NOTE

A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions

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Abstract

The ability of 13 strains of multi-stress-tolerant *Issatchenkia orientalis* yeast to produce ethanol was examined under different stress conditions, including conditions of elevated H_2SO_4 and Na_2SO_4 concentrations and increased heat. The MF-121 strain produced a significant amount of ethanol after the incubation in acidic media containing high concentrations of salt, e.g., 50 g/l Na_2SO_4 at pH 2.0, or at high temperatures, e.g., 43°C, when compared with other strains.

Bioethanol is a fuel derived from renewable sources of feedstock. Many studies have investigated low-cost bioethanol production from biomass that does not compete with food production, e.g., virgin wood, energy crops, agricultural residues, food waste, and industrial waste. Bioethanol production using stress-tolerant microorganisms has certain advantages with respect to both cost and convenience. Thermotolerant microorganisms can help reduce the cost of cooling and aid in the simultaneous saccharification and fermentation process for converting cellulosic biomass to ethanol (1). Specific wild-type and mutant yeast strains, e.g., *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, have already been reported to produce ethanol at temperatures between 40 and 45°C (2, 3). Ethanol fermentation by acid-tolerant microorganisms under acidic conditions, i.e., with a pH below 4.0, minimizes the risk of bacterial contamination and reduces the cost of sterilization (1, 4). Ma et al. reported that a mutant strain of *Zymomonas mobilis* produced ethanol from kitchen garbage under non-sterilized conditions at pH 4.0 (5). Even at pH 2.0, an industrial strain of *S. cerevisiae* has also been shown to produce ethanol (6). Furthermore, the use of microorganisms tolerant to salts, such as Na_2SO_4, is of industrial importance for

bioethanol production. Dilute H_2SO_4, e.g., 5–25 g/l, is often used for the pretreatment of lignocellulose or the hydrolysis of cellulose (4, 7), and Na_2SO_4 (e.g., 8–40 g/l) is formed when the product is adjusted to a weakly acidic or a neutral pH by adding NaOH for microbial fermentation. Ethanol fermentation under high salt concentrations using salt-tolerant microorganisms reduces desalting costs and decreases the possibility of contamination. Since the ethanol fermentation industry is considerably large, any small improvement in the efficiency of ethanol production could be economically significant. Therefore, microorganisms with tolerance for multiple types of stress are desirable for bioethanol production. Cakar et al. reported the evolutionary engineering of *S. cerevisiae* that exhibited an increased resistance to freeze–thaw stress and temperature, ethanol, and oxidative stresses (8). Recently, Benjaphokee et al. developed an *S. cerevisiae* strain exhibiting high ethanol productivity (more than 90%) under multiple stress conditions (41°C and pH 3.5) by breeding (9).

In a previous study, we isolated a yeast strain designated as MF-121 from a river of pH 3.0 flowing through the Manza hot spring area (10). Certain physiological and biochemical properties of the MF-121 strain and its rDNA sequences indicated that the strain belongs to *Issatchenkia orientalis,* also known as, *Pichia kudriavzevii* or *Candida krusei*. The MF-121 strain can ferment glucose to ethanol at high salt concentrations and low pH conditions, e.g., 50 g/l Na_2SO_4 at pH 2.0, which was adjusted with H_2SO_4. In addition, the MF-121 strain can grow at 42°C. We demonstrated that the MF-121 strain produces ethanol from starch hydrolysates prepared using dilute H_2SO_4; the pH of the hydrolysates was adjusted to 2.5 by adding NaOH, and desalting procedures were omitted. In another study, we reported that the MF-121 strain produced ethanol from lignocellulose hydrolysates, ranging from pH 2.5 to 2.8, that were passed through an

ion-exchange column (11). Recently, we constructed a transformation system for the MF-121 strain of *I. orientalis* and demonstrated that a recombinant strain expressing a heterologous β-glucosidase gene can produce ethanol from cellobiose under multiple stress conditions (40°C and pH 3.0) (12). Thus, the MF-121 strain of *I. orientalis* has multi-stress-tolerant properties and appears to be useful for low-cost bioethanol production under high stress conditions. Also, *I. orientalis* has been isolated from food, liquor, and soil (13–16). In this study, we examined whether other *I. orientalis* strains exhibited similar multi-stress-tolerant characteristics to the MF-121 strain.

