

Growth and Fermentation Characteristics of *Saccharomyces cerevisiae* NK28 Isolated from Kiwi Fruit

Jong-Sub Lee^{1†}, Eun-Hee Park^{1†}, Jung-Wan Kim², Soo-Hwan Yeo³, and Myoung-Dong Kim^{1*}

¹Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea

²Division of Life Sciences, University of Incheon, Incheon 406-772, Republic of Korea

³Fermented Food Science Division, National Academy of Agricultural Science, RDA, Suwon 441-853, Republic of Korea

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*Corresponding author
Phone: +82-33-250-6458;
Fax: +82-33-241-0508;
E-mail: mdkim@kangwon.ac.kr

†These authors contributed
equally to this work.

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The influences of glucose concentration, initial medium acidity (pH), and temperature on the growth and ethanol production of *Saccharomyces cerevisiae* NK28, which was isolated from kiwi fruit, were examined in shake flask cultures. The optimal glucose concentration, initial medium pH, and temperature for ethanol production were 200 g/l, pH 6.0, and 35°C, respectively. Under this growth condition, *S. cerevisiae* NK28 produced 98.9 ± 5.67 g/l ethanol in 24 h with a volumetric ethanol production rate of 4.12 ± 0.24 g/l·h. *S. cerevisiae* NK28 was more tolerant to heat and ethanol than laboratory strain *S. cerevisiae* BY4742, and its tolerance to ethanol and fermentation inhibitors was comparable to that of an ethanologen, *S. cerevisiae* D5A.

Keywords: *Saccharomyces cerevisiae*, fermentation, ethanol, tolerance, fermentation inhibitor

Introduction

The depletion of fossil fuels has led to an increasing interest in the use of renewable and sustainable resources, such as agricultural waste for the production of alternative fuels like bioethanol [12–14]. Bioethanol is ethyl alcohol, the same type of alcohol that is used in alcoholic beverages, and it is most often used as a motor fuel, mainly as a biofuel additive for gasoline [32]. Efficient ethanol production processes and inexpensive substrates are crucial for the economic production of bioethanol. Bioethanol can be produced from the fermentation of carbon-based feedstock or lignocellulosic biomass [12, 24]. The biochemical conversion of renewable feedstocks for ethanol has been investigated on a commercial scale for the production of biofuels [7, 28].

Saccharomyces cerevisiae is a well-characterized microorganism that has traditionally been used for ethanol production from hexoses, and it provides high yields and productivity in addition to having a high ethanol tolerance [10, 19]. Since the optimum growth temperature of this yeast usually

ranges between 25°C and 30°C, maximum ethanol yields are obtained by a conventional fermentation process with the operation temperature maintained at around 30°C [5]; however, ethanol fermentation at a higher temperature has a number of advantages, including reduction in cooling costs, avoidance of microbial contamination, and possible use of simultaneous saccharification and fermentation together with continuous stripping of ethanol [27].

Lignocellulosic biomass consists of polysaccharides and lignin [3, 19, 29]. Pretreatment of the polysaccharides either using acidic or basic conditions at elevated temperatures is indispensable, since ethanol production by microorganisms such as *S. cerevisiae* and *Zymomonas mobilis* occurs through fermentation of monosaccharides [25, 33]; however, pretreatment of lignocellulosic biomass often releases a variety of inhibitory compounds (fermentation inhibitors) that affect the microorganism's ability both to grow and to produce ethanol, depending on the microorganism employed [7, 18, 26]. Inhibitory compounds formed by pretreatment of lignocellulosic biomass can be classified into three categories [2, 3, 8, 11, 15, 22, 30]: weak acids (acetic acid, formic acid,

and levulinic acid), furan derivatives (furfural and 5-hydroxymethylfurfural), and phenolic compounds (coumaric acid, syringaldehyde, and vanillin). Accordingly, to ensure the successful production of ethanol from lignocellulosic biomass on an industrial scale, the choice of ethanologen may depend on its capability for surviving at high temperatures and tolerating fermentation inhibitors [9].

