Scientific Research Publishing

Advances in Microbiology, 2017, 7, 349-357
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

High Ethanol Production by Marine-Derived Yeasts-*Saccharomyces cerevisiae* under Stress Pressures

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How to cite this paper: Urano, N., Shirao, A., Okai, M. and Ishida, M. (2017) High Ethanol Production by Marine-Derived Yeasts-*Saccharomyces cerevisiae* under Stress Pressures. *Advances in Microbiology*, **7**, 349-357.
https://doi.org/10.4236/aim.2017.75029

Received: March 22, 2017
Accepted: May 15, 2017
Published: May 22, 2017

Abstract

For practical applications of bioethanol, the uses of both highly concentrated biomass materials and their effective fermentation by yeasts are indispensable in order to produce ethanol at low costs. However, as the saccharified products of those biomass generally contain abundant sugars, the yeasts are affected by the compounds and are inclined to decrease their physiological activities. In the process of fermentation, ethanol is gradually produced by the yeasts in the culture; the concentrated metabolic product also damages itself, and inhibition of the fermentation frequently occurs. The application of yeasts with high fermentative activities under stress pressures such as sugars and ethanol is thus desired for bioethanol production. In this study, various types of high-fermentative yeasts under stress pressures were isolated mainly from coastal waters in Japan and characterized. All yeast strains with high fermentative activities under 20% v/v ethanol were found to be *Saccharomyces cerevisiae*. The HK21 strain isolated from Tokyo Bay and identified as *S. cerevisiae* had the highest fermentation activity under 30% w/v sorbitol and under 20% v/v ethanol, and it produced approx. 70 g/l (9% v/v) ethanol from the 15% w/v glucose solution at 25˚C within 5 days.

Keywords

Marine-Derived Yeast, Fermentation, Bioethanol, Coastal Water, *Saccharomyces cerevisiae*, Stress Pressure

1. Introduction

Today, the United States is the largest bioethanol producer in the world, with corn and wheat as the main materials used to make bioethanol. The starch from

corn and wheat is saccharified with both α-amylase and glucoamylase, followed by yeast fermentation; the final ethanol concentration reaches 8% - 10% v/v [1] [2] [3]. The second biggest bioethanol producer in the world is Brazil. Its production has steadily increased over the last three decades. Sugarcane juice and molasses are used as substrates in Brazil, and a few days' fermentation of these materials provides a 6% - 8% ethanol concentration [4] [5]. However, the use of these crops as the materials for bioethanol usually competes with the food needs of people and domestic animals. In addition, the constancy of the crops' supply seems to be lacking due to the inadequate utilization of these agricultural products all year round.

Globally, various types of bioethanol production systems have been developed that use unutilized biomass such as seaweeds, *i.e.*, *Undaria pinnafida* [6] [7] [8], *Ulva* spp., and *Costaria costata* [6] [8], an alien aquatic plant in Japan, *Eichhornia crassipes* [9] [10]; and paper or wood scrap [7] [11]. In the case of seaweeds, these materials ordinarily contain rich salts, and the application of fermentative yeasts with salt tolerance is needed for the bioethanol production system. We identified *Citeromyces matritensis* M37 isolated from Tokyo Bay as a salt-tolerant yeast that produces ethanol from salted algae [12]. In addition, for the practical applications of all of the bioethanol production systems mentioned above, the uses of both highly concentrated biomass materials and their effective fermentation by yeasts are indispensable in order to produce ethanol at low costs.

However, as the first-step saccharified products from a biomass generally contain abundant sugars and yeasts are added for fermentation in the second step, the cells are affected by the concentrated compounds and their physiological activities are inclined to decrease. With the progress of fermentation, ethanol is gradually produced by the yeasts in the culture, and the cells are also seriously affected themselves by the concentrated ethanol, and inhibition of the fermentation frequently occurs. The application of yeasts with high fermentative activity under stress pressure such as that provided by sugars and ethanol is thus desired for bioethanol production. In our present study, we isolated various types of high-fermentative yeasts against both sugar and ethanol stresses, gathered mainly from Japan's coasts, and we characterized their fermentation behaviors in detail.

