had both high stress tolerance and high ethanol productivity.

Analysis of stress-tolerant yeasts

1. Acid-tolerant strains

Of the yeast strains evaluated, *Candida glabrata* NFRI 3164 is expected to be useful for ethanol production due to its superior lactate and high temperature tolerances, and its high ethanol productivity. We expect that the use of lactate-tolerant strain NFRI 3164 would facilitate ethanol fermentation in the presence of lactate, which suppresses the growth of various bacteria. Conventionally, antibiotics have been added to sugar solutions to prevent bacterial contamination, but their presence in residues after fermentation and distillation has been reported to damage livestock health when the residues are used as feedstuff (Basaraba et al. 1999). Moreover, the frequent use of antibiotics increases the risk of selecting antibiotic-resistant bacteria in the environment.

On the other hand, lactate is an acid produced by lactic acid bacteria that exist in large quantities in the natural world and are easily degraded in the environment; even if lactate is present in wastewater, it has little influence on the environment. We confirmed that adding lactate at a final concentration of 2% (v/v) to a synthetic medium largely prevented the growth of bacteria, such as Lactobacilli, that are known to contaminate fermentations. NFRI 3164 produced ethanol with high efficiency even in this lactate-supplemented condition (Watanabe et al. 2008). When yeast and lactic acid bacteria are inoculated and cultured at the same time, ethanol production decreases markedly. However, the inhibition of ethanol production by lactic acid bacteria was suppressed in 2% (v/v) lactate, and although fermentation with *S. cerevisiae* NBRC 0224 was greatly delayed, fermentation with NFRI 3164 in this medium was only slightly delayed.

In the process of ethanol production from molasses, contamination with *Bacillus* species is a serious problem. We therefore investigated the ability of acetate to prevent bacterial contamination. We found that *Schizosaccharomyces pombe* NFRI 3807, an acetate-tolerant yeast, was suitable for the production of ethanol from molasses in the presence of acetate (Saithong et al. 2009). NFRI 3807 produced ethanol even in a molasses medium containing 1% (v/v) acetate. The growth of *Bacillus* species was suppressed while ethanol was efficiently produced by fermentation with NFRI 3807 in the presence of 0.7% (v/v) acetate.

2. High sugar-tolerant strains

In the final stage of ethanol production, ethanol is distilled from the fermented mash (ethanol-containing fermentation liquor). The higher the ethanol concentration in the fermented mash, the more efficient the purification. Therefore, in industrial ethanol production, the fermentation of high-concentration sugar solutions is preferred in

Table 2. Yeast strains using in this review and their ethanol productivity

Strain	Sugar	Conc'n (%)	Stress	Ethanol production (%)*	Reference
S. cerevisiae NBRC 0224	Glucose	2	-	0.87	Watanabe et al. 2008
	Glucose	2	2% lactate	0.04	Watanabe et al. 2008
	Sucrose	3	-	1.32	Saithong et al. 2009
	Sucrose	3	1% acetate	ND	Saithong et al. 2009
	Glucose	35	High sugar	6.10	Watanabe et al. 2010
C. glabrata NFRI 3164	Glucose	2	-	0.78	Watanabe et al. 2008
	Glucose	2	2% lactate	0.53	Watanabe et al. 2008
Sc. pombe NFRI 3807	Sucrose	3	-	0.97	Saithong et al. 2009
	Sucrose	3	1% acetate	0.59	Saithong et al. 2009
S. cerevisiae NFRI 3225	Glucose	2	-	0.92	Watanabe et al. 2010
	Glucose	35	High sugar	8.00	Watanabe et al. 2010
Sche. shehatae JCM 18690	Glucose	2	-	0.59	Tanimura et al. 2012
	Xylose	2	-	0.25	Tanimura et al. 2012
Candida sp. NY 7122	Glucose	2	-	0.62	Watanabe et al. 2012
	Xylose	2	-	0.62	Watanabe et al. 2012
	Arabinose	2	-	0.10	Watanabe et al. 2012

*Ethanol concentration after 24 h of fermentation.
Conc'n: concentration, ND: not determined.

producing highly concentrated ethanol. A relatively high-concentration sugar solution (approx. 30%) is easily obtained from starch biomass, molasses, and similar sources. Thus, yeast strains tolerant to a high sugar concentration (over 35%) are required for industrial use.