This study was undertaken to examine the influences of growth conditions on the fermentation characteristics of *S. cerevisiae* NK28, which was isolated from kiwi fruit [25]. The optimum growth temperature, medium acidity (pH), and initial glucose concentration were determined in shake flask cultivations. In addition, the tolerance of *S. cerevisiae* NK28 to temperature, ethanol, and fermentation inhibitors was compared with that of industrial ethanol-producing strain *S. cerevisiae* D5A and laboratory strain *S. cerevisiae* BY4742.

Materials and Methods

Strain and Cultivation Conditions

S. cerevisiae NK28 [25], *S. cerevisiae* BY4742 [20], and *S. cerevisiae* D5A (ATCC 200062) were used in this study. The *S. cerevisiae* strains were grown in YEP (10 g/l bacto-yeast extract, 20 g/l bacto-proteose peptone) medium supplemented with various amounts of glucose. Shake flasks (500 ml) with a 100 ml working volume were used for batch cultivations. Initial medium acidity was adjusted to the desired pH using 2 N NaOH or 2 N HCl. All chemicals were purchased from Sigma-Aldrich (USA).

Analytical Methods

The cell mass concentration was determined by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Amersham Biosciences, USA) and a predetermined standard curve. Concentrations of ethanol and glucose were determined by a high-performance liquid chromatograph (Shimadzu, Japan) equipped with a Rezex Roa-Organic Acid H⁺ column (Phenomenex, USA)

and a refractive index detector (Shimadzu). H₂SO₄ solution (5 mM) at a flow rate of 0.6 ml/min was used as the mobile phase.

Plate Assay

S. cerevisiae strains were grown overnight in YEP broth containing 2% glucose (YEPD) and diluted to an OD_{600} of 0.2 with sterile water. Five-fold serial dilutions were spotted onto YEPD plate or YEPD plate supplemented with fermentation inhibitors and then incubated at 30°C for 3 days. To examine the thermotolerance, *S. cerevisiae* strains spotted on YEPD plate were incubated at various temperatures and cell growth was assessed after 2 days.

Statistical Analysis

Statistical analysis based on at least three independent experiments was performed using Student's *t*-test [6]. The results were considered to be statistically significant at $p < 0.05$.

Results and Discussion

In an attempt to examine the effects of initial glucose concentration on cell growth and ethanol production, YEP medium was supplemented with various concentrations of glucose, and *S. cerevisiae* NK28 was cultivated at 30°C for 24 h. As summarized in Table 1, at an initial glucose concentration of 20 g/l, approximately 5.34 ± 0.71 g/l of ethanol was produced with a yield of 0.30 ± 0.04 g ethanol/g glucose consumed. Cell growth and ethanol production were at a maximum at an initial glucose concentration of 200 g/l to produce 85.56 ± 1.13 g/l ethanol with a volumetric production rate of 3.46 ± 0.09 g/l·h. Neither cell growth nor ethanol production was significant at an initial glucose concentration of 400 g/l.

Subsequently, the influences of temperature and medium acidity on the growth and ethanol production of *S. cerevisiae* NK28 were examined in YEP medium supplemented with 200 g/l glucose (Table 2). Whereas optimum cell growth

Table 1. Effects of initial glucose concentrations on the growth and ethanol production by *S. cerevisiae* NK28.^a