2. Materials and Methods

2.1. Yeast Culture

Saccharomyces cerevisiae C-19 from Tokyo Bay with high fermentative activity [11] was used, as were *Citeromyces matritensis* M37 from Tokyo Bay and *Zygosaccharomyces rouxii* S11 from a soy sauce factory with salt-tolerant and high fermentative activities [12]. We also used a fermentative yeast screening from two types of yeast library in our marine biochemistry laboratory: No. 1 [13] and No. 2 [14]. From library No. 1, we selected 11 strains from the Sanriku district of Miyagi in Japan and 68 strains from Miura peninsula of Kanagawa in Japan as the fermentative yeasts. From library No. 2, we selected 3 strains from Hakkei-

jima of Kanagawa in Japan and 3 strains from Tokyo Bay in Japan as the fermentative yeasts. One strain, K6-38, was isolated from a pond at Tokyo University of Marine Science and Technology as a fermentative yeast. The total of 89 strains used had fermentative activities under a no-stress condition, assayed as described next in Section 2.2.

2.2. Assay of Fermentative Activity by the Yeast

We first assayed the yeast fermentation activity under a no-stress condition. Each yeast strain was precultured at 25˚C for 24 hr in 10 ml of YPD_2 liquid medium (D-glucose 20 g/l, peptone 20 g/l, and yeast extract 10 g/l). The growing cells were then centrifuged at 800 g for 5 min, and the cell pellet was gathered. A portion of each pellet was inoculated into a test tube containing 10 ml of YPD_2 with a Durham pipe and then statically incubated at 25˚C for 7 days. The yeast fermentative activity in the culture was examined by the naked eye based on the storage of gas evolving from the cells in the Durham pipe.

Next, the yeast fermentation activity under stress pressure was assayed. Yeast cells were precultured as described above, and a portion of each pellet was inoculated into a test tube containing 10 ml of YPD_2 plus 20% v/v ethanol or 30% w/v sorbitol with a Durham pipe and then statically incubated at 25˚C for 7 days. The yeast fermentative activity in the culture was also examined by naked eye based on the storage of gas evolving from the cells in the Durham pipe.

2.3. Yeast Identification

The 26S rRNA genes of the fermentative yeasts assayed under stress pressure as described above in Section 2.2. were amplified by polymerase chain reactions (PCRs) using the forward primer NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and the reverse primer NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The D_1/D_2 domain sequences of the 26S rRNA genes in the yeasts were deposited in DDBJ, EMBI, and GenBank.

2.4. Assay of Ethanol Production and Glucose Consumption by the Yeast

Each yeast was precultured at 25˚C for 24 hr in 10 ml of YPD_{10} liquid medium (D-glucose 100 g/l, peptone 20 g/l, and yeast extract 10 g/l). The growing cells were centrifuged at 800 g for 5 min, and the cell pellet was gathered. A portion of the pellet was cultured at 25˚C for 48 hr in 100 ml of YPD_{15} liquid medium (D-glucose 150 g/l, peptone 20 g/l, and yeast extract 10 g/l).

The yeast culture was centrifuged at 800 g for 5 min, and the cell pellet was washed with physiological saline and gathered by centrifugation three times. The pellet (0.1 g wet cells) was inoculated into 10 ml of 15% w/v glucose solution in the test tube. Fermentation by the yeast in the tube was anaerobically carried out at 25˚C for 5 days using the anaero Pack System (Mitsubishi Gas Chemical Co., Tokyo). The amounts of ethanol production and glucose consumption in the fermentative suspension were measured by a high-performance liquid chroma-

tography (HPLC) system (Prominence, Shimadzu, Kyoto, Japan). The HPLC conditions were as follows: a refractive index detector (RID-10A, Shimadzu), column (Shim-pack SPR-Pb, particle 8 μm, size 250 × 7.8 mm, Shimadzu); mobile phase: distilled water (Wako Pure Chemical Industries, Osaka, Japan), flow rate: 0.6 ml/min, column temperature: 80˚C.

