Fermentation of Molasses by Several Yeasts from Hot Spring Drain and Phylogeny of the Unique Isolate Producing Ethanol at 55℃

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Fermentation of Molasses by Several Yeasts from Hot Spring Drain and Phylogeny of the Unique Isolate Producing Ethanol at 55℃

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Abstract: The selected seven strains of the yeasts from hot spring drain were evaluated for their ethanol-producing abilities from sugarcane molasses at high temperatures. Maximum ethanol yields were obtained at 40℃ for the five strains and they reduced total organic carbon (TOC) in molasses during fermentation. In the early stage of molasses fermentation, use of the hot spring yeasts resulted in a 1.7-fold increase (at best) in the ethanol production rates compared to that observed in the culture of an industrial strain S. cerevisiae K-7, whereas they did not excel K-7 in the extent of ethanol yields. Therefore, use of the selected strains in the continuous fermentation was suggested as a possible application of these yeasts. Among the hot spring yeasts, phylogenetic position of the strain RND14, which fermented glucose at 55℃, was inferred from 18S rDNA sequence alignment: the strain was closely related to *Pichia fermentans*, though significant difference in the physiological characteristics was found between them.

Key words: *Ethanol production, Thermotolerant yeasts, Sugarcane molasses, 18S rDNA*

Introduction

Yeasts can be used in many industrial processes, such as the production of alcoholic beverages, biomass (baker's, food, and fodder yeasts), and various metabolic products.[1] Among these categories, fuel ethanol production from sugarcane molasses by thermotolerant strains (from terrestrial environments) has been studied extensively, because they are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates.[2–5] However, there had been no reports about the ethanol-producing ability of yeasts from aquatic environment with high temperatures. We found various thermotolerant, fermentative yeasts together with yeast-like unpigmented algae from a hot spring environment[6–10] and the ethanol-producing abilities of these eukaryotes have been reported already.[6–8] Nevertheless, we have not investigated the potentials of these yeasts for ethanol production from substrates other than glucose (i. e.; sugarcane molasses, sweet sorghum, and maize starch). In the present report, the seven yeast strains from hot spring drain[6] were evaluated for their ethanol-producing ability from sugarcane molasses at elevated temperatures. We also tested the ability of these yeasts to reduce total organic carbon (TOC) in molasses during fermentation, because increase of TOC, resulting from death of yeast culture, may cause environmental deterioration after the fermentation broth has been disposed. In taxonomic study of the yeast strains described above, morphological and physiological characteristics (comprises 68 tests) of the strain named RND 8, 12, 18, A2, and D4 perfectly coincided with those of RND 13, which was found to be a close relaive of *Kluyveromyces delphensis* on the basis of 18S rDNA sequence data,[6,7] and hence, the five strains

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were assumed to be taxonomically identical with RND 13. Apart from these strains, one strain named RND 14, featured by its distinctive fermentative ability at 55℃, was tentatively assigned to the genus *Dekkera*[6] on the basis of physiological characteristics alone and a definitive taxonomic conclusion of this strain requires further molecular characterization. There has been no report about the yeasts capable of fermentation over 50℃ and we are interested in the phylogenetic position of this strain. Therefore, in this study, we inferred the relative position of RND14 within ascomycetous yeasts from 18S rDNA sequence divergence.

Materials and Methods

Yeast strains and culture maintenance

The seven yeast strains (RND8, 12, 13, 14, 18, A2, and D4), that are capable of growth and fermentation at and above 40℃[6], were used throughout this study. All of them were isolated from drainage samples containing hot spring water at Rendaiji in Shizuoka Prefecture, located in the middle of Japan.[6] The yeast cultures were maintained on YPD agar plates (pH 6.2), which consisted of: glucose, 20 g; Difco BACTO™ Peptone, 20 g; Difco Yeast Extract, 10 g; all desolved in 1 L of distilled water, solidified with 20 g agar, and grown at 25℃.