Very-high-gravity simultaneous saccharification and fermentation using potato mash was carried out with three of our stock strains (*S. cerevisiae*) that had high sugar concentration tolerance and high ethanol productivity. We selected *S. cerevisiae* NFRI 3225, which showed high fermentative ability under high-temperature conditions. Fermentation using potato and sweet potato as substrates with NFRI 3225 resulted in ethanol yields over 92% (Watanabe et al. 2010). We concluded that NFRI 3225 is useful for ethanol production from a starchy biomass.

3. High-temperature-tolerant, pentose-fermenting strains

When a lignocellulosic biomass (e.g., rice straw, waste wood) is used, pentose sugars such as xylose and arabinose are important fermentation substrates. If these sugars could be used for ethanol fermentation, we would expect an increase in ethanol yield. We therefore attempted to isolate xylose-fermenting yeasts with high stress tolerance. We screened yeasts capable of xylose assimilation and then successfully isolated a strain that fermented xylose at 37°C (Tanimura et al. 2012). The strain, named JCM 18690 (formerly ATY839), belongs to *Scheffersomyces shehatae* (formerly known as *Candida shehatae*). JCM 18690 can grow at 39°C, albeit at a slower rate. Thus, JCM 18690 is one of the best-performing, high-temperature-tolerant strains of *S. shehatae*. Interestingly, this strain has high glucoamylase activity, and directly utilizes starch (Tanimura et al. 2015). JCM 18690 is useful for one-step ethanol production from a starchy biomass.

There are very few species of yeast capable of fermenting arabinose, and their ethanol production, as typified by the performance of *Candida arabinofermentans*, is very limited. In order to isolate yeasts with high arabinose-fermenting ability, we tested 36 strains of xylose-fermenting yeasts isolated from nature. Among those strains, three possessed arabinose-fermenting ability. In particular, NY 7122, which was isolated from a blueberry field, had high fermentation ability and a relatively high tolerance to high temperature. NY 7122 was presumed to belong to a species close to *Candida subhashii*, but its sugar assimilation characteristics are somewhat different (Watanabe et al. 2012). As NY 7122 can produce ethanol from xylose and arabinose, its ethanol productivity is higher than that of any previously reported arabinose-fermenting yeasts.

Molecular breeding of stress-tolerant yeasts

1. Acid-tolerant *S. cerevisiae*

Using our screen for stress-tolerant yeasts, we found a highly acetate-tolerant strain of *S. cerevisiae*. We compared the gene expression profile of an acetic acid-tolerant strain (*S. cerevisiae* ATCC 38555) with the profile of an industrial strain of *S. cerevisiae* (NBRC 0224), upon culture with acetate. DNA microarray analysis revealed that the genes regulated by transcription factors Aft1 and Haa1 were more highly expressed in ATCC 38555 (Haitani et al. 2012). Haa1 has been reported to be involved in weak acid tolerance, and we found that the expression of *HAA1* itself was also higher in ATCC 38555, which suggests that increasing the expression of Haa1 could enhance acetate tolerance. We therefore constructed a strain that overexpresses *HAA1* driven by the *TDH3* promoter, and then analyzed its acetate tolerance. The *HAA1*-overexpressing strain demonstrated superior growth and enhanced ethanol productivity in acetate-containing medium as compared to the wild-type strain (Tanaka et al. 2012, Inaba et al. 2013).