Twelve *I. orientalis* strains, NBRC 0011, 0012, 0013, 0155, 0201, 0584, 0841, 1162, 1279, 1395, 1664, and 10737, were obtained from the NITE Biological Resource Center in Chiba, Japan, and their characteristics were compared with those of the MF-121 strain. A brewing yeast strain, *S. cerevisiae* K-7, was obtained from the Brewing Society of Japan in Tokyo, Japan, and its characteristics were compared with those of the *I. orientalis* strain. All yeast strains were pre-cultured at 30°C for 24 h with shaking at 160 rpm in 3 ml of YPD medium containing 10 g/l yeast extract from Nacalai Tesque, Kyoto, Japan, 20 g/l peptone from Nacalai Tesque, and 20 g/l glucose. Cells were later diluted to OD_{600} = 0.4 in YPD medium and used as seed cultures.

First, we studied the effects of pH and salt concentrations on the ethanol production of each strain in YPGS medium containing 5 g/l yeast extract, 10 g/l polypeptone from Nihon Pharmaceutical in Tokyo, Japan, 100 g/l glucose, and various concentrations of Na_2SO_4, including 0, 10, 25, and 50 g/l. Na_2SO_4 was used for saline stress in this study for the reason described above. The pH of the medium was adjusted to multiple values, including 2.0, 2.5, and 3.0, using H_2SO_4. Fifty microliters of the seed cultures were inoculated into 5 ml of YPGS medium. The yeast strains were cultivated

using a rotary shaker under an aerobic condition of 160 rpm at 30°C. After 120 h of cultivation, OD_{600} and ethanol content were measured using a spectrophotometer (BioSpec-1600, Shimadzu, Kyoto, Japan) and an alcohol densitometer (Alcomate AL-2, Riken Keiki, Tokyo, Japan), respectively. All experiments were performed in duplicate, and the average values were reported.

Under all conditions, only a small change was observed in the pH of the medium before and after incubation (data not shown). A preliminary experiment demonstrated that aeration by shaking slightly accelerated or minimally affected the growth and ethanol production of these strains, which was probably because of the Crabtree effect, that is, stimulation of alcoholic fermentation under aerobic conditions (17). Nakayama et al. reported similar results for effects of aeration on ethanol fermentation in strains of *I. orientalis* (*C. krusei*) (18). In addition, mild aerobic or oxidative stress might improve tolerance to low pH and/or salt conditions because of a cross-tolerance acquisition as observed in *S. cerevisiae* (19).

Table 1 shows the ethanol content after incubation of each strain. The *S. cerevisiae* K-7 strain barely grew in the medium without salt at pH 2.0 and did not produce ethanol. In contrast, a high amount of ethanol was produced by individually culturing all of the *I. orientalis* strains in the same medium. No ethanol was detected after incubation of the K-7 strain in the medium containing 50 g/L Na_2SO_4 at pH 3.0; however, 10 of the 13 *I. orientalis* strains produced ethanol between 0.5 and 5.3% (v/v) under the same condition. Only 0.5% (v/v) ethanol was generated upon culturing the K-7 strain in the medium containing 25 g/l Na_2SO_4 at pH 2.5. However, all of the *I. orientalis* strains, excluding the NBRC 0011 strain, produced high amounts of ethanol, at concentrations between 4.9 and 5.3% (v/v), under the same condition. Thus, almost

all of the *I. orientalis* strains generated greater quantities of ethanol compared to the *S. cerevisiae* K-7 strain after incubation in acidic media containing high concentrations of Na_2SO_4.

We observed large differences in ethanol production among the *I. orientalis* strains cultured in media containing high concentrations of Na_2SO_4. The MF-121 strain produced a large amount of ethanol (5.3% (v/v)) when grown in a medium containing 50 g/l of Na_2SO_4 at pH 3.0. In contrast, the maximum percentage of ethanol produced by the NBRC 10737 strain, an *I. orientalis* strain, was 3.3% (v/v). In the medium containing 50 g/l of Na_2SO_4 at pH 2.0, which was the most severe condition used in this study, the MF-121 strain fermented glucose to ethanol at 1.3% (v/v). However, little or no ethanol was detected after incubation of the other *I. orientalis* strains under the same condition. Thus, in this experiment, the MF-121 strain produced the highest amount of ethanol under the combined stress of a low pH between 2.0 and 3.0 and a high salt concentration of 50 g/l Na_2SO_4. The NBRC 0201 and 0841 strains also generated a relatively large amount of ethanol under combined stress conditions. In contrast, the NBRC 0011 strain, which did not produce ethanol in medium containing more than 25 g/l of Na_2SO_4, was found to be the most susceptible to Na_2SO_4 among the *I. orientalis* strains.