Fermentation of molasses (made in Okinawa) with low glucose concentration at various temperatures

In the initial experiment, we studied the abilities of the yeasts to reduce TOC during fermentation of molasses as a function of temperature and substrate concentration. Molasses used in this experiment was kindly provided by Okinawa Seitou Co. (Okinawa, Japan). Total sugar in molasses was measured by colorimetry.[11] Glucose was enzymatically estimated using an F-kit Glucose (Boehringer Mannheim Co. Ltd, Mannheim, Germany) based on the method of Kunst *et al.* using hexokinase and glucose-6-phosphate dehydrogenase.[12] For fermentation tests, three sets of heat sterilized and diluted molasses with different total sugar concentrations (32.6, 24.5, and 19.6%, w/v) were prepared, to contain 2.5, 1.9, and 1.5 g/L of glucose, respectively. The initial pH of the molasses was within the range of 5.1–5.2. Large Durham tubes, each having a small inner Durham tube, were filled with 5 mL of diluted molasses. Each tube was inoculated with 100-μL cell suspension of the yeasts taken from an actively growing culture (washed twice in 0.85% NaCl solution in advance) to give an initial cell concentration of 10^8 cells/mL of molasses. The outer large tubes were fitted with aluminum caps and then incubated at 40, 42.5, and 45℃ for 8 days. Fermentation was recognized by the accumulation of CO_2 gas trapped in the inner Durham tubes. At the end of the incubation time, the fermentation broth was passed through a membrane filter (0.45 μm pore size, Nihon Millipore Co. Ltd, Tokyo, Japan) to remove yeast cells. A TOC 5000A analyzer (Shimadzu Co. Ltd, Kyoto, Japan) was used to quantify TOC in molasses.

Second experiment was carried out in order to clarify the effect of temperature on ethanol yields during batch fermentation of molasses. The diluted molasses used in this test was the same as used in the first experiment with 24.5% (w/v) total sugar (product of Okinawa Seitou Co.). Before the fermentation, a loopful of cells picked up from the culture was inoculated into a test tube containing 10 mL of a YPD liquid medium (pH 6.2), which includes the same components as YPD agar plates without solidification, and was pregrown at 37℃ for 16 h by reciprocal shaking at 120 rpm. A portion of this culture (200 μL) was inoculated into a 500 mL shaking flask containing 100 mL of a YPD liquid medium with 10% glucose (pH 6.1) and then cultured to the stationary phase again. The culture was centrifuged at 1600 × g for 5 min and washed with a 0.85% NaCl solution. A 1.0-g aliquot of the cells was used for inoculation in the following anoxic culture. Fermentations were conducted at 37.5, 40.0, 42.5, and 45℃ in 500 mL flasks containing 100 mL of the diluted molasses with reciprocal shaking at 120 rpm. The fermentation flasks were fitted with stoppers to vent the CO_2 through a water trap[7] and were allowed to ferment for 48 h. The retrieved fermentation broth was centrifuged at 1600 × g for 5 min

and the supernatant was heated at 80℃ for 15 min. Ethanol was enzymatically estimated using an F-kit Ethanol (Boehringer Mannheim Co. Ltd, Mannheim, Germany) by the method of Beutler.[13] Data are expressed as means of the produced ethanol derived from triplicate runs.

Fermentation of molasses with higher glucose concentration at 40℃

We further assessed the extent of ethanol production using different type of molasses containing more glucose. In this case, ethanol production levels were compared between the hot spring yeasts and a sake yeast strain Kyokai-7gou (K-7), which had been shown to be thermotolerant among industrial strains within *Saccharomyces cerevisiae*[14], but its instability of growth at 40℃ was confirmed previously by the authors (data not shown). Heat-sterilized and diluted molasses (Food Cycle Systems Co., Saitama, Japan) that contained 23.0% (w/v) total sugar (pH 4.8) was used in this experiment. Glucose in this diluted molasses was 8.9 g/L, which was much higher than that contained in the molasses (Okinawa) used in the first and second experiments. Precultures were grown in the same manner as the second experiment at 37℃ for the hot spring yeasts or at 30℃ for K-7. A 1.0-g aliquot of the cells was used for inoculation in the following anoxic culture. Fermentations were conducted at 40℃ in 500 mL flasks containing 100 mL of the diluted molasses with reciprocal shaking at 120 rpm for up to 48 h.

Phylogenetic Analysis of the Strain RND14 Based on the 18S rDNA Sequence Alignment

The procedures for extraction of genomic DNA, polymerase chain reaction and determination of sequences were previously provided.[7] The 18S rDNA sequence of the strain RND14 has been deposited in the DDBJ/EMBL/GenBank database under the accession no. AB112711. The obtained sequence was checked for similarities to the rDNA sequences in the databases using the BLAST program. Sequences were then aligned with those of the corresponding regions in ascomycetous yeasts and multiply aligned sequences were subjected to bootstrap resampling (1000 replicates),[15] genetic distance calculation by the 2-parameter method,[16] and phylogenetic tree construction by the neighbor-joining method,[17] all of which were computed using the multiple sequence alignment program Clustal W version 1.7.[18]