	Glucose (g/l)					
	20	50	100	200	300	400
Cell mass (g/l)	5.38 ± 0.85	9.91 ± 0.26	13.29 ± 0.32	16.55 ± 0.20	10.37 ± 0.72	0.08 ± 0.00
Maximum specific growth rate (h ⁻¹)	0.51 ± 0.01	0.56 ± 0.01	0.55 ± 0.01	0.53 ± 0.02	0.28 ± 0.01	0.03 ± 0.01
Cell mass yield (g cell/ g glucose)	0.31 ± 0.05	0.32 ± 0.03	0.18 ± 0.01	0.09 ± 0.01	0.05 ± 0.01	0.01 ± 0.01
Ethanol concentration (g/l)	5.34 ± 0.71	17.00 ± 0.38	39.29 ± 0.49	85.56 ± 1.13	46.58 ± 1.18	1.11 ± 0.03
Ethanol production rate (g/l·h)	0.22 ± 0.03	0.71 ± 0.02	1.64 ± 0.02	3.46 ± 0.09	1.91 ± 0.05	0.05 ± 0.00
Ethanol yield (g ethanol/g glucose consumed)	0.30 ± 0.04	0.47 ± 0.05	0.45 ± 0.01	0.45 ± 0.01	0.23 ± 0.01	0.14 ± 0.06

^aEach value shown is the average ± standard error from three independent cultivations performed at 30°C for 24 h. Initial medium acidity was adjusted to pH 6.0 at 30°C.

Table 2. Cell growth and ethanol production by *S. cerevisiae* NK28 at different growth temperatures and initial medium acidity (pH).^a

	Temperature (°C) ^a					Medium acidity (pH) ^b				
	20	25	30	35	40	3.0	4.0	5.0	6.0	7.0
Cell mass (g/l)	6.46 ± 0.09	17.86 ± 0.20	16.55 ± 0.20	13.62 ± 0.12	7.39 ± 0.07	8.84 ± 0.09	12.42 ± 0.14	13.19 ± 0.14	13.62 ± 0.12	13.38 ± 0.02
Maximum specific growth rate (h ⁻¹)	0.30 ± 0.02	0.47 ± 0.01	0.53 ± 0.02	0.51 ± 0.02	0.39 ± 0.03	0.47 ± 0.01	0.49 ± 0.02	0.50 ± 0.03	0.51 ± 0.02	0.53 ± 0.03
Ethanol concentration (g/l)	25.31 ± 2.01	89.37 ± 4.81	85.56 ± 1.13	98.96 ± 5.67	60.80 ± 1.73	76.98 ± 2.79	92.05 ± 0.91	93.10 ± 1.13	98.96 ± 5.67	95.70 ± 0.71
Ethanol production rate (g/l·h)	1.05 ± 0.08	3.72 ± 0.20	3.46 ± 0.09	4.12 ± 0.24	2.53 ± 0.07	3.21 ± 0.12	3.84 ± 0.07	3.88 ± 0.05	4.12 ± 0.24	3.99 ± 0.03
Ethanol yield (g ethanol/g glucose consumed)	0.46 ± 0.01	0.49 ± 0.01	0.45 ± 0.01	0.50 ± 0.03	0.47 ± 0.01	0.47 ± 0.02	0.48 ± 0.02	0.48 ± 0.01	0.50 ± 0.03	0.49 ± 0.02

^aGlucose at a final concentration of 200 g/l was used as the carbon source and initial medium acidity was adjusted to pH 6.0.

^bInitial medium acidity was adjusted to each desired value at 35°C. Each value shown is the average ± standard error from three independent cultivations performed for 24 h.

occurred at 30°C, maximum ethanol production was obtained at 35°C. An ethanol concentration of 98.96 ± 5.67 g/l was obtained at 35°C, and this corresponds to a volumetric ethanol production rate of 4.12 ± 0.24 g/l·h, which is comparable to the 93 g/l of ethanol produced from 200 g/l glucose by the thermotolerant *S. cerevisiae* VS₃ [9]. It was also interesting to note that *S. cerevisiae* NK28 produced ethanol at 40°C and the difference in ethanol yields between 35°C and 40°C was not significant; however, the ethanol production rate at 40°C was 61.4% of that obtained at 35°C. Accordingly, it appeared that the optimum growth temperature for ethanol production by *S. cerevisiae* NK28 is 35°C. Regardless of the initial medium acidity, which ranged from pH 3.0 to pH 7.0, *S. cerevisiae* NK28 yielded ethanol concentrations of above 90 g/l from 200 g/l glucose. Accordingly, at the optimum condition of pH 6.0 and 35°C, the ethanol concentration of 98.96 ± 5.67 g/l was obtained (Table 2). Profiles of cell growth and ethanol production at the optimum condition are shown in Fig. 1.