Next, we examined the effect of temperature on ethanol production by each strain. Fifty microliters of the seed culture was inoculated into 5 ml of YPG medium containing 5 g/l yeast extract, 10 g/l polypeptone, and 100 g/l glucose. After incubation at various temperatures between 30 and 44°C for 48 h with shaking at 160 rpm, the OD_{600} and ethanol content were measured as described above.

Table 2 shows the results of ethanol fermentation at high temperatures. The K-7

strain did not grow at temperatures exceeding 40°C. In contrast, all of the *I. orientalis* strains produced high amounts of ethanol at 41°C, which indicated that they are more thermotolerant than were the *S. cerevisiae* K-7 strain. The MF-121, the NBRC 0011, and the NBRC 10737 strains produced particularly large amounts of ethanol (between 4.0 and 4.1% (v/v)), even at 43°C. However, only a trace amount of ethanol was detected after their incubation at 44°C.

In conclusion, *I. orientalis* strains have a superior ability to ferment glucose to ethanol under high stress conditions, such as acid (H_2SO_4), salt (Na_2SO_4), or heat stress. The MF-121 strain produced a large amount of ethanol after incubation in acidic media containing high concentrations of Na_2SO_4 or at high temperatures compared to other strains investigated in this study. The NBRC 0011 strain, which is susceptible to high concentrations of Na_2SO_4, exhibited high thermotolerance. In contrast, the NBRC 1279 strain exhibited a relatively low tolerance to high temperatures but produced ethanol under combined stress conditions of H_2SO_4 and Na_2SO_4. Compared to the tolerance of other *I. orientalis* strains, the NBRC 0155 strain was susceptible not only to combined stress conditions of H_2SO_4 and Na_2SO_4 but also to heat. Thus, the ability of each strain to produce ethanol at a low pH and a high Na_2SO_4 concentration did not correlate with its ability to produce ethanol at high temperatures.

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References

1. **Olofsson, K., Bertilsson, M., and Lidén, G.:** A short review on SSF–an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels, **1**:7 (2008).

2. **Sridhar, M., Kiran Sree, N., and Venkateswar Rao, L.:** Effect of UV radiation on thermotolerance, ethanol tolerance and osmotolerance of *Saccharomyces cerevisiae* VS_1 and VS_3 strains. Bioresour. Technol., **83**, 199–202 (2002).

3. **Limtong, S., Sringiew, C., and Yongmanitchai, W.:** Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresour. Technol., **98**, 3367–3374 (2007).

4. **Balat, M., Balat, H., and Öz, C.:** Progress in bioethanol processing. Prog. Energy Combust. Sci., **34**, 551–573 (2008).

5. **Ma, H., Wang, Q., Qian, D., Gong, L., and Zhang, W.:** The utilization of acid-tolerant bacteria on ethanol production from kitchen garbage. Renewable Energy, **34**, 1466–1470 (2009).

6. **De Melo, H. F., Bonini, B.M., Thevelein, J., Simões, D.A., and Morais Jr, M. A.:** Physiological and molecular analysis of the stress response of *Saccharomyces cerevisiae* imposed by strong inorganic acid with implication to industrial fermentations. J. Appl. Microbiol., **109**, 116–127 (2010).

7. **Sarkar, N., Ghosh, S.K., Bannerjee, S., and Aikat, K.:** Bioethanol production from agricultural wastes: An overview. Renewable Energy, **37**, 19–27 (2012).

8. **Cakar, Z. P., Seker, U. O. S., Tamerler, C., Sonderegger, M., and Sauer, U.**: Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*. FEMS Yeast Res. **5**, 569–578 (2005).