3. Results and Discussion

3.1. Screening Fermentative Yeasts under Stress Pressures; Sugar and Ethanol

From the total of 89 strains tested as described above in Section 2.1, the screening results regarding the fermentative yeasts under stress pressures (sugar and ethanol) are summarized in Table 1 and Table 2**.** First, we selected 9 strains with fermentative activities under 20% v/v ethanol (Table 1). The strain HK6, HK21, HK27, K6-38, and C-19 had strong activity (*i.e.*, +++); strain M39 had moderate activity (++), and the strains RB59, RB60, and M46 had weak activity (+).

Second, we selected 24 strains with fermentative activity under 30% w/v sorbitol (Table 2). Strains M44 and T9 had strong activity (+++); strains M63 and M68 had moderate activity (++), and all other strains had weak activity (+). We carried out the identification of all of the species of the main strains with strong or moderate activity in this study, as described next.

3.2. Identification of the Yeast Species

Table 3 shows the identification results of the yeast species with stress-resistant characteristics with the accession numbers. First, under 20% v/v ethanol, we found that five strains with strong activity (HK6, HK21, HK27, K6-38, and C-19) were *Saccharomyces cerevisiae*. One strain with moderate activity, M39, was

Table 1. The yeasts with fermentative activities under 20%v/v ethanol.

Sampling area	Strain	Fermentation
Hakkeijima (Kanagawa)	HK6	+++
	HK21	+++
	HK27	+++
Tokyo University of Marine Science and Technology (Tokyo)	K6-38	+++
Tokyo Bay (Tokyo)	RB59	+
	RB60	+
Miura Peninsula (Kanagawa)	M39	++
	M46	+
Tokyo Bay (Tokyo)	C-19	+++

Yeast Fermentation in 1 day. +++: strong activity, CO_2 was completely pooled in the Durham pipe; ++: moderate activity, CO_2 was half pooled in the Durham pipe; +: weak activity, CO_2 was pooled a little in the Durham pipe.

Table 2. The yeasts with fermentative activities under 30% w/v sorbitol.

Sampling area	Strain	Fermentation
Hakkeijima (Kanagawa)	HK6	+
	HK21	+
	HK27	+
Tokyo University of Marine Science and Technology (Tokyo)	K6-38	+
Tokyo Bay (Tokyo)	RB59	+
Miura Peninsula (Kanagawa)	M5	+
	M6	+
	M7	+
	M10	+
	M22	+
	M39	+
	M43	+
	M44	+++
	M55	+
	M59	+
	M63	++
	M68	++
	M69	+
	M73	+
Shizugawa Bay (Miyagi)	T2	+
	T5	+
	T9	+++
	T13	+
Tokyo Bay (Tokyo)	C-19	+

Yeast Fermentation in 1 day. +++: strong activity, CO_2 was completely pooled in the Durham pipe; ++: moderate activity, CO_2 was half pooled in the Durham pipe; +: weak activity, CO_2 was pooled a little in the Durham pipe.

Table 3. Identification of the high fermentative yeast under stress pressure (Ethanol or sorbitol).

Sampling area	Strain	20% (v/v) ethanol	30% (w/v) sorbitol	Accession number
Hakkeijima (Kanagawa)	*Saccharomyces cerevisiae* HK6	R	R	LC215954
	Saccharomyces cerevisiae HK21	R	R	LC215955
	Saccharomyces cerevisiae HK27	R	R	LC215956
Tokyo University of Marine Science and Technology (Tokyo)	*Saccharomyces cerevisiae* K6-38	R	R	LC215947
Tokyo Bay (Tokyo)	*Candida tropicalis* RB59	R	R	LC215950
	Candida tropicalis RB60	R	S	LC215951
Miura Peninsula (Kanagawa)	*Kazachstania unispora* M39	R	R	LC215948
	Candida sake M44	S	R	LC215952
	Kazachstania unispora M46	R	S	LC215949
Shizugawa Bay (Miyagi)	*Metschnikowia bicuspidata* T9	S	R	LC215953
Tokyo Bay (Tokyo)	*Saccharomyces cerevisiae* C-19	R	R	AB767255

R: Fermentative Yeast under Stress Pressure; S: Non-Fermentative Yeast under Stress Pressure.