Recently, it was reported that a thermotolerant *S. cerevisiae* strain isolated from Thai fruits produces 38 g/l ethanol from 100 g/l glucose at 41°C [5], corresponding to a volumetric ethanol production rate of 1.58 g/l·h. *S. cerevisiae* NK28 produced 60.80 ± 1.73 g/l ethanol at a volumetric production rate of 2.53 ± 0.07 g/l·h at 40°C and an ethanol yield of 0.47 ± 0.01 g ethanol/g glucose consumed, which shows that *S. cerevisiae* NK28 is one of the most promising thermotolerant ethanologens.

Based on the plate assay, the thermotolerance of *S. cerevisiae* NK28 was compared with that of an industrial

ethanol producer (*S. cerevisiae* D5A) and a haploid laboratory strain (*S. cerevisiae* BY4742) (Fig. 2). Among these three yeast strains, *S. cerevisiae* D5A showed the highest growth rate at 25°C, 30°C, and 35°C. Although *S. cerevisiae* NK28 grew much faster than *S. cerevisiae* BY4742, its growth rate was notably lower than that of *S. cerevisiae* D5A at growth temperatures below 30°C. At growth temperatures above 35°C, the difference in the growth rates of *S. cerevisiae* NK28 and *S. cerevisiae* D5A was not significant, suggesting that *S. cerevisiae* NK28 might be as tolerant as *S. cerevisiae* D5A to heat. It was noteworthy that *S. cerevisiae* BY4742 did not grow at 40°C.

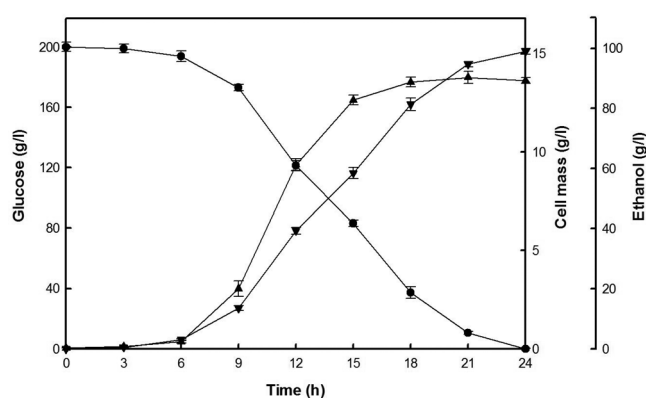


Fig. 1. Profiles of cell growth (▲), ethanol production (▼), and glucose consumption (●) of *S. cerevisiae* NK28 grown in YEP medium supplemented with 200 g/l glucose.

Initial medium acidity was adjusted to pH 6.0 and the temperature was maintained at 35°C.

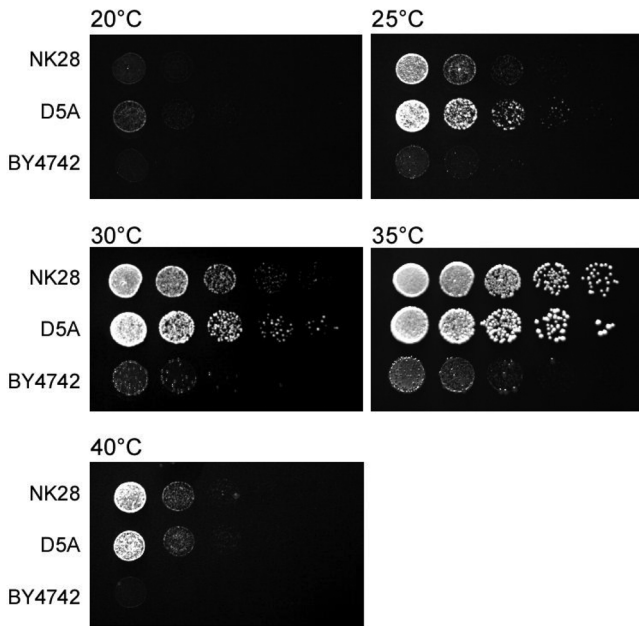


Fig. 2. Thermotolerance of *S. cerevisiae* strains D5A, NK28, and BY4742.