Results and Discussion

Fermentation of Molasses by Thermotolerant Yeasts

All strains produced gas from molasses and this was observed independent of temperature and substrate concentration. The changes of TOC during fermentation are shown in Table 1. All cultures were able to reduce TOC in molasses regardless of the initial total sugar content at 40℃, whereas three strains (RND 12, 14, and D4) increased the values under high substrate concentrations (24.5 and/or 32.6% total sugar) at 42.5℃. This phenomenon may be attributable to the death of cultures caused by the detrimental effect of high osmotic pressure at high temperature. However, the same did not apply to the cultures at 45℃; all of the strains (except for RND 12) increased TOC in response to the decrease in the initial total sugar amount in molasses. In this case, the detrimental effect of produced ethanol at elevated temperatures[2,7] may have been responsible for the death of cultures, which generally results in an increase of TOC.

On the basis of temperature profiles of the yeasts tested, the optimum temperature for ethanol production appears to be 40℃ for the five strains (RND 8, 12, 13, 14, and A2), as shown in Fig. 1. It is interesting that ethanol production level of RND A2 was not influenced markedly by temperature. Only one strain (RND D4) revealed a linear decrease in the ethanol yield with increasing the temperature, but its ethanol production level was comparable to those achieved by the remaining six strains at the temperatures tested. Fig. 2 shows the time courses of ethanol production by the thermotolerant yeasts and K-7 at 40℃. The ethanol production rates of the hot spring yeasts were higher than those of K-7 during the initial two hours of

Table 1. Changes of total organic carbon (TOC) in molasses (Product of Okinawa seitou Co.) during semi-anoxic culture for 8 days. Decrease or increase in the percentage of TOC, relative to the initial amount, are shown.*

Fermentation temperature (℃)	Initial total sugar contained in molasses (w/v) %	Strains						
		RND8	RND12	RND13	RND14	RND18	RND A2	RND D4
40.0	32.6	−9.1	−9.2	−4.1	−1.4	−7.6	− 3.3	− 5.8
	24.5	−7.1	−6.9	−0.5	−0.6	−4.6	−10.6	−10.0
	19.6	−5.5	−0.2	−0.5	−4.2	−8.8	− 9.0	− 9.2
42.5	32.6	−3.7	0.6	−0.2	2.1	−4.5	− 2.2	− 2.7
	24.5	−5.4	−5.9	−0.4	1.3	−2.2	− 1.4	1.4
	19.6	−3.4	−4.2	−0.2	−0.6	−1.5	− 2.4	− 2.6
45.0	32.6	−2.3	−0.5	−1.3	7.7	−2.7	− 2.1	4.3
	24.5	−0.1	−0.9	−1.5	5.5	<0.1	0.9	6.7
	19.6	0.7	−1.5	<0.1	8.4	2.1	5.2	7.2

* The underlined data represent increase of TOC.

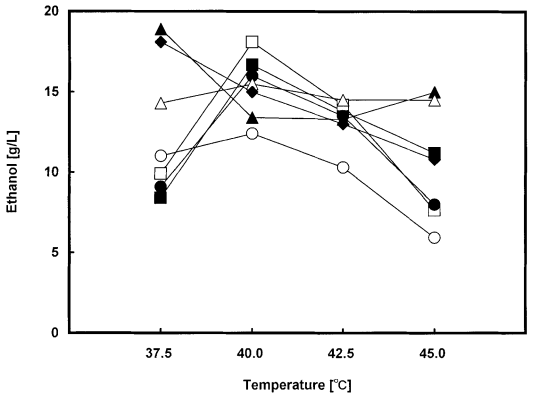

Fig. 1. Effect of temperature on ethanol yields of thermotolerant yeasts isolated from hot spring drainage during fermentation of molasses containing 24.5% (w/v) total sugar (product of Okinawa seitou Co.). Strains: ■, RND8; □, RND12; ●, RND13; ○, RND14; ▲, RND18; △, RND A2; ◆, RND D4.