Kazachstania unispora. The three strains with weak activities were identified as *Candida tropicalis* (RB59 and RB60) and *Kazachstania unispora* (M46).

Therefore, all yeast strains with high fermentative activity under 20% v/v ethanol were found to be *S. cerevisiae*. Under 30% w/v sorbitol, two strains with strong activity, *i.e.*, M44 and T9, were *Candida sake* and *Metschnikowia bicuspidata*, respectively. Therefore, all seven of the following strains had fermentative activity under both 20% v/v ethanol and 30% w/v sorbitol: HK6, HK21, HK27, K6-38, and C-19 identified as *S. cerevisiae*, M39 identified as *M. bicuspidata*, and RB59 identified as *C. tropicalis*. We measured the amounts of ethanol production and glucose consumption of these seven strains in 15% w/v glucose solution, as described next in Section 3.3.

3.3. Ethanol Production and Glucose Consumption by the Yeast Strains

Figure 1 illustrates the ethanol production by the selected seven strains during fermentation in the 15% w/v glucose solutions for 3 - 5 days. The *S. cerevisiae* strains HK6, HK21, HK27, K6-38, and C-19 produced 25 - 70 g/l ethanol in 3 - 5 days. The *C. tropicalis* strain RB59 and the *K. unispora* strain M39 produced only 5 - 10 g/l ethanol in 3 - 5 days. A good relationship concerning the fermentative activity results shown in Table 1 and Table 2 and those in Figure 1 was observed.

Figure 2 shows the glucose consumption by the seven strains during fermentation in the 15% w/v glucose solutions for 3 - 5 days. The *S. cerevisiae* strains HK6, HK21, HK27, K6-38, and C-19 consumed 50 - 90 g/l glucose in 3 - 5 days. The *C. tropicalis* strain RB59 and the *K. unispora* strain M39 consumed only 9 - 16 g/l glucose in 3 - 5 days. Thus, the fermentation activity shown by the *S. cerevisiae* species isolated from the coast of Japan was observed to be very high under stress pressure.

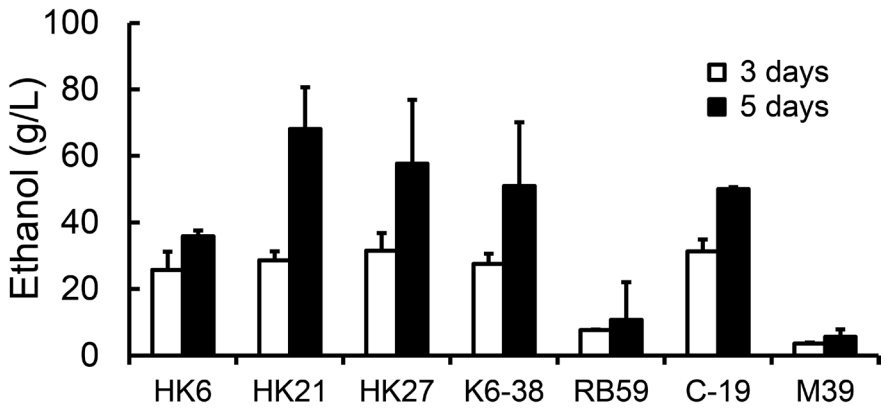

Figure 1. Ethanol productions by the seven selected strains during fermentation in the 15% w/v glucose solutions. The experimental conditions were as follows: yeast (0.1 g wet cells), glucose solution (15% w/v, 10 ml), anaerobic fermentation, temperature (25˚C), time (3 - 5 days). All experiments were conducted in triplicate. The data are the mean value. Error bars: the standard deviation (SD).