Each *S. cerevisiae* strain was harvested in the mid-exponential growth phase and diluted to an A_{600} of 0.2 with sterile water. Five-fold serial dilutions were spotted onto YEPD plates and incubated at the indicated temperature for 2 days.

The cell growth and ethanol production of the three different strains of *S. cerevisiae* were compared at elevated growth temperatures (Table 3). At 40°C, the specific growth rates and ethanol production rates of *S. cerevisiae* NK28 and *S. cerevisiae* D5A showed similar levels, but those of *S. cerevisiae* BY4742 were less than 10% of those levels at 5.52 ± 0.24 g/l ethanol. Meanwhile, at the relatively lower growth temperature of 35°C, *S. cerevisiae* NK28 showed a notably higher ethanol production rate than that of *S. cerevisiae* D5A (4.12 versus 3.52 g/l·h). The maximum ethanol concentration achieved by *S. cerevisiae* NK28 was 15% higher than that achieved by *S. cerevisiae* D5A. Accordingly,

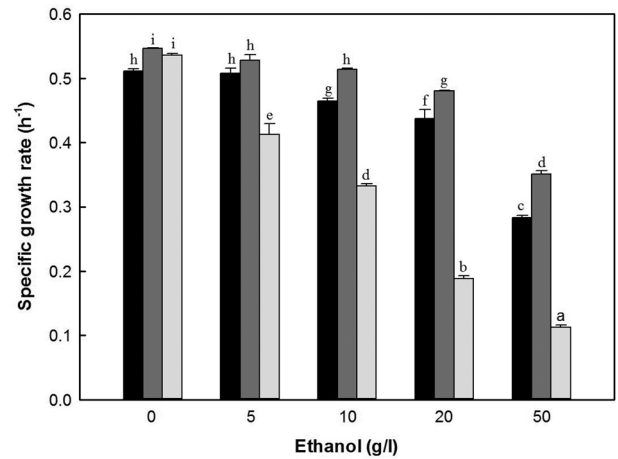


Fig. 3. Ethanol tolerance of *S. cerevisiae* strains D5A (black), NK28 (dark gray), and BY4742 (light gray).

Specific growth rate of each *S. cerevisiae* strain was determined in YEPD (2% glucose) medium supplemented with the indicated concentration of ethanol. Medium acidity was adjusted to pH 6.0. Different letters indicate significant difference between means.

S. cerevisiae NK28 was shown to be a promising yeast strain for ethanol production.

The tolerance of the *S. cerevisiae* strains to ethanol was compared by determining their specific growth rates in ethanol-supplemented YEPD broth (Fig. 3). When ethanol was added at a concentration of 50 g/l, the specific growth rate of *S. cerevisiae* BY4742 was 0.11 ± 0.01 h⁻¹. Up to 5 g/l ethanol concentration, there were no distinctive differences in the specific growth rates of *S. cerevisiae* NK28 and *S. cerevisiae* D5A; however, a difference in the ethanol tolerance of *S. cerevisiae* NK28 and *S. cerevisiae* D5A became clear as the ethanol concentration increased. At 50 g/l ethanol concentration, the specific growth rates of *S. cerevisiae* NK28 and *S. cerevisiae* D5A were 0.35 ± 0.01 h⁻¹ and 0.28 ± 0.05 h⁻¹, respectively. Accordingly, it was concluded that the ethanol tolerance of *S. cerevisiae* NK28 is higher than that of *S. cerevisiae* D5A.