2. High-temperature-tolerant *S. cerevisiae*

In order to elucidate the mechanism of high temperature tolerance, we screened the laboratory stock strains to identify *S. cerevisiae* strains with various degrees of high temperature tolerance (Fig. 2). Using these yeast strains, we identified genes involved in high temperature tolerance and bred high-temperature-tolerant strains using molecular genetics. We subjected three *S. cerevisiae* strains with differential high temperature tolerance to 37°C or 39°C, and then analyzed their genes expression levels by DNA microarray. We searched for genes whose expression levels were correlated with the degree of high temperature tolerance. After validation by real-time polymerase chain reaction (PCR), one candidate gene—*SDH8* (formerly *FMP21*)—was identified (Nakamura et al. 2014).

To evaluate the relationship between the expression of *SDH8* and the high temperature tolerance of yeast strains, we analyzed the growth of eight strains of *S. cerevisiae* under high temperature and examined their expression levels of *SDH8*. We observed a correlation between growth at 37°C and the expression of *SDH8* in the tested strains, although their genetic backgrounds differed. We confirmed that high temperature sensitivity increased when *SDH8* was deleted, and that high temperature tolerance was improved when *SDH8* was overexpressed under the control of the *TDH3* promoter. Sdh8 is required for the assembly of succinate dehydrogenase (Van Vranken et al. 2014). Thus, the assembly of succinate dehydrogenase is important for high temperature tolerance. A large quantity of Sdh8 in the *SDH8*-overexpressing cells may facilitate the assembly of functional succinate dehydrogenase under high-temperature

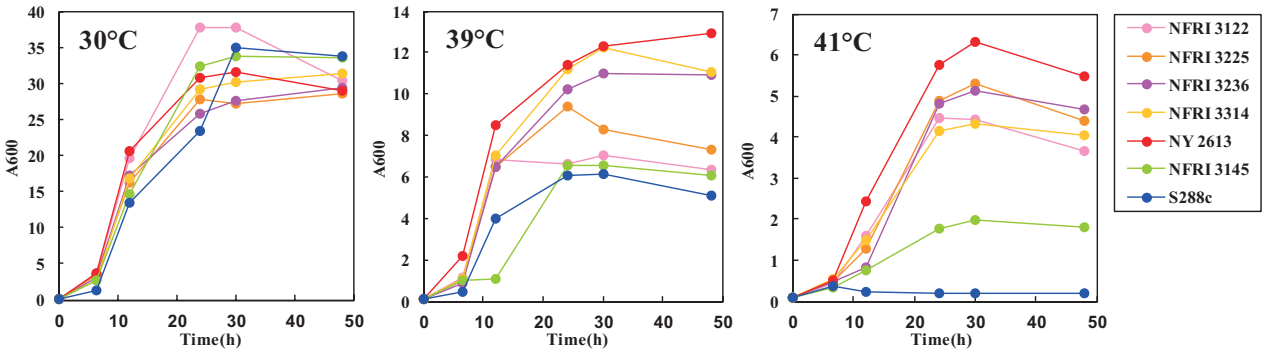

Fig. 2. Growth of *Saccharomyces cerevisiae* strains with differential high temperature tolerance

conditions.

Conclusions

Biofuels are expected to become increasingly important in the future, and determining how to produce biofuels from unused resources such as rice straw is a considerable challenge. In this review, we presented our efforts to develop and utilize stress-tolerant yeasts for industrial ethanol production. We have identified genes important for stress tolerance using DNA microarrays, and improved the strains' stress tolerance by overexpressing stress-related genes in yeasts. Research on high temperature tolerance is attracting attention as it can directly lead to reducing the cost of cooling the fermentation tanks. To develop stress-tolerant yeasts, it is necessary to clarify the adaptation mechanism of yeasts harboring superior stress tolerance.