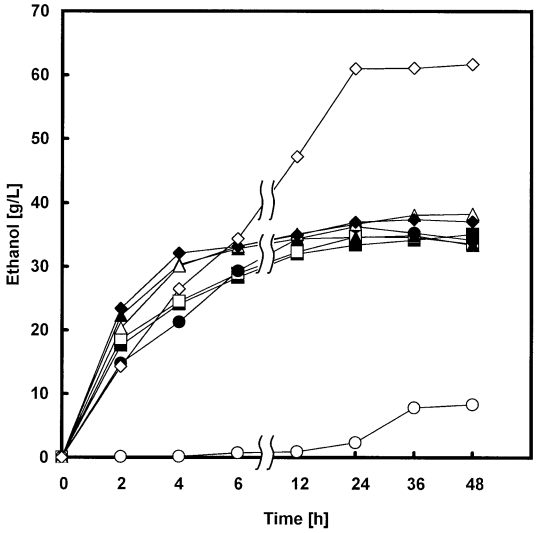

Fig. 2. Time courses of changes in ethanol concentration during the fermentation of molasses containing 23.0% (w/v) total sugar (pro-duct of Food cycle systems Co.) at 40℃. Strains: ■, RND8; □, RND12; ●, RND13; ○, RND14; ▲, RND18; △, RND A2; ◆, RND D4; ◇, K-7. K-7 represents a sake yeast strain Kyokai-7gou within *Saccharomyces cerevisiae.*

incubation with the exception of RND 14. Above all, three strains (RND18, A2, and D4) produced ethanol to the extent higher than K-7 even after four hours of the anoxic cultivation. However, the prolongation of the culture beyond four hours did not result in a marked increase in the extent of produced ethanol for the hot spring yeasts (up to 3.3–3.8%). In contrast, K-7 revealed the time-dependent ethanol production and the concentration of ethanol reached to the level of 6.2% after 48 h of fermentation. In this case, it would be possible to attribute the prolonged production of ethanol by K-7 to the capability of using sucrose and/or raffinose (generally contained in molasses) anaerobically.[19] However, we may pro-pose

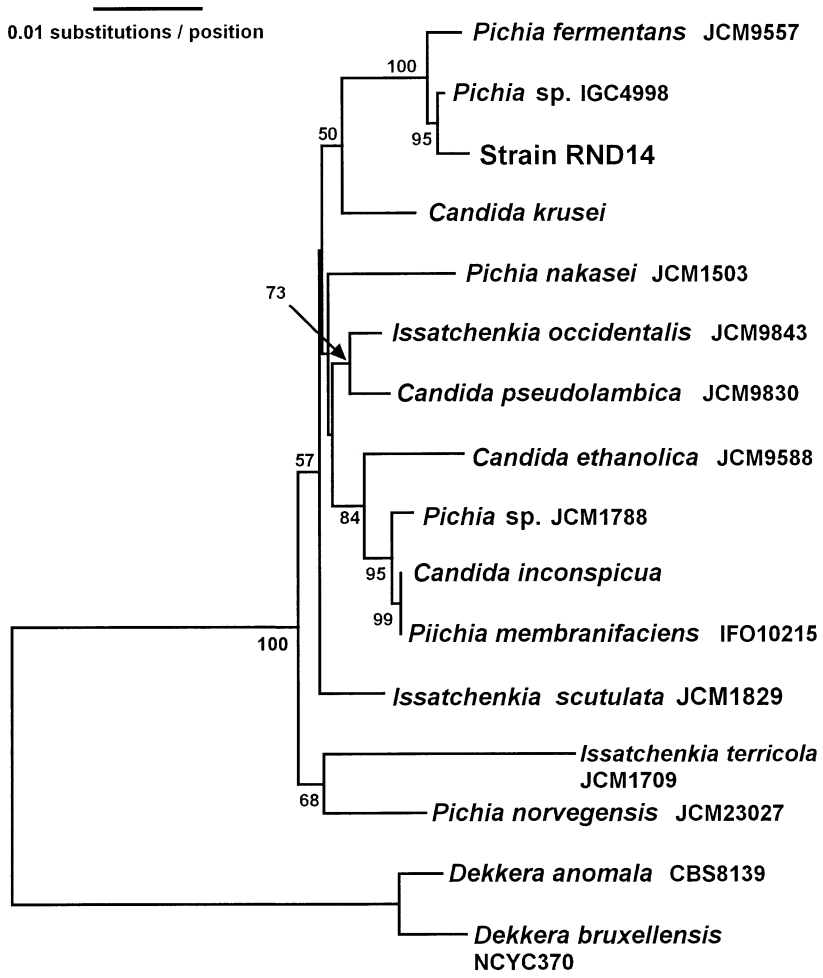

Fig. 3. A neighbor-joining tree resulting from analysis of 18S rDNA sequences of RND14 and related taxa. The strains used in the analysis are given after the scientific names of the organisms. Bootstrap values are shown at the internal nodes, when greater than 50%. The horizontal lengths are proportional to the evolutionary distances. A distance of 0.01 is indicated by the scale.

an application of the thermotolerant strains for continuous fermentation under the conditions of a reduced substrate concentration, because it should be of great economic benefit to utilize their enhanced activity of ethanol production repeatedly.