Table 3. Comparison of ethanol production by *S. cerevisiae* strains at high growth temperatures.^a

	<i>S. cerevisiae</i> NK28		<i>S. cerevisiae</i> D5A		<i>S. cerevisiae</i> BY4742	
	35°C	40°C	35°C	40°C	35°C	40°C
Cell mass (g/l)	13.62 ± 0.12	5.41 ± 0.02	7.31 ± 0.24	4.78 ± 0.07	5.46 ± 0.28	0.75 ± 0.01
Maximum specific growth rate (h ⁻¹)	0.51 ± 0.02	0.39 ± 0.03	0.55 ± 0.01	0.42 ± 0.01	0.37 ± 0.01	0.18 ± 0.01
Ethanol concentration (g/l)	98.96 ± 5.67	60.80 ± 1.73	84.54 ± 0.60	62.95 ± 1.37	28.98 ± 1.21	5.52 ± 0.24
Ethanol production rate (g/l·h)	4.12 ± 0.24	2.53 ± 0.07	3.52 ± 0.02	2.62 ± 0.06	1.21 ± 0.05	0.23 ± 0.01
Ethanol yield (g ethanol/g glucose consumed)	0.50 ± 0.03	0.47 ± 0.01	0.43 ± 0.01	0.45 ± 0.01	0.26 ± 0.01	0.22 ± 0.02

^aInitial glucose concentrations of 200 g/l were used and medium acidity was adjusted to pH 6.0 at a given temperature. Each value shown is the average ± standard error from three independent cultivations performed for 24 h.

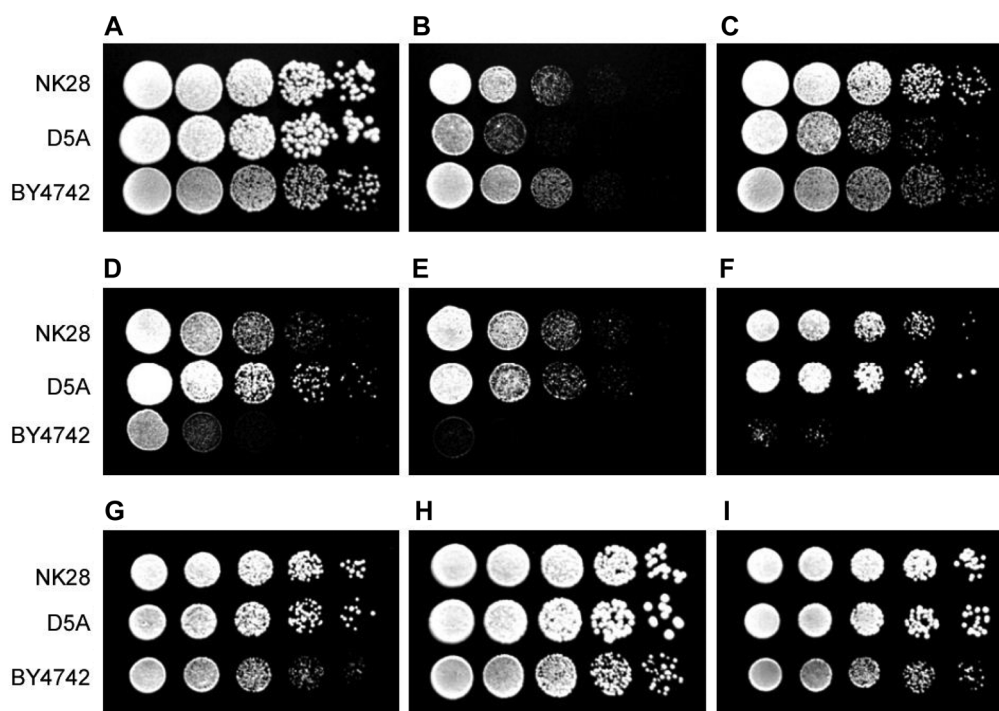


Fig. 4. Growth of *S. cerevisiae* strains in the presence of fermentation inhibitors.