In the fermentation process, there are general stresses such as ethanol and fermentation heat, and there are stresses related to the type of biomass used and the relevant pretreatment and saccharification methods. As industrial yeast must tolerate stresses corresponding to the nature of the sugar solution in addition to such general stresses as high temperature and ethanol, it is necessary to develop yeasts that are tolerant to multiple stressors. For the development of practical stress-tolerant yeasts, it is important to take into account research on pretreatment and saccharification. We will continue to contribute to improvements in industrial ethanol production by developing yeasts that efficiently produce ethanol.

Acknowledgment

Our work was supported by grants from Japan's Ministry of Agriculture, Forestry, and Fisheries (Rural Biomass Research Project, grants #BEC-BC050, #BEC-BC051).

References

Akbas, M. Y. & Stark, B. C. (2016) Recent trends in bioethanol production from food processing byproducts. *J. Ind. Microbiol. Biotechnol.,* **43** (11), 1593-1609.

Basaraba, R. J. et al. (1999) Toxicosis in cattle from concurrent feeding of monensin and dried distiller's grains contaminated with macrolide antibiotics. *J. Vet. Diagn. Invest.,* **11** (1), 79-86.

Haitani, Y. et al. (2012) Identification of an acetate-tolerant strain of *Saccharomyces cerevisiae* and characterization by gene expression analysis. *J. Biosci. Bioeng.*, **114** (6), 648-651.

Höök, M. & Tang, X. (2013) Depletion of fossil fuels and anthropogenic climate change—A review. *Energy Policy*, **52**, 797-809.

Inaba, T. et al. (2013) An organic acid-tolerant *HAA1*-overexpression mutant of an industrial bioethanol strain of *Saccharomyces cerevisiae* and its application to the production of bioethanol from sugarcane molasses. *AMB Express,* **3**, 74.

Kim, S. & Dale, B. E. (2004) Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioenergy*, **26**, 361-375.

Nakamura, T. et al. (2014) Identification of a gene, *FMP21*, whose expression levels are involved in thermotolerance in *Saccharomyces cerevisiae*. *AMB Express*, **4**, 67.

Saithong, P. et al. (2009) Prevention of bacterial contamination using acetate-tolerant *Schizosaccharomyces pombe* during bioethanol production from molasses. *J. Biosci. Bioeng.*, **108** (3), 216-219.

Sanchez, O. J. & Cardona, C. A. (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour. Technol.*, **99**, 5270-5295.

Shima, J. & Nakamura, T. (2015) Environmental stresses to which yeast cells are exposed during bioethanol production from biomass. *In* Stress Biology of Yeasts and Fungi, eds. Takagi, H. and Kitagaki, H., Springer Japan, Tokyo, Japan, 93-106.

Tanaka, K. et al. (2012) Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the *HAA1* gene, encoding a transcriptional activator. *Appl. Environ. Microbiol.*, **78** (22), 8161-8163.

Tanimura, A. et al. (2012) Isolation of a novel strain of *Candida shehatae* for ethanol production at elevated temperature.

SpringerPlus, **1**, 27.
Tanimura, A. et al. (2015) Direct ethanol production from starch using a natural isolate, *Scheffersomyces shehatae*: Toward consolidated bioprocessing. *Sci. Rep.*, **5**, 9593.
Van Vranken, J. G. et al. (2014) *SDHAF4* promotes mitochondrial succinate dehydrogenase activity and prevents neurodegeneration. *Cell Metab.*, **20** (2), 241-252.
Watanabe, I. et al. (2008) A strategy to prevent the occurrence of *Lactobacillus* strains using lactate-tolerant yeast *Candida glabrata* in bioethanol production. *J. Ind. Microbiol. Biotechnol.*, **35** (10), 1117-1122.
Watanabe, I. et al. (2012) Characterization of *Candida* sp. NY7122, a novel pentose-fermenting soil yeast. *J. Ind. Microbiol. Biotechnol.*, **39** (2), 307-315.
Watanabe, T. et al. (2010) Selection of stress-tolerant yeasts for simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash to ethanol. *Bioresour. Technol.*, **101** (24), 9710-9714.