9. **Benjaphokee, S., Hasegawa, D., Yokota, D., Asvarak, T., Auesukaree, C., Sugiyama, M., Kaneko, Y., Boonchird, C., and Harasima, S.:** Highly efficient bioethanol production by a *Saccharomyces cerevisiae* strain with multiple stress tolerance to high temperature, acid and ethanol. N. Biotechnol. (2011). (in press)

10. **Hisamatsu, M., Furubayashi, T., Karita, S., Mishima, T., and Isono, N.:** Isolation and identification of a novel yeast fermenting ethanol under acidic conditions. J. Appl. Glycosci., **53**, 111–113 (2006).

11. **Thalagala, T. A. T. P., Kodama, S., Mishima, T., Isono, N., Furujyo, A., Kawasaki, Y., and Hisamatsu, M.:** Study on ethanol fermentation using D-glucose rich fractions obtained from lignocelluloses by a two-step extraction with sulfuric acid and *Issatchenkia orientalis* MF 121. J. Appl. Glycosci., **56**, 7–11 (2009).

12. **Kitagawa, T., Tokuhiro, K., Sugiyama, H., Kohda, K., Isono, N., Hisamatsu, M., Takahashi, H., and Imaeda, T.:** Construction of a beta-glucosidase expression system using the multistress-tolerant yeast *Issatchenkia orientalis*. Appl. Microbiol. Biotechnol., **87**, 1841–1853 (2010).

13. **Prillinger, H., Molnár, O., Eliskases-Lechner, F., and Lopandic, K.:** Phenotypic and genotypic identification of yeasts from cheese. Antonie Van Leeuwenhoek, **75**, 267–283 (1999).

14. **Clavijo, A., Calderón, I. L., and Paneque, P.:** Diversity of *Saccharomyces* and non-*Saccharomyces* yeasts in three red grape varieties cultured in the *Serranía de Ronda* (Spain) vine-growing region. Int. J. Food Microbiol., **143**, 241–245 (2010).

15. **Katemai, W., Maneerat, S., Kawai, F., Kanzaki, H., Nitoda, T., and H-Kittikun, A.:** Purification and characterization of a biosurfactant produced by *Issatchenkia orientalis* SR4. J. Gen. Appl. Microbiol., **54**, 79–82 (2008).

16. **Quan, C-S., Fan, S-D., Zhang, L-H., Wang, Y-J., and Ohta Y:** Purification and properties of a phytase from *Candida krusei* WZ-001. J. Biosci. Bioeng., **94**, 419–425 (2002).

17. **De Deken, R. H.:** The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol., **44**, 149–156 (1966).

18. **Nakayama, S., Morita, T., Negishi, H., Ikegami, T., Sakaki, K., and Kitamoto, D.:** *Candida krusei* produces ethanol without production of succinic acid; a potential advantage for ethanol recovery by pervaporation membrane separation. FEMS Yeast Res., **8**, 706–714 (2008).

19. **Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S., and Young, R. A.:** Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell, **12**, 323–337 (2001).

Table 1. Ethanol production under combined stress conditions of low pH and Na_2SO_4