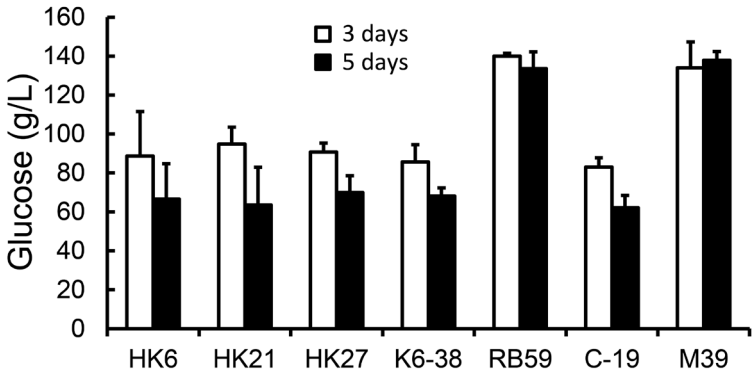

Figure 2. Glucose consumptions by the seven selected strains during fermentation in the 15% w/v glucose solutions. The experimental conditions were as follows: yeast (0.1 g wet cells), glucose solution (15% w/v, 10 ml), anaerobic fermentation, temperature (25˚C), time (3 - 5 days). All experiments were conducted in triplicate. The data are the mean value. Error bars: SD.

In general, bread has been made with baker's yeast since approx. 2000 BC, and beer has been made using brewer's yeast since approx. 1500 BC in Mesopotamia. The Japanese alcoholic beverage sake has also been made using sake yeast in Japan since ancient times. Suitable yeasts for various industries have been repeatedly isolated and bred separately according to the types of fermentation for many decades.

However, all industrial yeasts must have the same characteristics of high fermentative activity and high alcohol-tolerance activity. Identification methods for yeasts were developed after the 18th century, and most of the yeasts used in the fermentation industries were found to be *Saccharomyces cerevisiae* species. In modern times, bioethanol is usually produced by fermentation with *S. cerevisiae.* From ancient to modern times, *S. cerevisiae* has been the most important yeast species in the history of humans. However, in the bioethanol industry, various types of biomass materials are used and the screening of novel yeast strains with high fermentative activity under stress pressures is needed, again.

There also seems to be a variety of strains with different characters (even among the *S. cerevisiae* strains) that are dependent on their living environments. Although the *S. cerevisiae* species are generally isolated in fermentation factories or from natural terrestrial origins, we isolated highly fermentative yeasts from various marine origins and identified most of them as *S. cerevisiae* [6]-[11] [13] [14] [15]. In 2015, we reported the development of a simple isolation method for *S. cerevisiae* with marine origins [14]. In the present study, marine-origin strains of *S. cerevisiae* were found to have high fermentative activity under stress pressures. Thus, in the near future, a yeast library containing thousands of *S. cerevisiae* isolates with marine origins can be constructed and applied for practical bioethanol production using unutilized biomass.

4. Conclusion

From among the 89 strains tested, we isolated and characterized a variety of

yeast strains that were highly fermentative under stress pressure (sugar or ethanol), collected mainly from coastal waters in Japan. The seven strains—HK6, HK21, HK27, K6-38, and C-19 identified as *S. cerevisiae*, M39 identified as *M. bicuspidata*, and RB59 identified as *C. tropicalis*—had fermentative activity under both 20% v/v ethanol and 30% w/v sorbitol. Most notably, strain HK21 (isolated from Tokyo Bay and identified as *S. cerevisiae*) had high fermentation activity under 30% w/v sorbitol and under 20% v/v ethanol, and it produced approx. 70 g/l (9% v/v) ethanol from the 15% w/v glucose solution at 25˚C within 5 days. The fermentation activity shown by *S. cerevisiae* strains from marine origins was observed to be very high under stress pressure.

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