S. cerevisiae strains in the mid-exponential growth phase were harvested and diluted to an A_{600} of 0.2 with sterile water. Five-fold serial dilutions were spotted onto YEPD plates supplemented with fermentation inhibitors and then incubated at 30°C. (A) YEPD; (B) furfural (8 mM); (C) 5-HMF (10 mM); (D) vanillin (8 mM); (E) syringaldehyde (8 mM); (F) acetic acid (65 mM); (G) coumaric acid (10 mM); (H) formic acid (6 mM); and (I) levulinic acid (50 mM). Cell growth was assessed after 3 days.

Recently, different strategies have been approached to improve the ethanol tolerance of yeast by genetic engineering and process optimization [1, 4]; however, yeast cells have different mechanisms to respond to ethanol toxicity based on different strains, different cultivation conditions, and different genetic backgrounds. Thus, the ethanol tolerance of yeast is important for ethanol production with high yields and high productivity. As shown in Table 3 and Fig. 3, *S. cerevisiae* NK28 showed higher ethanol productivity and was more tolerant to ethanol than the industrial strain *S. cerevisiae* D5A.

The tolerance of *S. cerevisiae* NK28 to fermentation inhibitors was examined by plate assays. As shown in Fig. 4, did *S. cerevisiae* NK28 exhibited slightly higher tolerance to furfurals than *S. cerevisiae* D5A. It was interesting to note that *S. cerevisiae* BY4742, which is a haploid laboratory strain, was more resistant to furfurals than *S. cerevisiae* D5A. *S. cerevisiae* BY4742 was the least resistant to most fermentation inhibitors, except furfurals, among the three *S. cerevisiae* strains tested. *S. cerevisiae* NK28 was as tolerant as *S. cerevisiae* D5A to most of the fermentation inhibitors tested, with vanillin being the exception.

Pretreatment of lignocellulosic biomass is necessary to disrupt the plant cell wall structure and to facilitate the access of hydrolytic enzymes and thus hydrolysis of constituent polysaccharides to monosaccharides for fermentation [31]. Fermentation inhibitors released from pretreatment of lignocellulosic biomass include weak organic acids, furans, and phenolic compounds, all of which independently or synergistically interrupt the metabolism of *S. cerevisiae* and consequently limit the yield and productivity of bioethanol [17, 18, 25]. Previous efforts have described approaches to improve the fermentation performance of *S. cerevisiae* strains with respect to inhibitor tolerance [15, 19]. When an industrial strain of *S. cerevisiae* was cultured in increasing concentrations of furfurals, the length of the lag phase of the adapted strain was significantly reduced compared with that of the parental strain [15, 16].

Unfortunately, the mode of action of fermentation inhibitors on microbial physiology still remains unclear [23]. Although several detoxification methods such as alkali treatment, sulfite treatment, evaporation, anion exchange, and laccase treatment have been used to remove or to decrease the level of fermentation inhibitors [19], these

methods still have imperfections for bioethanol production because the detoxification process also results in a loss of fermentable sugars from the pretreated hydrolyzates [21]. One plausible way to circumvent inhibitor problems in the bioethanol industry might be to improve the tolerance of microorganisms to fermentation inhibitors. This might be accomplished by the isolation of tolerant strains [34] or evolutionary engineering of laboratory strains through long-term adaption to fermentation inhibitors [19]. *S. cerevisiae* NK28 showed tolerance to fermentation inhibitors comparable to that of the industrial strain *S. cerevisiae* D5A.

In conclusion, it is suggested in this study that *S. cerevisiae* NK28, which has an excellent ethanol production capability and tolerance to inhibitors, might be developed as a promising workhorse for bioethanol production through appropriate genetic manipulation. Further research is under way to understand the molecular mechanisms underlying the tolerance of this yeast strain to stress conditions.

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