Medium		Ethanol (%, v/v) [d]													
Na_2SO_4 (g/l)	pH	*I. orientalis*													*S. cerevisiae*
		MF-121	0011	0012	0013	0155	0201	0584	0841	1162	1279	1395	1664	10737	K-7
0	3.0	4.9[a]	5.1[a]	5.0[a]	5.3[a]	5.2[a]	5.1[a]	5.2[a]	5.1[a]	5.1[a]	5.1[a]	5.3[a]	5.4[a]	5.5[a]	5.4[a]
0	2.5	5.4[a]	4.7[a]	5.1[a]	5.3[a]	5.4[a]	5.2[a]	5.3[a]	5.3[a]	5.2[a]	5.3[a]	5.2[a]	5.3[a]	5.4[a]	5.3[a]
0	2.0	5.7[a]	5.1[a]	5.0[a]	5.3[a]	5.3[a]	5.3[a]	5.2[a]	5.0[a]	5.4[a]	5.7[a]	5.4[a]	5.7[a]	5.4[a]	0.0[c]
10	3.0	4.9[a]	5.0[a]	5.0[a]	5.1[a]	5.1[a]	5.1[a]	5.0[a]	5.0[a]	5.1[a]	5.0[a]	5.3[a]	5.2[a]	5.2[a]	5.3[a]
10	2.5	5.1[a]	4.8[a]	5.1[b]	5.2[a]	5.2[a]	4.8[a]	5.2[a]	4.9[a]	5.2[a]	5.2[a]	5.4[a]	5.3[a]	5.3[a]	5.1[a]
10	2.0	5.5[a]	4.4[a]	5.1[a]	5.1[a]	3.0[a]	5.2[a]	5.3[a]	5.2[a]	5.3[a]	5.5[a]	5.2[b]	5.5[a]	5.4[a]	0.0[c]
25	3.0	4.9[a]	0.0[c]	5.0[a]	5.0[a]	5.1[a]	5.1[a]	4.8[a]	4.6[a]	5.0[a]	4.9[a]	5.3[a]	5.3[a]	5.1[a]	3.5[a]
25	2.5	5.1[a]	0.0[c]	5.0[a]	5.1[a]	5.1[a]	5.0[a]	4.9[a]	4.9[a]	5.3[b]	5.1[a]	4.9[a]	5.1[a]	5.0[a]	0.5[c]
25	2.0	5.2[a]	0.0[c]	2.4[b]	4.6[a]	0.0[c]	5.2[a]	2.8[a]	4.9[b]	2.3[a]	5.3[a]	5.2[b]	5.3[a]	3.3[a]	0.0[c]
50	3.0	5.3[a]	0.0[c]	0.7[b]	3.1[a]	0.0[c]	2.6[b]	0.0[c]	3.0[b]	2.5[b]	1.9[b]	1.7[b]	0.5[b]	3.3[b]	0.0[c]
50	2.5	3.7[a]	0.0[c]	0.0[c]	0.1[b]	0.0[c]	1.5[b]	0.0[c]	1.1[b]	0.3[b]	1.0[b]	0.6[b]	0.1[c]	0.1[b]	0.0[c]
50	2.0	1.3[b]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.1[b]	0.0[c]	0.0[c]	0.0[c]	0.0[c]

[a] Final $OD_{600} \geq 5$
[b] $2 \leq$ Final $OD_{600} < 5$
[c] $0 \leq$ Final $OD_{600} < 2$
[d] The theoretical maximum of ethanol production from 100 g/l glucose is 6.4% (v/v).

Table 2. Ethanol production at various temperatures

Temperature (°C)	Ethanol (%, v/v) [d]													
	I. orientalis													*S. cerevisiae*
	MF-121	0011	0012	0013	0155	0201	0584	0841	1162	1279	1395	1664	10737	K-7
30	5.6[a]	5.6[a]	5.5[a]	5.5[a]	5.5[a]	5.3[a]	5.5[a]	5.4[a]	5.5[a]	5.5[b]	5.5[a]	5.5[b]	5.5[a]	4.3[a]
35	5.5[a]	5.4[a]	5.5[a]	5.6[a]	5.5[a]	5.4[a]	5.6[b]	5.4[a]	5.5[a]	5.5[b]	5.6[a]	5.6[a]	5.4[b]	4.5[a]
40	5.6[a]	5.6[a]	5.5[a]	5.6[a]	4.8[a]	5.5[a]	5.5[b]	5.5[a]	5.5[b]	5.6[b]	5.6[b]	5.5[a]	5.4[b]	0.0[c]
41	5.5[a]	5.4[a]	5.5[a]	5.5[a]	3.8[a]	5.0[b]	5.4[b]	5.4[a]	5.5[b]	5.6[a]	5.3[b]	5.5[a]	5.3[a]	0.0[c]
42	5.0[a]	4.3[a]	3.1[a]	3.5[a]	0.4[b]	2.2[b]	3.9[b]	5.5[a]	3.1[b]	1.5[b]	2.5[b]	5.6[a]	5.7[a]	0.0[c]
43	4.0[b]	4.1[a]	1.8[b]	2.2[b]	0.0[c]	0.7[c]	0.9[b]	4.1[b]	1.1[c]	0.0[c]	0.1[c]	3.2[a]	4.1[b]	0.0[c]
44	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.2[c]	0.0[c]	0.0[c]	0.0[c]	0.0[c]	0.2[c]	0.0[c]

[a] Final $OD_{600} \geq 5$
[b] $2 \leq$ Final $OD_{600} < 5$
[c] $0 \leq$ Final $OD_{600} < 2$
[d] The theoretical maximum of ethanol production from 100 g/l glucose is 6.4% (v/v).