Phylogenetic analysis of the strain RND14

When examined by BLAST similarity analysis, the 18S rDNA sequence from RND14 produced matches with several *Pichia* spp., while our physiological studies identified it as a member of the genus *Dekkera*.[6] A distance tree inferred from 18S rDNA sequences is shown in Fig. 3. The physiological differences between RND14 and its closely related strains (revealed from the sequence similarity and the phylogeny) are shown in Table 2. RND14 appears to be related to *P. fermentans*, however, in addition to several physiological differences shown in Table 2, the degree of thermotolerance differed from each other; RND14 grew well at 42℃ but *P. fermentans* did not grow at the tempera-

Table 2. The 18S rDNA nucleotide sequence similarities and physiological differences between the strain RND14 and related taxa.

Species/strain	18S rDNA sequence similarity (%)	Assimilation of carbon compounds					Other characteristics		
		Sucrose	Cellobiose	D-Glucosamine	Citrate	D-Xylose	Acetic acid production	Growth at 37℃	Growth at 40℃
Strain RND14	−	+	S	−	−	S	+	+	+
Pichia sp./IGC4998	99.5	*	*	*	*	*	*	*	*
Pichia fermentans/JCM9557	99.1	−	−	+	+	+	−	+	−
Pichia sp./IFO 1788	97.1	*	*	*	*	*	*	*	*
Pichia membranifaciens/IFO10215	97.1	−	−	V	V	V	−	+	−
Candida krusei/origin unknown	97.1	−	−	+	+/W	−	−	+	+

Physiological characteristics were examined as previously described[6]. Symbols: + = strongly positive, growth within seven days; s = slowly positive, growth observed slowly after more than seven days; w = weakly positive; - = negative, no growth; +/w = positive or weakly positive; v = variable response; * = data not available

tures over 37℃. Moreover, RND14 was clearly distinguished from its relatives within *Pichia* by its ability to assimilate sucrose and cellobiose, as well as its acetic acid production under aerobic condition, though both of the characteristics are considered to be fundamental in taxonomy of the yeasts. Such a discrepancy between phylogenetic position and the results of identification based on the physiological characteristics is unprecedented as far as the hot spring yeasts (and the yeast-like strains) are concerned[6–10], and therefore, the result demonstrated here should stimulate interests of the taxonomists.

In summary, we demonstrated that the selected strains of the yeasts from hot spring environment have a potential to reduce TOC in molasses during fermentation at high temperatures (Table 1) and that maximum ethanol yields were obtained at 40℃ for the five strains out of the seven tested in the temperature profile experiments (Fig. 1). Moreover, they could produce ethanol more rapid than K-7, which is known to be thermotolerant among industrial strains of *S. cerevisiae*, in the early stage of fermentation (Fig. 2). However, the extent of produced ethanol by the hot spring strains were lower than that of K-7, though one of them (RND 13) had a potential to produce high concentration of ethanol (6.6%) at 40℃ when 15% glucose was used as a substrate.[7] Hence, the successful application of the hot spring yeasts awaits further trials in the repeated batch/continuous fermentation at elevated temperatures.

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温泉排水由来の酵母による廃糖蜜からエタノール生産ならびに，55℃でエタノール発酵を認めた新規酵母株の分子系統解析

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温泉排水由来の耐熱性酵母 7 株が高温域でもつ，廃糖蜜を基質とした場合のエタノール生産能を調べた。沖縄産廃糖蜜（全糖 24.5%，グルコース 0.2%）を発酵させたところ，7 株中 5 株が 40℃ にエタノール生産の至適温度を有していた。次に，酵母が利用しやすいグルコースをより多く含む別種の廃糖蜜（全糖 23.0%，グルコース 0.9%）を基質として 40℃ で発酵させたところ，温泉由来株が発酵初期に示したエタノール生産速度は，産業酵母としては耐熱性に優れる日本酒酵母協会 7 号（*Saccharomyces cerevisiae*）に比較して最高で約 1.7 倍の値を示したが，エタノール生産量は協会 7 号より低かった。従って，温泉の耐熱性酵母の有効利用可能性として，連続培養によるエタノール生産が示唆された。本研究で用いた株のうち 55℃ の高温でグルコースの発酵能を示した 1 株（RND14 株）について，18SrDNA 塩基配列に基づく分子系統樹上の位置を推定したところ，子嚢菌酵母 *Pichia fermentans* に近縁であり，生理学的手法による同定結果とは大きく異なった。

キーワード：エタノール生産，耐熱性酵母，廃糖蜜，18SrDNA